The genetics of chloroquine resistance in the rodent malaria parasite *Plasmodium chabaudi*

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Abbreviations

ADA	adenosine deaminase
bp	base pair
CHEF	contour-clamped homogeneous electric field
cM	centiMorgan
CO_2	carbon dioxide
CQ	chloroquine
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5' triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dNTP	2'-deoxynucleoside 5'- triphosphate
d.p.	decimal place
DMSO	dimethyl sulphoxide
EDTA	ethylenediamine tetraacetic acid disodium salt
g	gramme
g	g force
HCl	hydrochloric acid
kb	kilobase
kDa	kiloDalton
LDH	lactate dehydrogenase
LMT	low melting temperature
m.u.	map unit
Mb	Megabase
MDR	multiple drug resistance
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide
N_2	nitrogen
NAD	nicotinamide adenine dinucleotide
NaOH	sodium hydroxide
O_2	oxygen
PCR	polymerase chain reaction
PFG	pulsed-field gradient gel
PFGE	pulsed-field gradient gel electrophoresis
6PGD	6-phosphogluconate dehydrogenase
PMS	phenazine methosulfate
PYR	pyrimethamine
RFLP	restriction fragment length polymorphism
sarcosyl	N-lauroylsarcosine sodium salt
SSC	saline-sodium citrate
TAE	Tris-acetate/EDTA buffer
TBE	Tris/borate/EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
TE	Tris/EDTA buffer
V	volt
v/v	volume for volume
w/v	weight for volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactosidase

Three letter amino acid code

A	Alanine	N	Asparagine
C	Cysteine	P	Proline
D	Aspartic acid	Q	Glutamine
E	Glutamic acid	R	Arginine
F	Phenylalanine	S	Serine
G	Glycine	T	Threonine
H	Histidine	V	Valine
I	Isoleucine	W	Tryptophan
K	Lysine	Y	Tyrosine
L	Leucine	*	Termination
M	Methionine		

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Finally, I would like to dedicate this thesis to the two most important men in my life, my Father and Angus.

I declare that apart from the assistance mentioned above, the work presented here is my own.

Jane M-R. Carlton
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Abstract

The aim of this work has been to use linkage analysis to determine the chromosomal location of genes involved in chloroquine resistance in the rodent malaria parasite *Plasmodium chabaudi*. Chloroquine resistance in the human malaria parasite *P. falciparum* has become a major problem in most areas where *P. falciparum* is prevalent. Attempts to uncover the mechanism responsible for the resistance have been complicated by the lack of *P. falciparum* isogenic mutants, and the difficulties of performing genetic crosses *in vitro*. *P. chabaudi* is an ideal laboratory model for *P. falciparum* because the complete life-cycle is possible under laboratory conditions.

Initially a cross was made between two *P. chabaudi* clones, one a chloroquine-resistant mutant, selected for reduced susceptibility to chloroquine at a low level, and the other a genetically distinct chloroquine-sensitive clone. Gametocytes of a mixture of both clones were fed to *Anopheles stephensi* mosquitoes to allow cross-fertilisation between the clones to take place. The infected mosquitoes were allowed to feed on an uninfected mouse, and the progeny of the cross collected from the mouse blood. Individual clones were made from these progeny by a dilution method.

The *P. chabaudi* genome was found to contain 14 chromosomes. A genetic map of each chromosome was made using DNA markers. Most of the markers were known genes from other species of *Plasmodium*. Other markers were developed by the RAPD-PCR (random amplified polymorphic DNA-polymerase chain reaction) technique, the first time this method had been developed for use with *Plasmodium* parasites. In total, more than 100 markers were mapped to individual *P. chabaudi* chromosomes. Two important markers were cloned from *P. chabaudi* DNA using PCR. (i) the *P. chabaudi* homologue (*pcmdr1*) of the multiple drug resistance gene of *P. falciparum* (*pfmdr1*); this has been implicated in the mechanism of chloroquine resistance in *P. falciparum*. (ii) a possible homologue of the *P. falciparum* marker pS590.7, which has been claimed to be linked to a chloroquine resistance locus in *P. falciparum*.

Thirteen genetically distinct clones from the cross were phenotyped for their susceptibility to chloroquine. Eight were found to be resistant and five sensitive. These clones were analysed for their inheritance of 46 polymorphic markers. This revealed that neither *pcmdr1* nor the putative pS590.7 homologue were linked to chloroquine resistance in this cross. Twelve of the thirteen progeny, however, appeared to show an association with chloroquine susceptibility, consistent with the presence of a chloroquine resistance locus on chromosome 11. Statistical analysis

revealed that, depending upon the method of calculation, the linkage was significant with a probability of $0.05 > Pr > 0.024$.

The work submitted here represents the first in-depth genetic analysis of a *P. chabaudi* cross and identifies a locus which may be involved in the genetic mechanism of chloroquine resistance.

1. Introduction

1.1 Aim of this project

The aim of this project has been to identify genes which may be involved in the mechanism of chloroquine resistance in the malaria parasite of rodents, *Plasmodium chabaudi*. The initial stage of the work has been to make a genetic map of the *P. chabaudi* genome, the first to have been produced for this parasite. Polymorphic markers from this map have subsequently been used in linkage analysis of the progeny of a cross between chloroquine-resistant and chloroquine-sensitive parasites, to determine the chromosomal location of the gene(s) determining resistance.

1.2 Introduction to the malaria parasite

Many species of malaria parasite have been described which infect a variety of vertebrate hosts, including reptiles, birds and mammals (Garnham, 1966). All are members of parasitic protozoa within the family Plasmodiidae, which is characterised by stages of asexual multiplication in the host cells, and sexual reproduction and transmission through species of mosquito vectors of the family Culicidae.

Four species of malaria parasite infect man: three species, *P. malariae*, *P. vivax* and *P. ovale* cause illness, but are rarely fatal. The fourth, *P. falciparum*, is the most common in tropical and sub-tropical areas and produces the most severe illness which may prove fatal, due to a condition known as cerebral malaria. This is characterised by rapid deterioration of the patient into coma, and immediate chemotherapeutic treatment is necessary to prevent death. It is estimated that 300 million people are infected with *P. falciparum* at any one time, and that 0.5-1.2 million deaths occur from infection with *falciparum* malaria each year in Africa (W.H.O., 1993).

It is not possible to maintain the complete life-cycle of *P. falciparum in vitro*. Consequently other species of *Plasmodium* which can be grown under laboratory conditions are important as laboratory models of human malaria. Examples of such species are *P. gallinaceum* which infects chickens, *P. cynomolgi* which infects monkeys, and species of rodent malaria which can be grown in laboratory mice. Four species of rodent malaria are available, *P. chabaudi*, *P. berghei*, *P. vinckei* and *P. yoelii*; the work in this study has been carried out using *P. chabaudi*. This parasite has many characteristics in common with *P. falciparum*; for example both species of parasite produce infections which are synchronous with a preference for mature red blood cells, and both species produce gametes late in an infection (Walliker, 1983).

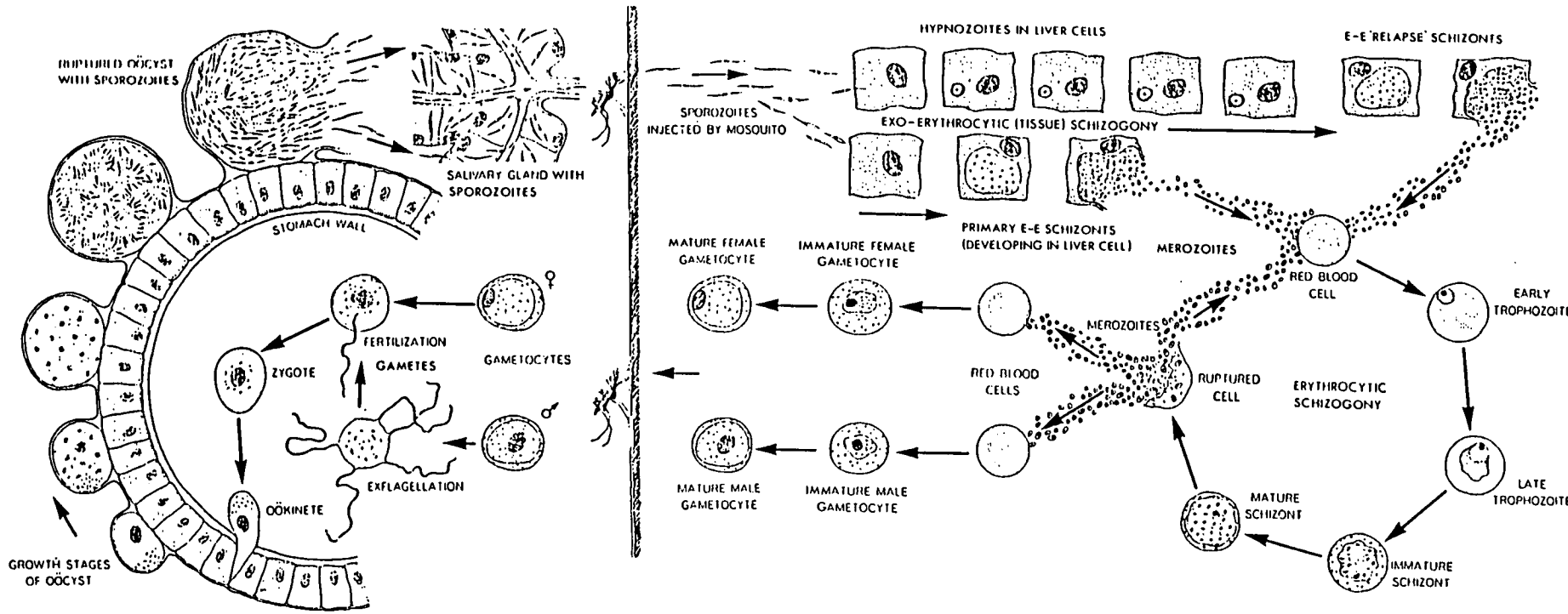
1.3. Origin and life-cycle of *P. chabaudi*

P. chabaudi was first isolated from thicket rats, *Thamnomys rutilans*, in the Central African Republic by Landau (1965). The life-cycle and infection pattern of the parasite have been described by Landau and Killick-Kendrick (1966), Wery (1968), Landau *et al.* (1970), Landau and Boulard (1978), Landau and Chabaud (1994). The parasite was temporarily reclassified as a subspecies of *P. vinckei* (*P. vinckei chabaudi*) by Bafort (1968), but then reinstated as a species (*P. chabaudi*) following later studies on the parasite's enzyme forms and morphology (Carter and Walliker, 1975). There are two subspecies, *P. chabaudi chabaudi* which is found in the Central African Republic, and *P. chabaudi adami* which is found in Brazzaville, The Congo (Carter and Walliker, 1976).

P. chabaudi has a typical mammalian malaria parasite life-cycle, as shown in **Figure 1**. The cycle starts when an infected female *Anopheles* mosquito takes a blood meal from a mammalian host. Sporozoites, inoculated from the salivary glands of the mosquito into the host, are carried to the liver where they invade liver parenchyma cells. The sporozoites undergo asexual development into exoerythrocytic schizonts, a process which takes a minimum of 52-53 hours. Each schizont contains 18,000 -20,000 merozoites, and these are released into the blood upon rupture of the schizonts. The free merozoites invade mature erythrocytes as ring forms. These grow to form intraerythrocytic trophozoites, and then nuclear division occurs to produce schizonts. Each schizont contains 4-8 merozoites which are released upon rupture of the infected erythocyte. Merozoites invade further red blood cells and the cycle is repeated. Each cycle takes 24 hours and the development of schizonts is synchronous, depending upon the circadian rhythm of the host. Some merozoites may develop into macro- or micro-gametocytes following invasion of a red blood cell. These are the sexual forms of the parasite, which do not develop further unless ingested by a female mosquito during a blood meal.

Development of gametocytes within the mosquito midgut occurs as follows: the macro-gametocyte transforms into the female gamete, and the micro-gametocyte undergoes exflagellation, releasing male gametes. Fertilisation occurs between male and female gametes, producing a zygote which develops as a motile ookinete. This penetrates the midgut wall of the mosquito and develops as an oocyst on its outer surface. After 8-10 days the mature oocysts rupture, releasing several thousand sporozoites into the haemocoele. These migrate to the salivary glands where they are injected into a new mammalian host the next time that the mosquito takes a blood meal.

Figure 1. Life-cycle of a typical mammalian malaria parasite.



Insect host stages are shown to the left of the picture, and mammalian stages to the right.

Note: the presence of *P. chabaudi* hypnozoites in the host liver has yet to be demonstrated conclusively.

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1.4 Genetics of *Plasmodium*

The malaria parasite has three genomes : (i) a large nuclear genome, which contains up to 7,500 genes (Reddy, 1995); (ii) a linear 6 kb (kilobase) mitochondrial genome, which contains genes necessary for an electron transport system (for a review see Wilson *et al.* (1991)); and (iii) a circular 35 kb organellar genome, which contains several ribosomal RNA, transfer RNA and DNA-dependent RNA polymerase genes (Wilson *et al.*, 1991). It is likely that the products of some of the extranuclear genes are sites of action for several antibiotics and 8-aminoquinolines (e.g. Strath *et al.*, 1993; Vaidya *et al.*, 1993), and mutations in these genes may ultimately be shown to produce resistance to these drugs. Genes involved in chloroquine resistance are most likely to be nuclear-encoded for reasons stated later; the rest of this chapter is limited to a discussion of this genome.

1.4.1 Genome size and base composition

Estimates of the size of the malaria parasite nuclear genome vary within a range of 2-4 x 10^7 bp (base pairs) per haploid genome. The different estimates are probably due to the method of determination used by different workers; for example Dore *et al.* (1980) produced a value of 2 x 10^7 bp per haploid *P. berghei* genome using reassociation kinetics, whereas Wellems *et al.* (1987) produced a value of 2.5-3 x 10^7 bp per haploid *P. falciparum* genome by measurement of chromosomal DNA mobilities during electrophoresis. This DNA content is comparable to that of yeast and is 5-6 times more than that of the bacterium *Escherichia coli.*

Plasmodium genomes are very rich in the nucleotide bases A + T. Estimates of the base composition of *P. falciparum* include 18% G + C by McCutchan *et al.* (1984), and 17-19% G + C by Pollack *et al.* (1982). The *P. chabaudi* genome has been estimated as 18% G + C (McCutchan *et al.*, 1984). One of the consequences of such a high A + T content is that the codon bias is remarkably skewed (Hyde and Sims, 1987; Saul and Battistutta, 1988), and this can produce problems when using heterologous probes from more G + C-rich genomes (Hyde *et al.*, 1989). Moreover, it may be responsible for the instability of parasite DNA fragments in *E. coli*, which has prevented the cloning of large segments of *P. falciparum* DNA (Weber, 1988).

1.4.2 Karyotype and karyotypic rearrangements

The chromosomes of malaria parasites cannot be seen by conventional light microscopy, as they do not condense during mitosis. This is unexpected because histone proteins and nucleosomes are present in the parasite nucleus (Wunderlich *et al.*, 1980; Creedon *et al.*, 1992; Cary *et al.*, 1994). However, electron microscopy

has identified 14 pairs of kinetochores, the regions of chromosomes to which spindle microtubules attach during division, in serial sections of *P. falciparum* mitotic spindles (Sinden and Strong, 1978; Prensier and Slomianny, 1986). This suggested that the genome of *P. falciparum* consists of 14 chromosomes. Confirmation of this has come with the advent of pulsed-field gradient gel electrophoresis (PFGE) (Carle and Olson, 1984; Schwartz and Cantor, 1984), which has enabled the visualisation of all 14 *P. falciparum* chromosomes on agarose gels (Kemp *et al.*, 1987; Wellems *et al.*, 1987).

The karyotype of the four rodent malarias has been studied in depth, although the exact chromosome number has been difficult to ascertain due to problems in separating all the chromosomes by PFGE. Langsley *et al.* (1987) reported separating 11 *P. chabaudi* chromosomes but considered it likely that the organism contained 14. Sharkey *et al.* (1988) were able to separate 10 *P. chabaudi* bands, but concluded that it was not possible to determine the exact number of chromosomes, because each DNA band could represent more than one chromosome. Finally, Sheppard *et al.* (1989b) reported that *P. chabaudi*, *P. vinckei* and *P. berghei* appeared to contain 14 chromosomes, although this was speculative considering the quality of the pulsed-field gels (PFGs) at the time the work was carried out.

Malaria parasite chromosomes are structurally similar to those of lower eukaryotes. Each chromosome is compartmentalised into a conserved, transcribed, central domain and polymorphic, transcriptionally silent, chromosome ends (Lanzer *et al.*, 1994). Individual chromosome ends consist of telomeric repeat sequences (Vernick and McCutchan, 1988; Ponzi *et al.*, 1985) and subtelomeric repeat regions. The subtelomeric regions have conserved and polymorphic features. In *P. berghei*, a series of 2.3 kb repeats lie proximal to the telomere, but not every chromosome hybridises to these repeats and the pattern of hybridisation varies in different isolates (Dore *et al.*, 1990). The subtelomeric regions of *P. falciparum* chromosomes contain complex repeat sequences adjacent to a series of tandemly repeated 21 bp repeat sequences, the so-called 'rep20' repeats (Oquendo *et al.*, 1986).

Genetic linkage maps of several *Plasmodium* chromosomes have been constructed by probing Southern blots of separated chromosomes with known gene probes. The linear order of the genes has also been determined by long-range restriction mapping, involving digestion of individual chromosomes with rare-cutting restriction enzymes (Sinnis and Wellems, 1988; Ponzi *et al.*, 1990; Triglia *et al.*, 1992). Recent advances in cloning large fragments of DNA into the yeast *Saccharomyces cerevisiae* have also enabled the construction of yeast artificial chromosome (YAC) libraries containing the complete *P. falciparum* genome (Triglia and Kemp, 1991), and the arrangement of

such YACS into overlapping clones representing complete chromosomes (Lanzer *et al.*, 1993).

Chromosomes of *P. falciparum* show considerable size variation among parasite isolates taken directly from patients' blood (Corcoran *et al.*, 1986; Langsley *et al.*, 1988), and following asexual mitotic division in culture (Wellems *et al.*, 1988). Chromosomes of *P. berghei* have been shown to change size following serial passage through mice (Janse *et al.*, 1989). The size polymorphisms may arise as a result of various processes, such as (a) unequal crossing-over between homologous chromosomes during meiosis (Corcoran *et al.*, 1988; Sinnis and Wellems, 1988); (b) deletion and insertion of repeat sequences such as rep20 (Patarapotikul and Langsley, 1988) and the 2.3 kb repeats of *P. berghei* (Ponzi *et al.*, 1990); (c) gene amplification (Foote *et al.*, 1989; Triglia *et al.*, 1991); and (d) the addition of telomeric DNA sequences (Pologe and Ravetch, 1988).

Most of these large scale rearrangements affect only the subtelomeric, transcriptionally silent, regions of chromosomes, rather than the internal gene-rich domains (Lanzer *et al.*, 1993). However, chromosome translocations resulting in exchange of genes between non-homologous chromosomes have been reported (Janse *et al.*, 1992), as well as duplication and translocation of coding sequences, resulting in the creation of parasites with 15 chromosomes instead of 14 (Cowman and Lew, 1989; van Dijk *et al.*, 1994). It is not clear whether these types of rearrangements play a significant role in changes in the location and linkage of genes on chromosomes of parasites in natural populations (although see Janse *et al.* (1994) and **Chapter 6**).

1.4.3 Ploidy, mitosis and meiosis

The malaria parasite is haploid throughout the vertebrate host stages. Studies on the inheritance of isoenzyme markers in *P. chabaudi* (Walliker *et al.*, 1975) and in *P. falciparum* (Walliker *et al.*, 1987) showed that the erythocytic stages were haploid. Subsequent studies on the inheritance of exoerythrocytic stage antigens (Szarfman *et al.*, 1988) demonstrated exoerythrocytic haploidy.

The parasite undergoes mitotic division during erythrocytic and exoerythrocytic schizogony, sporogony and microgametogenesis. Evidence for this comes from studies by Janse *et al.* (1986) who showed that *P. berghei* sporozoites, ring forms, young trophozoites and mature microgametes possess a similar quantity of DNA, assumed to be the haploid amount. Also, DNA has been localised to mitotic spindles in the nuclei of sporulating *P. falciparum* oocysts (Vanderberg, 1967). It has been calculated that each mitotic division takes 6 to 8 hours (Sinden and Strong, 1978).

The only diploid phase in the parasite life-cycle is the zygote (ookinete) in the mosquito stomach. In electron microscope studies of *P. berghei* ookinetes, meiotic division was detected within 3 hours of fertilisation (Sinden and Hartley, 1985), and synthesis of 4 times the haploid DNA quantity has been found to occur at this time, consistent with duplication of the diploid chromosome set at the first stage of meiosis (Janse *et al.*, 1986). Moreover, the presence of synaptonemal complexes has been demonstrated (Sinden and Hartley, 1985), which are characteristic of meiotically dividing cells and appear to be necessary for crossing-over to occur.

1.4.4 Genetic markers

Genetic studies depend upon the availability of characters which are polymorphic. Several types of marker which exhibit such variation have been exploited for use in genetic studies in *Plasmodium* :-

1) Protein variants

Many enzymes and other proteins detectable by electrophoretic methods, show variant forms which are distinguished by their size and/or charge (for reviews see Kemp *et al.*, 1987a; Beale and Walliker, 1988). Electrophoretic forms of enzymes have proved to be particularly useful genetic markers, because they are usually the products of single genes and because they are stable during blood passage and mosquito transmission. Enzyme variation has been much studied in species of rodent malaria (Carter, 1970; Carter and Walliker, 1975; Carter, 1978), primate malaria (Carter and Voller, 1973) and *P. falciparum* (Carter and McGregor, 1973; Carter and Voller, 1975; Sanderson *et al.*, 1981; Thaithong *et al.*, 1981).

2) Antigens

Plasmodium antigens are known to possess variant forms among organisms of a given population (so-called 'antigenic diversity'). Antigenic diversity in *P. falciparum* has been demonstrated by McBride *et al* (1982) using monoclonal antibodies and immunofluorescence techniques, and in *P. yoelii* by crossed immunoelectrophoresis (Panton *et al.*, 1984). Several genetic studies involving the use of variant antigens as markers have been carried out with *P. falciparum* (e.g. Walliker *et al.*, 1987; Ranford-Cartwright *et al.*, 1993). The only studies on the inheritance of blood stage antigens in rodent malaria species are a cross between clones of *P. yoelii*, which differed in the variant forms of a single antigen (Panton *et al.*, 1984), and a study of inheritance of a merozoite surface antigen in *P. chabaudi* by McLean *et al.* (1982).

3) Drug susceptibility

Drug resistance can be caused by a number of mechanisms, non-genetic as well as genetic (Beale, 1980). Resistance due to non-genetic mechanisms is usually unstable, whereas mechanisms involving the spontaneous mutation of genes generally result in a stable, heritable genotype (see **Section 1.5**). It is possible to select lines of malaria parasites with decreased susceptibility to certain drugs, by one of two ways: (i) a single high dose treatment which eliminates most of the parasites, and only resistant mutants survive (the 'single-step' method); or (ii) continuous low dose treatments which cover many passages and may gradually increase in concentration (the 'multi-step' method). Laboratory mutants obtained by both these methods have been exploited in genetic studies of *P. gallinaceum* (Greenberg and Trembley, 1954a) and the rodent malarias (Walliker *et al.*, 1971; Walliker *et al.*, 1973; Walliker *et al.*, 1975). Drug-resistant *P. falciparum* clones isolated from the field have also been used in genetic crossing experiments (Walliker *et al.*, 1987; Wellems *et al.*, 1990).

4) DNA markers

Recent progress in molecular genetic techniques has produced numerous *Plasmodium* DNA markers which can be used in genetic studies. These are of various types:

(i) *Plasmodium* gene markers. Over 230 *Plasmodium* genes have been cloned and many mapped to specific chromosomes by PFGE (see Triglia *et al.*, 1992; Reddy, 1995). Moreover, DNA sequencing has enabled variants (alleles) of specific genes to be identified.

(ii) RFLP markers. Probing Southern blots of restricted parasite DNA with known *Plasmodium* genes or anonymous DNA fragments has enabled the identification of restriction fragment length polymorphisms (RFLPs) of loci between different parasite clones (Botstein *et al.*, 1980). RFLPs are the result of changes in genomic DNA, such as the substition, insertion or deletion of DNA sequences. They have been extensively used in the analysis of a *P. falciparum* cross by Walker-Jonah *et al.* (1992).

The size polymorphisms of homologous chromosomes in different cloned lines (**Section 1.4.2**), although not precise DNA markers, have also been used as genetic markers in *Plasmodium* crosses (Sharkey *et al.*, 1988; Sinnis and Wellems, 1988).

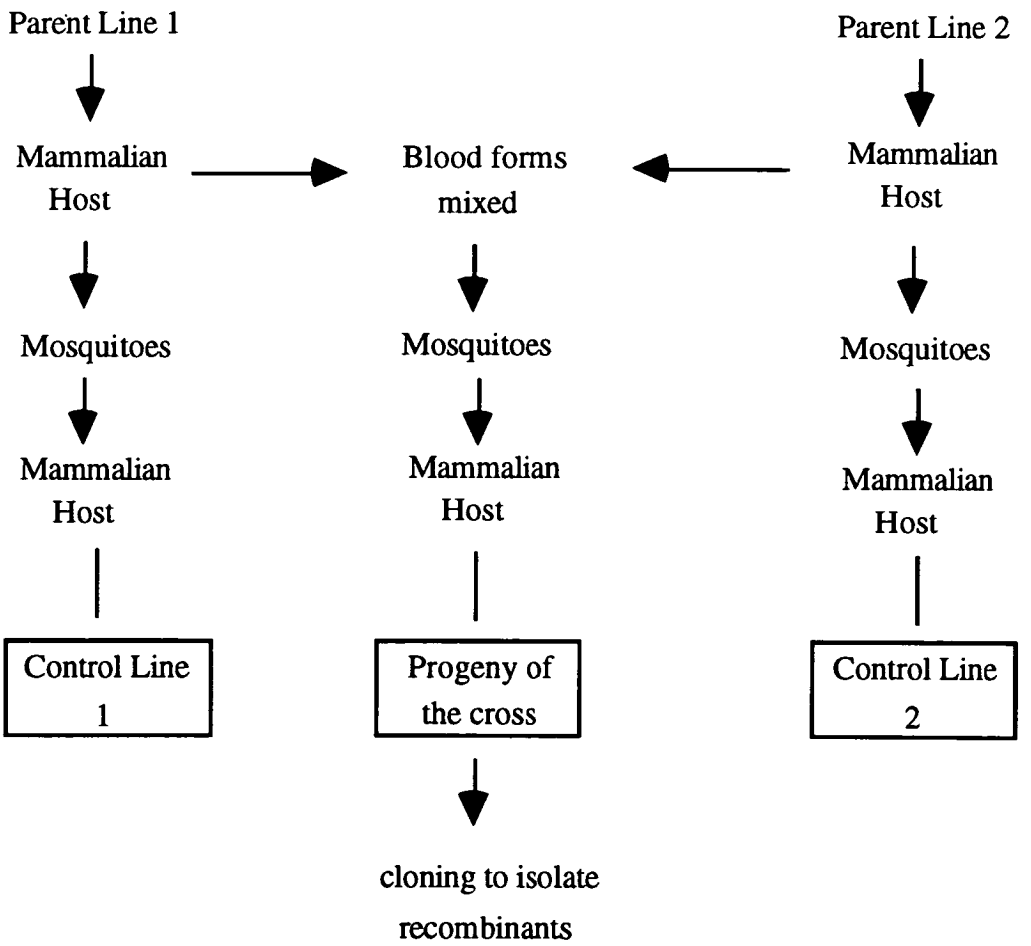
1.4.5 Genetic crossing experiments

(i) Methods used, and evidence of recombination

Crosses between malaria parasites can be carried out under laboratory conditions. An outline of the method used is shown in **Figure 2**. Mosquitoes are allowed to feed

on a mixture of gametocytes of two cloned parasite lines differing in a number of genetic markers. This enables cross-fertilisation to occur between gametes of each clone in the mosquito stomach, resulting in the production of hybrid zygotes. Self-fertilisation events are also expected between gametes of the same clone, resulting in production of parental-type zygotes. Assuming that each clone produces equal numbers of gametes, and that fertilisation is random, equal numbers of hybrid (heterozygous) and parental-type (homozygous) zygotes should be produced. The zygotes immediately undergo meiosis, all the haploid products of each zygote being retained in each resulting oocyst. Evidence that random mating does occur has been obtained by Ranford-Cartwright *et al.* (1993), who showed that the numbers of homozygous and heterozygous oocysts obtained in crosses between *P. falciparum* clones were in accordance with Hardy-Weinberg expectations (discussed in further detail in **Section iv**).

Figure 2. The procedure used in making a cross between mammalian malaria parasites.



Important genetic mechanisms occur during meiosis of the zygotes. Each chromatid of a pair of homologous chromosomes segregates at random into the progeny. Such independent assortment of chromosomes is one way in which recombinant parasites can be produced. Furthermore, crossing-over events may occur at this stage between non-sister chromatids of a pair of homologous chromosomes. Crossing-over between linked genes (i.e. genes found on the same chromosome) which usually segregate together is thus a second mechanism by which recombinant parasites are produced. Hence the term 'recombinant' is used here to refer to a parasite with novel combinations of parent genes, produced either through independent assortment of unlinked genes, or through crossing-over between linked genes.

Recovery of the products of the cross is achieved by allowing the parasites to develop into sporozoites, which are then used to infect a new host. The resultant blood forms can be cloned, and examined for the inheritance of the original polymorphic markers.

Genetic crosses have been made between malaria parasites that infect birds, rodents and humans. Greenberg and Trembley (1954b) were the first to attempt to cross lines of *Plasmodium*, using two lines of *P. gallinaceum* differing in their response to pyrimethamine and in their ability to produce exoerythrocytic schizonts (described in Walliker, 1983). Initially they were disappointed as no evidence of recombination was found, but later experiments reported success (Greenberg and Trembley, 1954a). However, one of the genetic markers, the development of exoerythrocytic schizonts, was known to be unstable.

In the early 1970s, Walliker *et al.* (1971; 1973) demonstrated conclusively genetic recombination in rodent malaria parasites by crossing two lines of *P. yoelii* which differed in their response to treatment with the antimalarial drug pyrimethamine, and in the forms of the isoenzyme glucose phosphate isomerase (GPI). The segregation and re-assortment of the isoenzyme forms and drug susceptibilities were in agreement with typical Mendelian inheritance of unlinked markers.

The first cross between two clones of *P. chabaudi* was made in 1975 (Walliker *et al.*, 1975). Both clones differed by the forms of two isoenzyme markers, 6-phosphogluconate dehydrogenase (6PGD) and lactate dehydogenase (LDH), and in their susceptibility to pyrimethamine. Analysis of this cross enabled recombination between two enzyme markers to be demonstrated for the first time, and showed that the drug resistance character segregated independently of either enzyme marker. Moreover, the experiments showed that recombination and segregation of the parental enzyme forms had occurred before the emergence of parasites into the blood, proving that the blood forms were haploid.

(ii) Recombination due to chromosome re-assortment

Although the first genetic crosses detected recombinant parasite forms, they did not distinguish between recombinant parasites formed due to chromosome re-assortment and those formed due to crossing-over events; this was because the chromosomal locations of the genes studied were not known. Sharkey *et al.* (1988) were able to demonstrate independent assortment of chromosomes in the recombinant progeny of a cross between *P. chabaudi* clones AS and CB. These clones show polymorphism in the size of their chromosomes; in clone AS, chromosome 4 is larger than in clone CB, while chromosome 5 is smaller. Of six recombinant clones analysed by PFGE, three possessed karyotypes identical to parental clone AS, two possessed karyotypes identical to parental clone CB, and one had chromosome 4 characteristic of clone CB and chromosome 5 characteristic of clone AS.

(iii) Recombination due to crossing-over events

Crossing-over between homologous chromosomes has been demonstrated by analysis of the progeny of two *P. falciparum* crosses. These are the only crosses to have been made using human malaria parasites. Walliker *et al.* (1987) performed the first crossing experiment, using the *P. falciparum* clones HB3 (from Honduras) and 3D7 (probably from Africa, see Collins *et al.*, 1986). Crossing-over between homologous chromosomes was shown to account for the appearance of chromosomes among the progeny which were of a different size from those of the parents (Sinnis and Wellems, 1988). For example, chromosome 4 was 1280 kb in clone HB3 and 1490 kb in clone 3D7, but one progeny clone XP5 possessed a 1400 kb chromosome 4. Long-range restriction mapping showed that the XP5 chromosome 4 contained restriction sites characteristic of one end of the HB3 chromosome and of one end of the 3D7 chromosome. It was concluded that a crossing-over event must have occurred in the central region of the parent chromosomes, and most probably during meiosis of a HB3/3D7 heterozygote (Sinnis and Wellems, 1988).

The presence of 'hot-spots' of recombination in the chromosomes of malaria parasites was first proposed by Vernick *et al.*(1988), who investigated the inheritance of a telomeric sequence among the progeny of the HB3/3D7 cross. The sequence was radiolabelled and hybridised to Southern blots of restricted DNA of the progeny clones. Novel non-parental sized fragments were shown to hybridise to the sequence, some of which were found to be located at internal chromosome sites, as well as at subtelomeric locations. It was proposed that such sites were genetically unstable at meiosis, and could represent 'hot-spots' of recombination.

Wellems *et al.* (1990) performed a second *P. falciparum* cross using the clones HB3 and Dd2 (from Indochina). Through analysis of the inheritance of more than 80 RFLP markers among 16 recombinant progeny clones, it was possible to show that each progeny clone possessed at least one chromosome which was the result of a crossing-over event between linked loci (Walker-Jonah *et al.*, 1992).

Crossing-over within a single locus has also recently been shown to occur by Kerr *et al.* (1994). Allele-specific PCR (polymerase chain reaction) primers were used to demonstrate the presence of a novel form of the merozoite surface protein (MSP-1) gene, within the uncloned progeny of the *P. falciparum* HB3/3D7 cross. The area in which the recombination event had occurred between the two parental clones was narrowed down to a 250 bp section of this gene.

(iv) Frequency of recombination

Genetic crossing work using *P. falciparum* has also enabled studies on the frequency of recombinant forms among progeny of crosses to be undertaken. Some of the crossing experiments outlined above produced recombinant progeny at a greater frequency than expected. 50% of the progeny of a cross are expected to derive from self-fertilisation events. However, in the HB3/3D7 *P. falciparum* cross, only 3 of 22 progeny clones tested for 10 markers had the parental-type characteristics (Walliker *et al.*, 1987), and in the HB3/Dd2 *P. falcipaum* cross, none of the 76 progeny clones tested for the inheritance of 30 RFLP markers exhibited parental combinations of markers (Wellems *et al.*, 1990).

Work by Ranford-Cartwright *et al.* (1993) has involved making crosses between *P. falciparum* clones HB3 and 3D7, and typing individual oocysts for homo- or heterozygosity of two polymorphic genes. The results showed no evidence for cross-fertilisation being favoured over self-fertilisation in these laboratory crosses. Moreover, the work produced direct evidence of cross-fertilisation between genetically distinct parasites in the mosquito vector. The high numbers of recombinants found in the two *P. falciparum* crosses are therefore thought to be due to selection within the vertebrate host or during *in vitro* culturing of asexual blood forms.

Similar findings on typing *P. chabaudi* oocysts in laboratory crosses have been obtained by L. Groves (Division of Biological Sciences, Edinburgh University; personal communication).

1.5 Drug resistance

Drug resistance is a common problem associated with infectious organisms. Beale (1980) has proposed a number of different mechanisms, genetic and non-genetic,

which could account for changes in the ability of such organisms to grow in the presence of drugs. The non-genetic mechanisms included (a) physiological adaptations of the organism to particular drugs; and (b) expression of latent chromosomal genes caused by alterations in environmental factors. The genetic mechanisms included (a) spontaneous mutation of nuclear or extra-nuclear genes and their selection under the influence of drug pressure; (b) gene mutation induced by mutagenic drugs; and (c) resistance associated with extrachromosomal factors such as plasmids, viruses or transposons.

Many of these mechanisms have been shown to operate in bacteria (for a review see Neu, 1992) and some parasitic protozoa (reviewed in Saklatvala, 1993). The most important as regards drug resistance in *Plasmodium* is probably spontaneous gene mutation and the selection of mutants from mixed parasite populations. Physiological adaptation has been shown to occur in some laboratory selected lines (see **Section 1.6.4**), but these are less important because of their temporary and unstable nature. The involvement of extrachromosomal elements, such as plasmids, in transfer of resistance has never been demonstrated.

1.5.1 Antimalarial drugs and the spread of drug resistance

There are 10 main groups of antimalarial drugs in use (**Table 1**). Examples of each group are given under their international non-proprietary names. All of these drugs are primarily active against the blood forms of the parasite. Primaquine and other 8-aminoquinolines are the only drugs effective against exo-erythrocytic stages.

The first cases of drug resistance shown by *P. falciparum* parasites were reported as early as 1910 against the antimalarial quinine (noted by Foote and Cowman, 1994), but it was not until the end of the 1950s that drug resistance was found to be a major problem. This necessitated a precise definition of drug resistance, in order that monitoring the spread of resistance could be standardised. The World Health Organisation (W.H.O.) defines drug resistance as "the ability of a parasite strain to multiply or to survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication" (W.H.O., 1963). This is not to be confused with an absence or inadequacy of drug action, which is caused by host factors such as impaired drug absorption, and which may complicate the recognition of resistance.

There have been several reported incidents of failed chemotherapy in patients with *P. ovale*, *P. malariae* and *P. vivax* infections, discussed by Peters (1987). Reports of chloroquine-resistant *P. vivax* in Papua New Guinea and the Solomon Islands by Rieckmann *et al.* (1989) and Whitby *et al.* (1989) respectively, as cited by

Table 1. Ten main groups of antimalarial drug in use.

	Generic Chemical Group	Example
1	antifolic drugs	pyrimethamine, proguanil
2	sulpha drugs	a) sulphones e.g. dapsone b) sulphonamides e.g. sulphadoxine
3	cinchona alkaloids	quinine
4	4-aminoquinolines	chloroquine, amodiaquine
5	8-aminoquinolines	primaquine
6	4-quinoline methanols	mefloquine
7	9-phenanthrene methanols	halofantrine
8	sesquiterpene lactones	a) artemisinin b) artemisinin derivatives e.g. artemether
9	antibiotics	tetracycline, chloramphenicol
10	drug combinations	pyrimethamine + sulphadoxine ('Fansidar') pyrimethamine + sulphadoxine + mefloquine ('Fansimef')

Wernsdorfer (1994), and latterly in Irian Jaya (Baird *et al.*, 1991) are disconcerting, but so far they appear to be confined to Western Indonesia. However, it is drug resistance in *P. falciparum* which is of much greater importance, because of its higher rate of incidence and the significant mortality associated with cerebral malaria caused by this species.

P. falciparum isolates resistant to almost every antimalarial drug and combination of drugs are now known to exist (Peters, 1987). Parasites exhibiting resistance to more than one drug are proving a serious problem in some areas, particularly the Thai/Cambodia and Thai/Myanmar borders (Thaithong and Beale, 1992). However, such multidrug resistant parasites have not become as prevalent as was once expected (Peters, 1987). Cross-resistance to different drugs may be due to the shared chemical structure of the compounds. Thus resistance to chloroquine has been noted to produce reduced sensitivity to amodiaquine, which is in the same chemical class (Wernsdorfer, 1994). Other examples of cross-resistance between less closely related drugs include mefloquine resistance which has been associated with reduced susceptibility to halofantrine (Basco and Le Bras, 1992; Rojas-Rivero *et al.*, 1992), and quinine resistance which has been correlated with resistance to mefloquine (Suebsaeng *et al.*, 1986; Brasseur *et al.*, 1991; Brasseur *et al.*, 1992a; Brasseur *et al.*, 1992b).

Outbreaks of drug resistance in the field are identified by failed programmes of chemotherapy *in vivo*. The levels of resistance are classified as three types according to the initial clearance of parasites from the blood and their later recrudescence (W.H.O., 1973):-

RI- after initial clearance, parasites reappear after seven or more days despite continuing treatment.

RII- the number of parasites is reduced initially, but rises again after seven or more days.

RIII- the drug has little effect on parasite numbers.

Resistance can be monitored *in vitro* also, through the examination of cultures exposed to serial drug dilutions over a set time period, for example, the W.H.O microtest (W.H.O., 1979). Drug susceptibilities can be presented in a number of ways, the most common being: (a) MIC values: minimum inhibitory concentration of the drug at which all, or almost all, parasites are killed; or (b) $IC_{50/90/99}$ values: the drug concentration at which 50/90/99% of the parasites die, obtained through monitoring the drug-induced inhibition of [^{3}H]-hypoxanthine uptake by the parasite (Desjardins *et al.*, 1979).

The relationship between *in vitro* and *in vivo* drug tests is not straightforward because of the interaction between host immunity, host nutrition, drug metabolism and parasite development. These factors may contrive to produce a 'resistant' outcome from an *in vitro* test which does not match the clinical result. This is especially relevant because it is known that natural *P. falciparum* populations frequently contain mixtures of parasites (Thaithong *et al.*, 1984), some of which may not survive the culture conditions.

1.5.2 Genetic mechanisms of drug resistance

Understanding the genetic mechanisms of antimalarial drug resistance is important for two reasons: (1) in order to design new drugs which target different parasite molecules; and (2) to enable predictions to be made on the efficacy of a particular drug regime within a parasite population. Unfortunately, the identity of parasite genes involved in the majority of types of drug resistance remain unknown. Indeed the mode of action, which might give an indication of the basis of resistance, is not understood for many of the drugs.

However, the mode of action of, and mechanism of resistance to, the antifolate drugs is better understood. In particular, the mechanism of resistance to pyrimethamine has been successfully studied, and this is discussed in the following section (reviewed by Hyde, 1990a). It is of direct relevance to understanding the

genetic mechanisms underlying chloroquine resistance, because of the contrasting manner in which resistance to the two drugs originated and spread.

1.5.3 Pyrimethamine resistance

Malaria parasites are unable to scavenge pyrimidines and rely almost exclusively on *de novo* synthesis through the folic acid pathway (Ferone, 1977). Part of this pathway involves the conversion of dihydrofolate to tetrahydrofolate by the enzyme dihydrofolate reductase (DHFR), which is one component of a bifunctional enzyme molecule including thymidylate synthetase (TS). The antifolate drug pyrimethamine binds to the parasite enzyme several hundred times more tightly than to the equivalent enzyme in the mammalian host (Ferone *et al.*, 1969). This disrupts folate metabolism, which prevents pyrimidine synthesis and ultimately stops the production of DNA. Thus pyrimethamine is most effective at the erythocytic stage of schizont formation, although it is slow acting and therefore mostly used for prophylaxis.

Pyrimethamine was introduced as an antimalarial agent in the field in 1952. The first indication that *P. falciparum* parasites were becoming pyrimethamine resistant came shortly after, in a report by McGregor and Smith (1952), and subsequently by Jones (1954; 1958; cited by Peters, 1987). Many other reports of pyrimethamine resistance followed, and these have also been summarised by Peters (1987). Typically, resistant isolates tested *in vitro* are able to grow in concentrations of pyrimethamine hundreds of times greater than those which can be tolerated by sensitive parasites.

The speed by which resistance to pyrimethamine arose suggested that a single mutation at a single locus might be sufficient to produce resistance. Experimental evidence for this came with the selection of pyrimethamine-resistant laboratory lines of *P. gallinaceum* (Bishop, 1962) and *P. berghei* (Diggens, 1970), as described by Foote and Cowman (1994). Bishop (1962) found that the rate of development of resistance seemed to be independent of the size of the drug dose used to induce it, and Diggens (1970) was able to select a resistant line using a single-step, high dose method. Other workers too were able to produce pyrimethamine-resistant rodent malaria lines using a single, high dose of drug (Morgan, 1972; Walliker *et al.*, 1973; 1975).

However, it was not until evidence from genetic crossing studies became available that the resistance character was shown to be inherited in the manner expected for a genetic mutation (Walliker *et al.*, 1973). Similar conclusions were drawn from the results of further crosses (Walliker *et al.*, 1975; Rosario, 1976b; Knowles *et al.*, 1981; Sharkey *et al.*, 1988). Subsequently, the *P. falciparum* HB3/3D7 cross first identified

the DHFR gene as being involved, or closely linked to a gene involved, in pyrimethamine resistance (Peterson *et al.*, 1988). All the pyrimethamine-resistant progeny from this cross possessed an RFLP associated with the resistant parent DHFR gene, while all the sensitive progeny possessed an RFLP associated with the sensitive parent gene.

Further evidence that the DHFR gene determined the response of the parasite to pyrimethamine came from sequencing studies on the gene from the 3D7 and HB3 clones. A single amino acid difference at position 108, which was serine in clone 3D7 and asparagine in clone HB3, was found to be the only difference in the amino acid sequence of the gene of each clone (Peterson *et al.*, 1988). This amino acid change is thought to be within the cleft for substrate binding, and so may affect the binding of pyrimethamine to the enzyme (see Hyde (1989) for molecular graphic images of the structure of the *Lactobacillus casei* DHFR enzyme used as a model for the *P. falciparum* molecule). Sequencing of the gene from different isolates has shown that, with few exceptions, pyrimethamine-sensitive parasites possess serine or threonine at position 108, while resistant parasites possess asparagine at this site (Cowman *et al.*, 1988; Peterson *et al.*, 1988; Basco *et al.*, 1995).

Mutations at other sites in the DHFR gene may also be involved in resistance to pyrimethamine (Cowman *et al.*, 1988; Snewin *et al.*, 1989; Zolg *et al.*, 1989; Basco *et al.*, 1995). For example, a mutation at position 59 has been found to affect the affinity of the DHFR enzyme for pyrimethamine (Sirawaraporn *et al.*, 1990) and this may be necessary for high-level pyrimethamine resistance.

Other causes of resistance to pyrimethamine cannot be ruled out. Thaithong *et al.* (1992) have reported selecting a pyrimethamine-resistant *P. falciparum* clone, which showed no mutation of the DHFR gene. They suggest that the resistance may be caused by a change in the promoter region of the gene which causes its over-expression. Additional mechanisms involving mutations in genes other than DHFR could also play a rôle in resistance, for example, by decreased uptake of the drug into the parasite, or conversion of pyrimethamine into an inactive form.

Of particular interest to this project is the selection of pyrimethamine-resistant mutants from rodent malaria parasites, and their mechanisms of resistance. Pyrimethamine-resistant mutants from all the rodent malaria species have now been selected; from *P. yoelii* (Diggens, 1970; Morgan, 1972; Walliker *et al.*, 1973), *P. chabaudi* (Walliker *et al.*, 1975; MacLeod, 1977; Cowman and Lew, 1989), *P. berghei* (Rollo, 1952; van Dijk *et al.*, 1994) and *P. vinckei* (Yoeli *et al.*, 1969). Most were selected using the single-step method, although a few were selected using a continuous low-dose of drug.

Molecular studies of several of these mutants have shown the mechanisms of resistance to be remarkably similar to those seen in pyrimethamine-resistant *P. falciparum*. Cheng and Saul (1994) sequenced the DHFR gene from two pyrimethamine-resistant clones derived from *P. chabaudi* (line AS) and *P. yoelii* (line 17X), and showed the only difference between the resistant and sensitive clones to be a point mutation which causes an amino acid change of serine to asparagine at position 106, thought to be equivalent to the serine to asparagine change at position 108 in *P. falciparum*. Also, duplication of the DHFR gene has been found to be associated with changes in drug susceptibility. Cowman and Lew (1989) selected a pyrimethamine-resistant clone from *P. chabaudi* (line DS) using a continuous low-pressure method, and found it to have doubled the copy number of the DHFR gene by a partial chromosome duplication. Similar results have been reported for a low-level resistant line selected from *P. berghei* (line ANKA) (van Dijk *et al.*, 1994). Further drug selection on the *P. chabaudi* DS clone resulted in a point mutation in one copy of the gene and subsequent loss of the second copy (Cowman and Lew, 1990).

These results illustrate a possible tendency for pyrimethamine resistance mechanisms to be linked to differences in the method of selection of resistant parasites. Treatment of parasites with low doses of pyrimethamine may select for parasites with increased expression of the gene, whereas selection with high amounts of pyrimethamine may select for parasites with functional mutations in the DHFR gene (Cowman and Foote, 1990). This generalisation may also be important when considering mechanisms of resistance to other antimalarial drugs such as chloroquine.

Mutations at other sites in the DHFR gene of *P. falciparum* have been associated with resistance to a second antifolate drug, proguanil (Foote *et al.*, 1990a; Peterson *et al.*, 1990, 1991; Basco *et al.*, 1995). Crossing studies to confirm the rôle of these mutations in proguanil resistance have not be done.

1.6 Chloroquine resistance

Chloroquine is a derivative of quinine, one of the four alkaloids extracted from the bark of the *Cinchona* tree, and it contains the quinoline ring characteristic of all quinine derivatives. It is one of the cheapest and most widely used antimalarial drugs. Developed in Germany during the 1930s, it was brought into widespread use in the field by the Americans during the following decade. Its popularity is due to several characteristics: it has a low incidence of severe side-effects, it is cheap and chemically stable, it is administered on a weekly basis, and it has a rapid onset of action which makes it effective against cerebral malaria. Resistance to chloroquine however has

now been reported in 73 of the 95 countries afflicted by *P. falciparum* (Wernsdorfer, 1991), exceptions being Central America and Haiti.

1.6.1 Origin and spread of chloroquine resistance

Chloroquine-resistant *P. falciparum* was initially reported in two geographically distant foci, one in Colombia (Moore and Lanier, 1961; Young and Moore, 1961) and the other in Thailand (Harinasuta *et al.*, 1962). Since then, many cases have been documented, as described by Peters (1987). Chloroquine resistance first appeared in Africa in the late 1970s (Campbell *et al.*, 1979; Fogh *et al.*, 1979), and has now spread to all regions where malaria is endemic. Typically, resistant isolates tested *in vitro* are able to grow in concentrations of chloroquine 5-10 times greater than can be tolerated by sensitive parasites.

Resistant parasites are believed to be selected through drug treatment programmes which are only partially effective and do not eliminate all the parasites of an infection (reviewed by Wernsdorfer, 1994). A new population of parasites which is less sensitive compared to the population prior to drug exposure, is selected. Further selection takes place upon renewed drug pressure, resulting in enhanced resistance. These situations are known to occur frequently with mass drug administration, especially if sub-therapeutic doses are used. Such administration was previously carried out by the use of medicated salt, as part of malaria eradication programmes in South East Asia, Africa and South America (for a review see Payne, 1988). Thus, it is not surprising that the first cases of chloroquine-resistant *P. falciparum* infections originated from areas where chloroquinized salt was used (Payne, 1988). Whereas pyrimethamine-medicated salt regimes produced almost instantaneous resistance and were withdrawn immediately, chloroquine-medicated salt was used for longer because of the slow spread of resistance, which was exacerbated by clandestine supplies of non-medicated salt (Wernsdorfer, 1994).

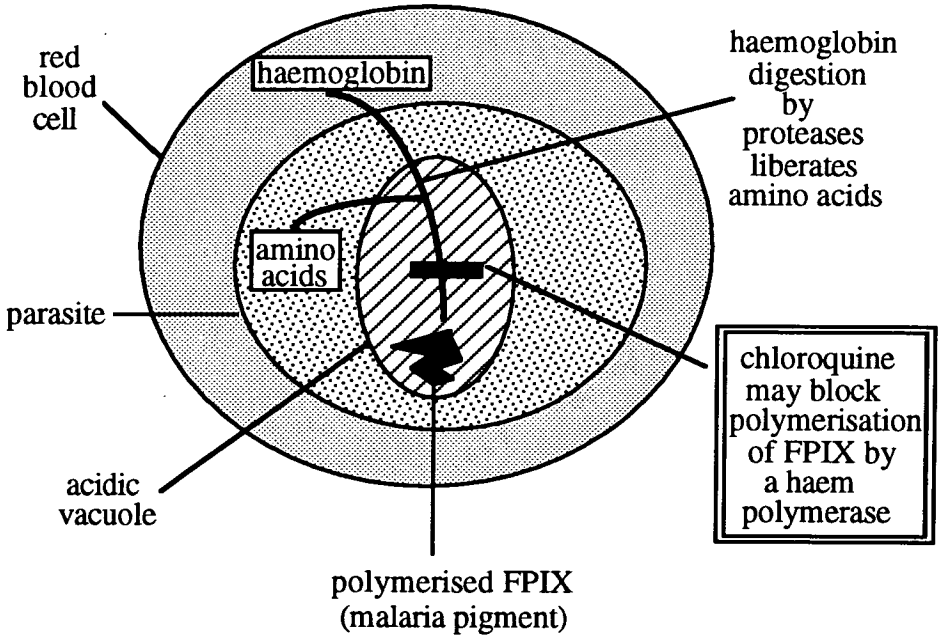
The slow spread of chloroquine resistance contrasts sharply with the spread of pyrimethamine-resistant *P. falciparum.* The difference would seem to indicate that, whereas a single defective gene appears to be sufficient to produce pyrimethamine resistance, several mutations at several loci might be necessary before the malaria parasite is resistant to chloroquine.

1.6.2 Mode of action of chloroquine

The mode of action of chloroquine, and other quinoline-ring antimalarial drugs such as quinine, is not fully understood. They are effective only against the intraerythrocytic stages of pigment-producing malaria parasites (for recent studies on the stage-

specificity of chloroquine, see Kuile *et al.*, 1994). A brief explanation of pigment-formation is as follows: in order to meet their nutritional requirements for essential amino acids, malaria parasites ingest and degrade host erythrocyte haemoglobin (reviewed in Sherman, 1979; Slater, 1992). This occurs in the food vacuole, alternatively known as the digestive vacuole, acidic vesicle or lysosome (**Figure 3**), which is present during all intraerythrocytic stages of the parasite life-cycle.

Figure 3. Schematic diagram of haemoglobin digestion in the malaria parasite. The black bar represents the possible disruption of FPIX polymerisation by chloroquine, as discussed later.



During breakdown of haemoglobin, large amounts of a haem moiety ferriprotoporophyrin IX (FPIX) are released, which is toxic and able to lyse the parasite cell and affect the function of lysosomal enzymes. The parasite overcomes this potential hazard by sequestering FPIX within the food vacuole as malaria pigment or haemozoin. Chemical studies have revealed haemozoin to be a polymer of haem units held together by iron-carboxylate bonds (Slater *et al.*, 1991).

Historically, three mechanisms of chloroquine action have been proposed:-

a) Chloroquine raises the vacuolar pH above that required for the function of parasite enzymes involved in haemoglobin digestion- the 'Lysosomotropic Hypothesis'

Chloroquine is a lysosomotropic agent, i.e. a weak base which concentrates in the acidic vesicles of both host and parasite cells (Aikawa, 1972; Yayon *et al.*, 1984). It crosses the vesicle membrane as a free base and is rapidly di-protonated, becoming

impermeable to passage out of the vacuole. The accumulation of chloroquine is dependent upon the pH gradient between the acid vesicle and the extracellular medium (Aikawa, 1972; Yayon *et al.*, 1984). Thus at pharmacological concentrations of chloroquine (10^{-9}M), drug concentrations in the food vacuole can reach millimolar levels at physiological pH (Geary *et al.*, 1986). The lysosomotropic theory of chloroquine action proposed that the accumulation of chloroquine raises the pH of the food vacuole above that required for the function of parasite enzymes involved in haemoglobin digestion, resulting in parasite death (Homewood *et al.* (1972), as reported by Krogstad *et al.* (1985) and Ginsburg (1990)).

Subsequently, it was shown that the pH is only altered slightly at pharmacological concentrations of chloroquine (Ginsburg *et al.*, 1989), so that alkalinisation of the food vacuole is insufficient to explain the mode of chloroquine action. The temporary alkalinisation of the vesicles is probably counteracted by the vacuolar proton pump which rapidly restores the pH and allows more chloroquine to accumulate (reviewed by Ginsburg and Krugliak, 1992). Moreover, it has been shown that chloroquine concentrates up to 800-fold more in parasite vesicles than is predicted from its properties as a weak base (Krogstad and Schlesinger, 1987), suggesting that the parasite vacuole has high-affinity chloroquine-binding sites which are not present in mammalian cells, and which account for the specificity of the drug (for a review, see Krogstad *et al.* (1992b)).

b) Chloroquine binds to FPIX, forming a complex which is highly toxic to the parasite

Chloroquine has been shown to bind to FPIX (Chou and Fitch, 1980a), preventing its polymerisation into haemozoin and leading to the formation of chloroquine-FPIX complexes which are highly toxic to the parasite (Chou and Fitch, 1980b and 1981; as reviewed by Foote and Cowman, 1994). However, evidence against FPIX being the receptor for chloroquine comes from the lack of correlation between the affinities of various antimalarial drugs for FPIX and their rank order of activities (Warhurst, 1987).

c) Chloroquine binds to DNA preventing DNA synthesis

Chloroquine inhibits DNA synthesis in bacteria, viruses and mammalian cells at concentrations of 1-2mM (Parker and Irvin (1952) as reviewed by Peters (1987)). It has been proposed that chloroquine might manifest its antimalarial activity by preventing DNA synthesis in *Plasmodium*. Some evidence came from studies showing inhibition of DNA and RNA synthesis in *P. knowlesi in vitro* (Gutteridge *et al.*, 1972). However, the theory fails to account for the fact that serum chloroquine

concentrations reach a peak of only 1-2μM *in vivo* (Peters, 1987). Recent work has shown that chloroquine binds more avidly to specific regions of DNA and inhibits the transformation of B-form to Z-form DNA at concentrations as low as 20μM (Kwakye-Berko and Meshnick, 1990). Although it is not clear whether parasite DNA is exposed to similar concentrations *in situ*, DNA intercalation could play some part in the antimalarial activity of chloroquine (for a review see Meshnick, 1990).

A fourth possible mechanism for the mode of chloroquine action has been proposed recently:-

(d) Chloroquine prevents the polymerisation of haem

A novel haem polymerase activity has been shown to be present in malaria parasites, which is capable of polymerising haem *in vitro*, and that is inhibited by chloroquine at pharmacological concentrations (Slater and Cerami, 1992) (**Figure 3**). This inhibition could result in the inability of the parasite to detoxify haem and render it susceptible to the lytic effects of FPIX. Further evidence that this haem polymerase may be the target of chloroquine includes the estimated intravesicular chloroquine concentration in the parasite (Krogstad and Schlesinger, 1987) which is great enough to inhibit haem polymerase activity *in vitro* (Slater and Cerami, 1992). Haem polymerase isolated from several chloroquine-resistant isolates was found to retain full sensitivity to chloroquine (Slater, 1992). This agrees with the widely held view that the mechanism of chloroquine resistance is distinct from the mode of action of chloroquine, suggested because chloroquine-sensitive parasites accumulate greater levels of the drug than do chloroquine-resistant parasites (Fitch, 1970; Verdier *et al.*, 1985; Yayon *et al.*, 1985). A haem polymerase activity has also been extracted from chloroquine-sensitive *P. berghei* clone NYU-2, and shown to be down-regulated in the presence of chloroquine (Chou and Fitch, 1992)

Dorn *et al.* (1995) have recently produced evidence that haem polymerisation is not enzyme-mediated but rather a chemical process dependent only upon the presence of haem-derived material associated with haemozoin, and not on the presence of protein. The authors state that this does not invalidate haem polymerisation as a target for the action of chloroquine; however, if a protein activity does exist, they suggest that it will have a structural function, for example as a scaffold for the initiation of polymerisation, rather than a haem polymerase function.

Further *in situ* studies are required to isolate the target of chloroquine. It may be that all of the mechanisms proposed above could play minor rôles in chloroquine action.

1.6.3 Mechanisms of chloroquine resistance

As mentioned above, the only feature which distinguishes chloroquine-resistant parasites from sensitive parasites is that the former accumulate less chloroquine compared with the latter (Fitch, 1970; Verdier *et al.*, 1985; Yayon *et al.*, 1985). Such reduced accumulation could be caused by (a) reduced uptake of the drug in resistant parasites; (b) enhanced efflux of the drug from resistant parasites; or (c) a combination of reduced uptake and enhanced efflux in resistant parasites.

There is much debate regarding the relative merits of reduced uptake/enhanced efflux. Some of the experimental results from different laboratories are in direct conflict with each other. For example, the uptake of chloroquine into acid vacuoles has been reported to be similar between chloroquine-resistant and chloroquine-sensitive parasites (Krogstad *et al.*, 1987; Krogstad *et al.*, 1992a; Bayoumi *et al.*, 1994), but mathematical models (Geary *et al.*, 1990; Ginsburg and Stein, 1991) and some experimental evidence (Bray *et al.*, 1992a) from other laboratories suggest that resistant parasites accumulate reduced levels of chloroquine compared with sensitive parasites. The reasons for these discrepancies are three-fold. Firstly, experimental techniques differ between laboratories. Secondly, no chloroquine-resistant *P. falciparum* clones have been obtained which are isogenic with chloroquine-sensitive clones; analysis of the resistant phenotype has thus been carried out on different genetic backgrounds which may influence the expression of the phenotype. Finally, it remains a matter of debate as to whether resistance is a simple trait due to mutations in a few genes, or a complex trait due to many mutant genes.

Several theories of chloroquine resistance have been suggested as follows:-

(i) The MDR theory of chloroquine resistance

A rapid efflux phenotype in chloroquine-resistant parasites was first demonstrated by Krogstad *et al.* (1987), who showed that although the initial rate of chloroquine uptake in resistant and sensitive parasites was the same, resistant parasites released chloroquine 40-50 times more rapidly than susceptible parasites, and that this was responsible for the lower steady-state accumulation levels and hence resistance to chloroquine. This process was found to be dependent upon ATP (adenosinetriphosphate) (Krogstad *et al.*, 1992a). Subsequently, the resistant phenotype was found to be reversed by a calcium channel blocker, verapamil (Martin *et al.*, 1987), by tricyclic antidepressants (Bitonti *et al.*, 1988) and by antihistamines (Peters *et al.*, 1989). Resistance to other quinoline-containing antimalarials was also found to be modulated by calcium antagonists (Kyle *et al.*, 1990). This led to the

theory that chloroquine resistance might be similar to the multidrug resistance (MDR) phenotype seen in mammalian tumour cells (review by Endicott and Ling, 1989).

In mammalian tumour cells, the MDR phenotype is characterised by the cells pumping out drugs which may be structurally unrelated. This process reduces their intracellular concentration, thereby rendering the cells resistant to the effects of the drugs. The molecule which pumps out the drugs is an ATP-dependent transporter, the P-glycoprotein (P-gp), which is membrane-associated and has affinity for a wide range of drugs. In humans, P-gp is coded for by the MDR gene, *MDR1*, which is excessively amplified in drug-resistant cell lines, thereby resulting in increased production of the protein. The MDR phenotype can be reversed by calcium channel blockers such as verapamil. Thus, chloroquine resistance in *P. falciparum* appeared to exhibit similarities with the MDR phenotype of mammalian cancer cells.

Genes encoding P-glycoproteins have been found in a variety of organisms, although not all are involved in drug resistance (see review by Ouellette *et al.*, 1994). They make up a large group within an even larger family of proteins called ATP-binding cassette (ABC) transporters (reviewed in Higgins, 1992), so-called because of their characteristic ATP-binding domains. ABC transporters consist of two similar halves, each containing six putative transmembrane domains and one hydrophilic domain containing two short motifs associated with nucleotide binding (the 'Walker motifs' (Walker *et al.*, 1982), as described in Higgins (1992)). Most ABC proteins are known to be involved in the ATP-dependent transport of a variety of substrates. For example, the mammalian cystic fibrosis trans-membrane regulator (CFTR) is a chloride channel (Gregory *et al.*, 1990), and the *S. cerevisiae* ABC transporter STE6 is involved in transport of a pheromone (McGrath and Varhavsky, 1989), as cited by Ouellette *et al.* (1994)).

Two MDR genes have been cloned from *P. falciparum*, *pfmdr1* on chromosome 5, and *pfmdr2* on chromosome 14 (Foote *et al.*, 1989; Wilson *et al.*, 1989). The 160 kDa (kilo-Dalton) product of *pfmdr1*, Pgh1, is found on the surface of the lysosome (Cowman *et al.*, 1991), is predicted to have 12 transmembrane domains and two ATP binding sites (Foote *et al.*, 1989), and has been shown to bind nucleotides, indicative of the protein being involved in nucleotide-regulated transport (Karcz *et al.*, 1993a). Amplification of *pfmdr1* has been noted in some chloroquine-resistant isolates (Foote *et al.*, 1989; Wilson *et al.*, 1989), suggesting that overproduction of Pgh1 may enable the parasite to expel chloroquine. A survey of 26 chloroquine-resistant isolates showed 16 of them to contain amplified copies of *pfmdr1*, the majority of which were thought to have arisen as independent events (Triglia *et al.*, 1991). This result

suggested that the area of the genome containing the *pfmdr1* gene was under strong selective pressure.

The observation that both resistant and sensitive parasites have been found to contain equivalent numbers of copies of *pfmdr1* led Foote *et al.* (1989) to suggest that alleles of the gene, rather than gene amplification, might render the parasite competent for chloroquine resistance. Evidence for this theory came from studies showing that a point mutation in human P-gp altered the drug resistance pattern of tumour cells (Choi *et al.* (1988) as described by Karcz and Cowman (1991)). Foote *et al.* (1990b) claimed that two alleles were involved in chloroquine resistance, the so-called 'K1-type' of South East Asia and the '7G8-type' of South America. In their study, the chloroquine susceptibility of 34 out of 36 *P. falciparum* isolates exhibiting different chloroquine susceptibilities were predicted correctly from the presence of these alleles. More recent studies have found no correlation between these *pfmdr1* alleles and chloroquine resistance in field isolates from Africa (Awad-El-Kariem *et al.*, 1992), Thailand (Wilson *et al.*, 1993), and elsewhere (Haruki *et al.*, 1994).

Wellems *et al.* (1990) analysed 16 independent recombinant progeny from a cross between *P. falciparum* clones HB3 (chloroquine-sensitive) and Dd2 (chloroquine-resistant), for inheritance of the *pfmdr1* gene. Dd2 was known to contain 4 copies of the gene and HB3 to contain one copy. Examination of the progeny produced evidence for amplified copies in both chloroquine-sensitive progeny and chloroquine-resistant progeny. Interestingly, the levels of *pfmdr1* amplification varied among progeny inheriting the Dd2 gene; this was put down to the loss of amplified elements due to unequal crossing-over during meiosis. Analysis of the uncloned, chloroquine-treated progeny of the cross, which were expected to contain only chloroquine-resistant parasites, showed that an RFLP associated with the Dd2 gene did not segregate with the resistance phenotype. The authors concluded from this work that neither *pfmdr1* amplification nor inheritance of the Dd2 allele of *pfmdr1* correlated with chloroquine resistance (Wellems *et al.*, 1990). This work is described further in **Section iii**.

Thus the rôle of *pfmdr1* in chloroquine resistance is far from clear (reviewed by Ginsburg, 1991; Karcz and Cowman, 1991). Recent work suggests that *pfmdr1* may be involved in the uptake of chloroquine. Two theories have been proposed for the mechanism of chloroquine uptake by the food vacuole: (a) the weak base effect of chloroquine is sufficient for its concentration (a corollary of the lysosomotropic theory mentioned above); and (b) a permease must supplement the passive diffusion of chloroquine into the food vacuole (Warhurst, 1986), supplemented by evidence from studies by Krogstad and Schlesinger (1987) and Krogstad *et al.* (1992b). Initially it

was thought that Pgh1 might be such a permease. Transfection of Chinese hamster ovary (CHO) cells with wild-type *pfmdr1* produced cells expressing Pgh1 which showed increased chloroquine sensitivity due to elevated chloroquine uptake into the cells (van Es *et al.*, 1994a). Interestingly, expression of Foote *et al's.* (1990b) 7G8-type *pfmdr1* allele, which produced mutant Pgh1 protein in CHO cells, did not produce an increase in chloroquine sensitivity as chloroquine uptake remained the same. However, recent work suggests that Pgh1 does not directly transport chloroquine, but may influence chloroquine accumulation by modulating the pH of acidic organelles, perhaps by functioning as a chloride channel which regulates chloride permeability of the vacuolar membrane (van Es *et al.*, 1994b).

Finally, a correlation has been noted between over-expression of Pgh1 and mefloquine resistance in some *P. falciparum* laboratory clones. Chloroquine-resistant parasites selected under chloroquine pressure *in vitro* for increased resistance, were found to exhibit decreased mefloquine resistance and reduced *pfmdr1* amplification and Pgh1 expression (Barnes *et al.*, 1992; reviewed by Martin, 1993). Chloroquine-resistant clones selected for mefloquine resistance were found to have amplified and over-expressed *pfmdr1* (Wilson *et al.*, 1989; Cowman *et al.*, 1994; Peel *et al.*, 1994); these clones showed a decrease in their level of chloroquine resistance after selection (Cowman *et al.*, 1994; Peel *et al.*, 1994). Peel *et al.* (1994) have also shown that *P. falciparum* clones selected for different levels of mefloquine resistance contained equivalently amplified and over-expressed *pfmdr1* genes, suggesting that additional genetic changes may have occurred to produce different resistance levels.

However the correlation of over-expressed Pgh1 and mefloquine resistance is not clear cut. Analysis of the HB3 (mefloquine-sensitive)/Dd2 (mefloquine-resistant) *P. falciparum* cross showed no correlation between mefloquine-response and *pfmdr1* copy number among the 16 progeny clones (Wellems *et al.*, 1990). Moreover there are conflicting reports from the field. Whereas some mefloquine-resistant field isolates from Thailand have been reported to show amplification and over-expression of *pfmdr1* (Wilson *et al.*, 1993), *pfmdr1* copy number did not correlate with mefloquine resistance in 42 isolates from sub-Saharan Africa (Basco *et al.*, 1993). Interestingly, a correlation has been made between mefloquine resistance and decreased susceptibility to halofantrine and quinine, in parasites selected *in vitro* (Cowman *et al.*, 1994; Peel *et al.*, 1994) and in field isolates (Basco and Le Bras, 1992; Rojas-Rivero *et al.*, 1992; Wilson *et al.*, 1993).

A molecular model has been proposed for the part that Pgh1 might play in chloroquine resistance (Barnes *et al.*, 1992). Pgh1 is present in small amounts on the parasite plasma membrane (Cowman *et al.*, 1991). At low external concentrations of

chloroquine, over-expression of Pgh1 could result in a limited ability of the cell to remove chloroquine. At high external chloroquine concentrations, the surface Pgh1 may not be able to control the movement of chloroquine into the parasite. Chloroquine would accumulate in the food vacuole due to the increased presence of Pgh1 on this organelle, and chloroquine susceptibility would increase. Thus, over-expression of Pgh1 would be detrimental to the parasite, and strong drug pressure would select for deamplification of *pfmdr1*.

It is more difficult to devise models reconciling the inverse relationship between chloroquine and mefloquine resistance, and *pfmdr1* amplification. If Pgh1 transports mefloquine, its increased presence on the plasma membrane rather than the food vacuole membrane, could result in increased transport of mefloquine out of the parasite, producing mefloquine resistance (Cowman *et al.*, 1994). It is difficult to explain how this could increase susceptibility to chloroquine, unless Pgh1 were orientated towards mefloquine export and chloroquine import (Peel *et al.*, 1994). Alternatively, the inverse relationship could be explained if Pgh1 were the target of mefloquine action (Cowman *et al.*, 1994). Finally, Pgh1 could mediate ionic movements in and out of the parasite vacuole, which might influence mechanisms of resistance to a variety of different drugs (Martin, 1993). In this respect it is interesting to note that human P-gp encoded by *MDR1* is bifunctional and can act as a volume-regulated chloride ion channel in epithelial cells as well as a drug transporter, as noted by van Es *et al.* (1994b).

The second MDR gene cloned from *P. falciparum*, *pfmdr2*, does not appear to be involved in chloroquine resistance. The product of *pfmdr2*, Pgh-2, has one nucleotide binding domain and ten transmembrane domains, and shows significant homology to the *S. pombe* HMT1 protein, an ABC transporter molecule which is involved in heavy metal tolerance (Zalis *et al.*, 1993; Rubio and Cowman, 1994). It is localised to the plasma membrane of parasites and one theory suggests that it may be involved in metal homeostasis (Rubio and Cowman, 1994). Initial reports that *pfmdr2* transcripts were overexpressed in chloroquine-resistant isolates (Ekong *et al.*, 1993) have since been shown to be unfounded (Zalis *et al.*, 1993; Rubio and Cowman, 1994). Analysis of the progeny of Wellems *et al.*'s HB3/Dd2 cross also showed no segregation of an RFLP of an anonymous probe which maps near *pfmdr2*, with chloroquine susceptibility (Wellems *et al.*, 1990).

(ii) The impaired vacuolar acidification theory

Other work to identify possible chloroquine resistance gene(s) has centred on regulation of the pH of the acidic vacuole. The pH gradient across the membrane of

the vacuole is thought to provide the driving force for accumulation of chloroquine (Yayon *et al.*, 1985). The vacuolar pH is presumed to be maintained by a vacuolar-H^+ adenosinetriphospatase pump (V-ATPase or proton pump) located on the surface of the vacuole. A model to explain its function has been proposed (Ginsburg and Stein, 1991): as chloroquine accumulates in the vacuole, it titrates some of the protons present there, raising the internal pH which reduces further drug uptake. The V-ATPase continues pumping in H^+ ions, with the result that the vacuole pH becomes more acidic, and the uptake of chloroquine is resumed. This chloroquine titrates the recently entered proton(s), causing a rise in pH, and the cycle continues. Other molecules besides the proton pump are known to regulate proton gradients, such as the P-type ATP-ases.

(a) Vacuolar ATP-ase pump

It has been hypothesised that the lower levels of chloroquine observed in chloroquine-resistant parasites may be due to a weakened vacuolar proton pump, which causes higher intravacuolar pH levels (Geary *et al.*, 1990; Ginsburg and Stein, 1991). Evidence for this comes from Bray *et al.* (1992b), who have shown that a proton pump inhibitor reduces [^{3}H]chloroquine uptake to a greater extent in resistant parasites than sensitive parasites, suggesting that the former have a pump which is already weakened compared to the latter (reviewed by Bray and Ward, 1993). V-ATPases have been found to consist of two subunits, A and B. The genes coding for subunits A and B of the *P. falciparum* pump, *VAP A* and *VAP B*, have been cloned from *P. falciparum.* Sequence analysis from three resistant and two sensitive clones has shown no differences which are associated with chloroquine resistance (Karcz and Cowman, 1991; Karcz *et al.*, 1994). The authors suggest other explanations to account for elevated vacuolar pH in resistant parasites; for example there could be changes in the level of expression of vacuolar ATPase subunits which could limit the number of active pumps on the vacuole membrane. Alternatively, alterations in the counter ion permeability of the membrane could play a rôle in the regulation of vacuolar pH; this is discussed next.

(b) P-type ATPase pumps.

P-type ATPases are a ubiquitously distributed class of membrane proteins which contribute to electrochemical gradients by pumping cations, using energy derived from the hydrolysis of ATP (for a review see Krishna and Robson, 1991). The transporters can form proton gradients which affect parasite pH (Krishna and Ng, 1989), and hence are of interest because of the part they may play in regulating the accumulation

of chloroquine in the food vacuole. Several P-type ATPases have been cloned from species of *Plasmodium.* An organellar-type Ca^{2+}-ATPase gene has been cloned from *P. yoelii* (Murakami *et al.*, 1990) and its homologue from *P. falciparum* (Kimura *et al.*, 1993), both of which are thought to regulate the parasites' cytoplasmic concentration of Ca^{2+} in calciosomes and the endoplasmic reticulum.

Four other related genes have been cloned from *P. falciparum. PfATPases 1-3* (Krishna *et al.*, 1993; Krishna *et al.*, 1994; Trottein and Cowman, 1995) and *PfATPase4* (Trottein and Cowman, 1995) have been examined for amplification in several mefloquine and chloroquine-resistant *P. falciparum* lines (Trottein and Cowman, 1995). *PfATPases1* and *3* were of particular interest because of their location on chromosome 5, which also carries the *pfmdr1* gene, and their possible involvement in amplification events in response to drug pressure. However, none of the genes were found to be amplified in the parasite clones tested. The authors concluded that these P-type ATPases are not likely to play a rôle in drug resistance.

(iii) Chromosome-7 chloroquine resistance locus

A locus on chromosome 7 of *P. falciparum* has been found to segregate with chloroquine susceptibility in the progeny of a cross between chloroquine-resistant (clone Dd2) and chloroquine-sensitive (clone HB3) parasites (Wellems *et al.*, 1991). Sixteen recombinant progeny clones from the cross were phenotyped for chloroquine susceptibility and efflux, and reversal of resistance by verapamil (Wellems *et al.*, 1990). Eight clones were shown to possess a susceptible phenotype identical with the HB3 parent, and the remaining clones exhibited a resistant phenotype identical with Dd2. No clones demonstrated intermediate phenotypes, which would have indicated that the trait was multigenic. The authors concluded that a single genetic locus governs chloroquine susceptibility in *P. falciparum.* Linkage analysis of the progeny clones with 85 RFLP markers revealed a single locus on chromosome 7 to be linked to chloroquine susceptibility (Wellems *et al.*, 1991). Mapping of chromosome 7 has localised the region containing the putative chloroquine resistance gene to an area of 100 kb (personal communication from J. Ravetch, The Memorial Sloan-Kettering Cancer Center, New York), and further studies promise to pinpoint candidate genes.

A recent report suggests that both resistant and sensitive parasites can exhibit a rapid chloroquine efflux (Bray *et al.*, 1992a). These results cast doubt on the validity of the rapid efflux phenotype as a predictor of chloroquine resistance, but they do not challenge the unigenic trait as proposed by Wellems.

Recently, a gene coding for a heat-shock protein was cloned from the locus, and it was found to be linked to chloroquine response in the HB3/Dd2 cross (Su and

Wellems, 1994). However, examination of three chloroquine-resistant and six sensitive clones from different geographical regions for the resistant allele of the gene, failed to show any correlation.

(iv) Other proposed mechanisms

Certain other genes and systems have been put forward as candidate chloroquine resistance mechanisms, and a brief description of these follows:-

(a) Over-expression of calmodulin

Calmodulin is a calcium binding protein, which changes its conformation on binding calcium. This enables it to modulate a number of important enzymes such as kinases, phosphatases and a calcium ATPase pump which regulates the flow of Ca^{2+} (see review by Krishna and Squire-Pollard, 1990). Calmodulin was first suggested as playing a rôle in drug resistance by Scheibel *et al.* (1987), who found that calcium modulators acted more antagonistically in a multidrug resistant clone than a sensitive one. This implied that a common site of action, i.e. calmodulin, was more important in the resistant parasites compared with the sensitive parasites. The *P. falciparum* calmodulin gene has been cloned (Robson and Jennings, 1991), but it is not amplified or overexpressed in the chloroquine-resistant isolates which have been examined, suggesting that it plays no part in the mechanism of chloroquine resistance (Cowman and Galatis, 1991).

(b) Chloroquine modification

Cytochrome P-450 is the terminal oxidase in the eukaryotic mono-oxygenase systems responsible for the metabolism of a wide variety of structurally unrelated drugs. It has been proposed that malaria parasites may convert chloroquine to a less active metabolite and that this activity is increased in chloroquine-resistant strains (Salganik *et al.*, 1987; cited by Ndifor *et al.*, 1990). Evidence is accumulating that this difference is due to higher levels of cytochrome P-450 messenger RNA (mRNA) in chloroquine-resistant compared to chloroquine-sensitive parasites (Surolia *et al.*, 1993). Whether this represents a true resistance mechanism or merely reflects an overall increase in metabolic activity in resistant parasites, remains to be seen (Ndifor *et al.*, 1990). Studies are in progress to isolate the P-450 gene(s) from *Plasmodium* (Surolia *et al.*, 1993).

1.6.4 Chloroquine resistance in species of rodent malaria

The most important feature of studies involving chloroquine resistance in species of rodent malaria is the ability to select chloroquine-resistant parasites from chloroquine-sensitive lines. A number of different selection procedures have been used, but it appears that only a continuous low dose method produces stable chloroquine resistance. Few selection experiments have been carried out using cloned sensitive parasites as starting material. An exception to this is the work on chloroquine resistance in *P. chabaudi*, which has utilised cloned lines of resistant and susceptible parasites. Studies carried out on each of the four rodent malaria species can be summarised as follows:-

(i) *P. yoelii*

All *P. yoelii* isolates so far examined are innately resistant to high doses of chloroquine (Warhurst and Killick-Kendrick (1967); Carter (1972), as cited by (Walliker, 1983)). However, the stage most sensitive to chloroquine action in *P. falciparum* is the trophozoite, and recent work has suggested *P. yoelii* trophozoites are normally sensitive to the drug too (Beauté-Lafitte *et al.*, 1994). The authors suggest that innate chloroquine-resistance is a result of the asynchronicity of *P. yoelii* infections, as certain parasite forms are more resistant to chloroquine than others, and these may not be eliminated by the drug.

(ii) *P. berghei*

Naturally occuring isolates of *P. berghei* are sensitive to chloroquine. Many attempts have been made to select chloroquine-resistant mutants (as documented by Peters, 1987), employing a number of different techniques. A chloroquine-resistant ('RC') line of *P. berghei* was made by Peters (1965), but found to be unstable in the absence of the drug. This resistance may have been due to physiological adaptations of the parasites to the drug. Interestingly, this line did not produce malaria pigment when under chloroquine pressure, but was able to do so after removal of the drug (Peters, 1965). Subsequent claims that a stable, chloroquine-resistant line had been selected from a sensitive isolate of *P. berghei* by a single-step selection method were found to be unreliable; the resistant 'NS' line obtained in this work was found to possess enzyme forms characteristic of the innately resistant *P. yoelii* (Peters *et al.*, 1978).

(iii) *P. vinckei*

Only one successful attempt to produce chloroquine resistance in this species has been reported. A resistant *P. vinckei* line, which was stable in the absence of drug, was selected by Powers *et al.* (1969) using a continuous low pressure method. The line proved to be resistant to 200 mg of chloroquine per kg mouse of body-weight (200 mg/kg) after 44 weeks of treatment, and appeared not to produce malaria pigment. Earlier attempts to select a resistant line from sensitive parasites had failed, and chloroquine resistance could only be developed from parasites that were already resistant to pyrimethamine. No genetic studies have been carried out using this line.

(iv) *P. chabaudi*

Initial studies showed isolates of *P. chabaudi* to be chloroquine-sensitive (Peters; 1987; cited by Rosario, 1976a). Subsequent work has concentrated on selecting lines for low, intermediate and high levels of resistance, and carrying out genetic crosses to determine the number of genes involved.

(a) Selection of chloroquine-resistant mutants *in vivo*

Rosario (1976b) selected a chloroquine-resistant *P. chabaudi* clone using a low pressure method. The AS(3CQ) clone was found to be resistant to chloroquine at a concentration of 3 mg/kg administered to mice over a period of 6 days. The resistance was stable following six months' passage without drug, and after mosquito transmission. An interesting observation is that Rosario was only able to select clone AS(3CQ) from parasites already resistant to pyrimethamine; this mirrors the situation found in *P. vinckei* (see above). In this regard, it is also of interest that mefloquine resistance is easier to establish in *P. falciparum in vitro* if the parasites are already chloroquine-resistant (Foote and Cowman, 1994).

Competition studies have shown that the chloroquine-resistant AS(3CQ) clone of *P. chabaudi* possesses a selective advantage over the sensitive clone AS from which it was derived, even in the absence of drug (Rosario, 1978). This is surprising because most mutations are disadvantageous in the absence of selection pressure in their favour. Rosario (1976a) suggested that the earlier schizogony noted to occur in AS(3CQ) parasites compared with AS could account for this; chloroquine-resistant merozoites would be produced earlier which would be able to invade more red blood cells than the sensitive parasites, particularly if the numbers of red cells were low, as at the peak of an infection. As gametocytes are produced towards the end of an infection, i.e. when the supply of red blood cells is limited, the resistant line would also be at an advantage in cyclical transmission.

Further selection studies were carried out by Padua (1981). The AS(3CQ) clone of Rosario was exposed to gradually increasing doses of chloroquine over a period of ten months, and three new resistant lines were established, AS(15CQ), AS(20CQ) and AS(30CQ). All lines were found to be resistant to a drug dose of 30 mg/kg administered to mice over six days, but parasites recrudesced at different rates following drug treatment; parasites of the most resistant line always emerged before parasites from more susceptible lines. The resistance of the lines was found to be stable following mosquito transmission and after multiple blood passage.

(b) The chloroquine resistance phenotype

The chloroquine resistance phenotype of *P. chabaudi* clone AS(3CQ) has been studied in some detail. Tanabe *et al.* (1990) have shown that Ca^{2+} antagonists, such as verapamil, reverse resistance in AS(3CQ). Verapamil increased the susceptibility to chloroquine in the chloroquine-sensitive parent line AS, a phenomenon reported in sensitive *P. falciparum* clones (Wellems *et al.*, 1990). Ohsawa *et al.* (1991) have studied the ultrastructural changes associated with this reversal and report finding swelling of the food vacuoles and clumping of electron-dense material in the nucleus, consistent with an increase in the accumulation of chloroquine in the resistant parasites. Similar changes have been reported to occur in cultures of chloroquine-resistant *P. falciparum* after incubation with chloroquine and verapamil (Jacobs *et al.*, 1988). Padua (1980) also noted morphological differences in AS(30CQ) in the presence of chloroquine, such as the presence of highly vacuolated trophozoites which gave the parasite a foamy appearance.

Finally, Miki *et al.* (1992) reported reduced accumulation of chloroquine in AS(3CQ) parasites compared with the sensitive parasites from which they were derived. The authors attributed this to enhanced efflux of the drug in the resistant line, although no efflux studies have yet been carried out on this parasite. Such studies may be irrelevant as a phenotypic indicator of chloroquine resistance, as mentioned previously (Bray *et al.*, 1992a; Bray and Ward, 1993).

(c) Genetic studies

Rosario (1976b) crossed the chloroquine-resistant clone AS(3CQ) with a chloroquine sensitive line AJ, which was pyrimethamine sensitive and possessed different forms of the isoenzymes LDH and PGD. Clones exhibiting recombination between all the markers were obtained, which indicated that the chloroquine resistance trait underwent a typically Mendelian form of inheritance expected of a nuclear gene. Moreover, the two drug-resistance characters, for chloroquine and pyrimethamine,

segregated independently, indicating that mutations at different loci were responsible for each type of resistance.

70 clones were isolated from the progeny of this cross and typed for chloroquine susceptibility. The drug tests used to type the clones were based upon the rate of appearance of parasites following drug treatment of 3 mg/kg chloroquine for 6 days; those appearing on or before Day 8 were typed as resistant and those appearing after this day as sensitive. Thirty-two clones were classified as chloroquine-resistant and thirty-eight as chloroquine-sensitive. The levels of parasitaemia reached by some resistant parasites was found to vary independently of their susceptibility status. It is not clear whether these intermediate levels of resistance were due to the multigenic nature of the resistance or to host factors affecting the susceptibility tests (Rosario, 1976a).

Padua (1981) examined the progeny of two crosses between her highly chloroquine-resistant line AS(30CQ) and the drug-sensitive line AJ. In each cross, progeny were obtained showing various grades of susceptibility, from complete sensitivity, to low (3CQ), intermediate (15CQ), and high (30CQ) levels of resistance. Padua concluded that the high level of resistance seen in AS(30CQ) was probably due to an accumulation of mutations at different loci, each conferring a low level of resistance. Following the cross, the genes had segregated into different parasite clones in various combinations which conferred different levels of resistance. The exact number of mutations responsible for the trait was not clear, because the drug tests used to type the clones were also based upon the rate of appearance of parasites following drug treatment, and this was subject to variation as mentioned above.

1.7 The identification of loci through linkage analysis

The identification of genetic loci through linkage to other markers was proposed as early as 1932 (Haldane, 1932). The objective is to detect a marker closely linked to the gene which causes the trait under study (for a review, see Weatherall, 1991). Loci are linked if they are close together on the same chromosome, in which case they are likely to be passed on together into the same gamete. Thus, there is a high probability that linked loci will pass through successive generations together, unless they are separated by a cross-over at meiosis. If the marker and gene are on separate chromosomes, random assortment can occur, and the gene and the marker will be found as often together as they are apart. Once the chromosomal location of the linked marker is known, then by inference, the gene can be assigned to the same area of the chromosome.

Linkage analysis involves the collection of genetic cross data or pedigrees in which the responsible gene is segregating. The parents and progeny are studied for inheritance of the trait and for inheritance of multiple polymorphic markers, until evidence for linkage is found. Problems can be encountered if the trait is caused by mutations at more than one locus, but the use of data from large 'families' over several generations can overcome this. Mathematical methods exist to determine whether a given set of data contains sufficient information to give a high likelihood of positive linkage, the most common method in human linkage analysis being the LOD score.

Fine mapping can then be applied to narrow down the region containing the gene, but this is limited to the number of informative meioses available. In linkage analysis of human disease loci using family tree data, it is unusual to have more than 100 informative meioses available, and fine mapping is often limited to intervals of 1 Mb (mega base) (Collins, 1992). Direct localisation of the gene ultimately requires physical mapping and cloning.

Linkage analysis relies upon the availability of informative markers, i.e. markers that detect variation between organisms at the DNA level. Ideally they should also be abundant, evenly distributed throughout the genome and easily typed. RFLP markers have been used extensively for mapping human disease loci, for example Huntington's chorea (Gusella *et al.*, 1983), but often they show a low rate of polymorphism among specific individuals or strains of interest, and are time-consuming to type. Other markers which have been developed recently include: (i) multi-locus minisatellites (DNA fingerprints) (Jeffreys *et al.*, 1985); (ii) single-locus minisatellites (variable number of tandem repeat (VNTR) markers) (Nakamura *et al.*, 1987); (iii) microsatellites (see review by Queller *et al.*, 1993); and (iv) RAPDs (random amplified polymorphic DNA) (Welsh and McClelland, 1990; Williams *et al.*, 1990).

Linkage analysis is one part of the approach used to identify and clone genes causing a particular phenotype, an approach referred to here as 'positional cloning' (for a review, see Collins, 1992). Once a candidate gene has been located by positional cloning, it is necessary to confirm that a mutant form of it causes the trait, for example through identification of mutations in DNA from individuals exhibiting the phenotype. Absolute proof is obtainable from transfection studies which show the mutant gene to cause the phenotype of the trait in question (Wicking and Williamson, 1991).

1.7.1 Linkage analysis studies of *Plasmodium*

Two linkage analysis studies in species of *Plasmodium* have been reported; the first study identified a chloroquine resistance locus on chromosome 7 of *P. falciparum*

(Wellems *et al.*, 1991), as described in **Section 1.6.3**. The second study used data from 11 progeny clones of the HB3/Dd2 cross of Wellems *et al.* (1991), and identified a locus on *P. falciparum* chromosome 12 which may contain genes that determine mosquito-infectivity and male gametogenesis (Vaidya *et al.*, 1995). Linkage analysis in crosses between species of *Plasmodium* is helped considerably by the haploid nature of the parasite genome.

1.8 Outline of the present study

The object of this study has been to investigate the genetic basis of chloroquine resistance in the rodent malaria parasite *P. chabaudi*, by linkage analysis of a cross between chloroquine-resistant and chloroquine-sensitive parasites.

P. chabaudi is an ideal model for studying genetic mechanisms of resistance to chloroquine. It is possible to produce isogenic clones of *P. chabaudi* which differ only in their susceptibility to drugs. Moreover, the molecular mechanism of resistance to at least two drugs in *P. chabaudi*, pyrimethamine (**Section 1.5.3**) and mefloquine (Bisoni, 1994), are likely to be the same as that found in *P. falciparum*. Genetic crosses are more easily accomplished using *P. chabaudi* than *P. falciparum* because of the ethical issues involved in using chimpanzees as vertebrate hosts, and the time and cost of *in vitro* culturing.

The chloroquine-resistant clone AS(3CQ) was chosen for this work because of its low level of resistance (Rosario, 1976b). The resistance was more likely to be the result of a single mutant gene (i.e. a single gene trait), than to multiple mutant genes (i.e. a multigenic trait). Also, linkage analysis of a trait becomes more complicated the greater the number of genes are involved. A genetically distinct parasite clone AJ, was chosen as the chloroquine-sensitive parent.

In the first section of this study, a description is given of the construction of a chromosome map of *P. chabaudi*. The map includes markers which were developed from a novel technique, RAPD-PCR, and also anonymous markers, and *P. falciparum* genes which were found to cross-hybridise to *P. chabaudi* chromosomes.

The location of homologous genes between *P. chabaudi* and *P. falciparum* enabled a report of the synteny relationship between the two genomes to be made. This is presented in the second part of this study.

In the final section, a cross made between the two *P. chabaudi* parental clones is described. The cross progeny were cloned and tested for their susceptibility to chloroquine. Chromosome markers found to be polymorphic between the parents were then used in the linkage analysis of the progeny clones. Evidence was obtained that a gene on chromosome 11 of this parasite is involved in chloroquine resistance.

2. Materials and methods

Abbreviations of reagents are listed in **Abbreviations**, and the composition of buffers, solutions and media are given in **Appendix 1**.

2.1 Definition of terms

Four terms describing the type of parasite infection are used throughout this work, as follows:-

(a) Isolate - a sample of parasites collected from a wild-caught animal on a single occasion and preserved as deep-frozen material. An isolate may not be genetically homogeneous and may contain parasites of more than one species.

(b) Line - a collection of parasites which have undergone a particular laboratory passage. In this sense, every laboratory manipulation of parasites creates a new line, but usually parasites are described as belonging to a line only after special treatment, such as selection for drug resistance. All the parasites in a line have certain characteristics in common, but they need not be genetically identical.

(c) Clone - an infection derived from a single parasite by asexual multiplication. All parasites within a clone are assumed to be genetically identical.

(d) Stabilate- a population of viable parasites preserved on a unique occasion. *Plasmodium* parasites are routinely stored under liquid nitrogen.

2.2 Cloned lines of *P. falciparum*

The *P. falciparum* clones used in this work are shown in **Table 2**.

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

Asexual parasite forms were cultured *in vitro* following a modified protocol of Trager and Jensen (1976) and Haynes *et al.* (1976). Human red blood cells (RBCs; group O and Rhesus group positive, obtained from the Edinburgh and South-East Scotland Blood Transfusion Service) were washed and centrifuged at 1500 **g** three times in incomplete medium (**Appendix 1**) and the 'buffy coat' of white blood cells removed. The RBCs were then resuspended in complete medium (**Appendix 1**) to give a packed red cell volume ('haematocrit') of 50%, and stored at 4^{o}C. Newly thawed parasites were resuspended in this complete medium in 5 ml culture flasks

Table 2. Clones of *P. falciparum* used in this work.

Isolate	Origin	Clone	Pyrimethamine MIC[b]	Chloroquine MIC[b]	Reference
NF54[a]	The Netherlands	3D7	10^{-7}M	0.2 x 10^{-6} M	Walliker *et al.* (1987)
H1	Honduras	HB3	10^{-5} M	0.2 x 10^{-6} M	Bhasin and Trager (1984)
Indochina III	Indochina	Dd2	10^{-5} M	1.6 x 10^{-6} M	Wellems *et al.* (1988)

[a] Derived from a patient living near Schipol Airport, Amsterdam, who was probably infected by a mosquito imported on an aircraft from a tropical country. There is evidence that the parasite is of African origin (Collins *et al.*, 1986).

[b] Determined using the method of Thaithong and Beale (1981), by Babiker (1994).

(J.Bibby Science Products Ltd.) at a final haematocrit of 5%. Flasks were gassed with a mixture of 1% O_2, 3% CO_2 and 96% N_2, and maintained at 37°C. Medium and gas were replaced daily.

The parasitaemia and health of the parasites were monitored by microscopic examination of thin blood films (blood smears) taken from cultures each day. Smears were fixed with methanol and stained with Giemsa's stain (Gibco BRL) at pH 7.2. The parasitaemia was calculated by counting the number of parasites found in three different fields of the same Giemsa-stained blood smear, and the total number of red blood cells in those fields. Double and triple parasite infections were counted as two and three parasites respectively. The percent parasitaemia was calculated as follows:-

$$\% \text{ parasitaemia} = \frac{\text{number parasitized red blood cells}}{\text{total number red blood cells}} \times 100$$

Parasites were subcultured into larger 25 ml flasks also containing red blood cells, at 5% haematocrit when the parasitaemia reached 8-9%.

2.2.2 Cryo-preservation of parasites

Parasites were preserved in liquid nitrogen (Jensen *et al.*, 1979). Cultures at a parasitaemia of at least 2% and containing predominantly ring forms were centrifuged at 1500 **g** for 5 minutes. After removal of the supernatant, an equal volume of deep freeze solution (**Appendix 1**) was added slowly to the packed RBCs, which allowed

the glycerol to penetrate parasitized RBCs. 0.3 ml aliquots of the cell suspension were pipetted into polypropylene ampoules (Nunc) which were immersed immediately in liquid nitrogen.

Parasites were recovered from storage by thawing the ampoules at room temperature and treating the parasites using a method based on Aley *et al* (1984), as described by Ranford-Cartwright (1992). Briefly, three thawing solutions containing decreasing concentrations of NaCl (**Appendix 1**) were added in turn to the thawed parasites and mixed, the mixture centrifuged at 1500 **g** for 5 minutes and the supernatant removed. The RBC pellet containing thawed parasites was then placed into culture with new medium.

2.3 Lines and clones of *P. chabaudi* and their hosts

2.3.1 Parasite lines and clones

The parasite lines used in this study are shown in **Table 3**. The two lines AS(sens) and AJ were originally obtained from separate, wild-caught thicket-rats of the Central African Republic (Carter and Walliker, 1975). They were subsequently cloned by dilution of blood forms by the method described by Walliker *et al* (1975). They differed from each other in the electrophoretic form of three enzymes, lactate dehydrogenase (LDH; E.C. 1.1.1.27), 6-phosphogluconate dehydrogenase (6PGD; E.C. 1.1.1.44) and adenosine deaminase (ADA; E.C. 3.5.4.4) (Carter, 1978; Sanderson *et al.*, 1981). Both AJ and AS(sens) are pyrimethamine- and chloroquine-sensitive, as defined in **Table 3**.

AS(0CQ) was derived from the AS(sens) line and is resistant to 4 daily doses of pyrimethamine at 15 mg/kg mouse body weight (Walliker *et al.*, 1975). This resistance was established in a single step using a high pressure method. Briefly, 50 infected mice were treated with pyrimethamine at 50 mg/kg for 4 days. Parasites appeared in the blood of one animal 14 days later and were then passaged through undrugged rodents and mosquitoes to ensure the stability of the resistance. The line was cloned before being cryo-preserved.

The chloroquine resistant line, AS(3CQ), was derived from the AS(0CQ) line by Rosario (1976a; 1976b) and is resistant to 6 daily doses of chloroquine at 6mg/kg mouse body weight. This resistance was established in multiple steps using a continuous low pressure method. Briefly, groups of infected mice were treated with chloroquine at 2 mg/kg for 5 days. Parasites from the mouse which exhibited the highest parasitaemia were injected into a second group of mice and the treatment repeated. After five similar passages, during which the dose of chloroquine was increased to 3 mg/kg, the surviving parasites were transmitted through mosquitoes

Table 3. Cloned lines of *P. chabaudi* used during this work.

Cloned Lines	Enzyme Types			Drug Response	
	ADA	6PGD	LDH	Pyrimethamine	Chloroquine
AJ	9	3	2	S	S
AS(sens)	6	2	3	S	S
AS(0CQ)	6	2	3	R	S
AS(3CQ)	6	2	3	R	R

Different electrophoretic forms of each respective enzyme ADA, 6PGD and LDH are shown as numbers. For example, AJ parasites have electrophoretic form 9 of the enzyme ADA, whereas AS parasites have electrophoretic form 6 of the same enzyme (Carter, 1978; Lainson, 1983). Drug responses for the lines are based upon the following drug tests:

Pyrimethamine : S, sensitive to treatment with 15 mg/kg pyrimethamine for 4 days
R, resistant to treatment with 15 mg/kg pyrimethamine for 4 days

Chloroquine : S, sensitive to treatment with 3 mg/kg chloroquine for 6 days
R, resistant to treatment with 3 mg/kg chloroquine for 6 days

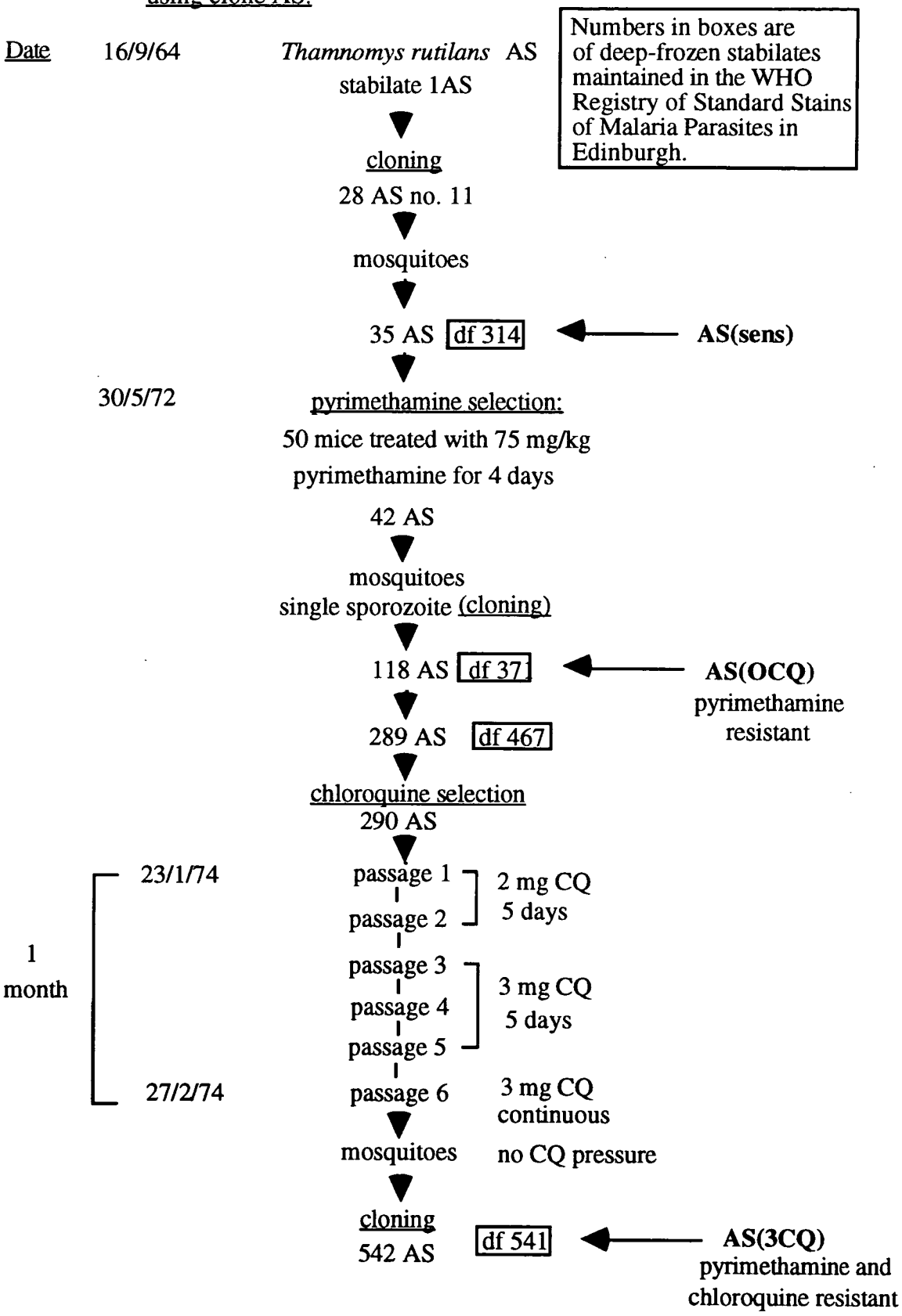
The preparation of drug tests is described in **Section 2.6.3.**

into mice and found to be resistant. The relationships between these three AS lines is summarised in **Figure 4.**

2.3.2 Mammalian and insect hosts

Laboratory mice (*Mus musculus*) and rats (*Rattus norvegicus*) were used throughout this project as mammalian hosts. Male and female outbred strains of mice (University of Edinburgh) were used for the routine maintenance and cloning of parasite lines, and 7-8 week old CBA/Ca male mice (B & K Universal, Hull) for chloroquine susceptibility tests and genetic crossing work. Newly weaned and splenectomised Wistar rats (B & K Universal, Hull) were used for inducing gametocytogenesis (MacLeod and Brown, 1976). Rats were splenectomised at least 24 hours before required, following normal procedures. All rodents were kept in polypropylene cages with wood shavings for bedding and fed with SDS Formula

Figure 4. Pyrimethamine and chloroquine resistance selection experiments using clone AS.



Number 1 (Special Diets Services Ltd., Essex, England). Drinking water was supplemented with 0.05% PABA (*para*-aminobenzoic acid), which may be an essential requirement for parasite growth (Hawking, 1953; Jacobs, 1964). Animal rooms were kept at a constant 22-25^{o}C with a cycle of 12 hours light and 12 hours dark.

Anopheles stephensi mosquitoes were used as the insect host for crossing studies. They were maintained at 24-26^{o}C and 70-90% humidity with alternating 12 hour sequences of light and darkness, and fed with a 10% solution of glucose containing 0.05% PABA.

2.4 Maintenance of *P. chabaudi* in the laboratory

2.4.1 Blood passage

P. chabaudi infections were maintained by blood passage from infected to uninfected mice. Blood from mouse tail-snips was collected in either citrate saline or heparinized serum Ringer solution (**Appendix 1**), and 0.1ml injected intraperitoneally into each uninfected mouse. The day of inoculation was called Day 0 (D_0), and subsequent days Day 1 (D_1), Day 2 (D_2) *etc.* Infections were monitored by examining thin blood films (blood smears) taken from tail-snips and stained with Giemsa's stain as described for *P. falciparum.*

2.4.2 Cryo-preservation

Mice on days 3-4 of infection were anaesthetized with ether, and blood collected from the brachial vessels directly into citrate saline. The blood was subsequently treated as described for *P. falciparum* (**Section 2.2.2**). The cell suspension was dispensed into glass capillaries which were sealed and plunged into liquid nitrogen for storage. Parasites were recovered from storage by thawing frozen capillaries, diluting the contents with citrate saline, and inoculating uninfected mice with 0.1ml each. Infections became patent 5-10 days after inoculation and were passaged once before undergoing a particular laboratory procedure.

2.4.3 Preparation of standard inocula

Inocula containing known numbers of parasites were required for drug resistance tests and for establishing clones by dilution of infected blood. Parasitaemias were calculated as described for *in vitro* cultures of *P. falciparum*, and a haemocytometer used to calculate the number of red blood cells/ml of infected mouse blood. The number of parasites present in a given volume of blood was then determined.

(i) Inocula containing 10^5 or 10^6 parasites

An infected donor mouse was bled from the tail into fixed-volume glass micro-pipettes (Camlab). The blood was diluted to the appropriate concentration in heparinized serum Ringer solution, so that each 0.1ml contained 10^5 or 10^6 parasites as required. Diluted blood was kept on ice at all stages. Recipient mice were inoculated with 0.1ml of diluent within an hour of the preparation of the inocula.

(ii) Inocula containing 0.5 parasite (cloning)

Clones of *P. chabaudi* were established using a modified limiting dilution technique (Walliker *et al.*, 1975). Inocula containing 0.5 parasite/0.1ml of heparinized serum Ringer solution were prepared from donor mice. Mice with low but rising infections were selected, to minimize the number of double and triple parasite infections in a single erythrocyte. 0.1ml aliquots were injected intraperitoneally into groups of 20-40 mice, and thin blood films examined after 10-14 days. The numbers of clones established from one or more parasites could be estimated from the proportion of mice which became infected, by means of the Poisson distribution. By inoculating 0.5 parasite per mouse, approximately 40% of the animals should become infected. Of these, 75% are predicted to be clones. These calculations are approximate but adequate for this work, because non-clonal infections were readily detected by examining genetic markers.

2.5 Preparation and administration of drugs and standard drug tests

2.5.1 Chloroquine

Chloroquine sulphate, commercially known as 'Nivaquine', was obtained in the form of a solution of 40mg chloroquine base/ml (May and Baker Ltd., Dagenham, England). The required dilutions of drug were made in distilled water and stored at 4^oC. The drug was administered to mice orally by intubation in 0.1ml amounts.

2.5.2 Pyrimethamine

Pyrimethamine, commercially known as 'Daraprim', was obtained in powder form from the Wellcome Research Laboratories, Kent, England. The required dilutions of drug were made in warmed DMSO (dimethyl sulphoxide), due to the insolubility of the drug in water, and stored at room temperature. The drug was administered orally by intubation in 0.1ml amounts.

2.5.3 Standard drug resistance tests

Drug doses were expressed as mg of drug per kg mouse body weight (mg/kg). All drug tests involved the prior inoculation of 10^6 parasites into 4 or 5, 7-8 week-old, CBA/Ca male mice. Two mice served as controls, and the remainder as the mice to be treated with drug. Three hours after inoculation, the first drug dose was administered (D_0) and repeated at the same time thereafter (on D_1, D_2 *etc.*). The mice were weighed individually and daily to take account of any changes in body weight, and the dose adjusted accordingly. Blood smears were taken from each mouse on appropriate days and examined for the presence of parasites.

(i) Standard test for chloroquine resistance

Mice were subjected to 3mg chloroquine/kg for 8 days and tail smears examined for parasites on D_{11}, D_{13}, D_{15} and, when necessary, D_{17}. This test produced a time lag between the emergence of resistant parasites (on or before D_{13}) and sensitive parasites (D_{15} onwards), and was used for all drug tests.

(ii) Standard test for pyrimethamine resistance

Mice were subjected to 15mg pyrimethamine/kg body weight for 4 days and tail smears examined on D_8 and D_{10}. Those parasites which appeared on or before D_{10} were classified as resistant, and those which did not appear up to D_{20}, as sensitive.

2.6 Extraction of parasites from host cells

2.6.1 Extraction of *P. chabaudi* parasites

Mice with a parasitaemia of 60-80% were anaesthetized with ether and blood collected from the brachial vessels directly into citrate saline. The blood was passed down a column of CF11 cellulose powder (Whatman), pre-wetted with citrate saline, to remove host leucocytes (Fulton and Grant, 1956) and then lysed with a solution of 0.15% saponin in 1 x PBS (**Appendix 1**). After washing twice in 1 x PBS, the parasites were pelleted by centrifugation at 1500 **g** for 10 minutes and stored at -20°C.

2.6.2 Extraction of *P. falciparum* parasites

In vitro cultures with a parasitaemia of 10-15% were centrifuged to pellet the erythrocytes. As *P. falciparum* cultures contain few human white blood cells, removal of the cells by a CF11 column is not necessary. Erythrocyte lysis was completed following the method outlined above and parasites stored at -20°C.

2.7 Alloenzyme analysis

Electrophoretic variants of the enzymes LDH and ADA were examined by horizontal cellulose acetate electrophoresis (Carter, 1978; Sanderson *et al.*, 1981; Babiker, 1994). Parasites freed from their host red blood cells by saponin lysis were lysed with a small quantity of water and loaded onto Titan ISO-VIS cellulose acetate plates pre-soaked in Supra-heme buffer for ADA or Electra-HR buffer for LDH (**Appendix 1**). Electrophoresis was carried out according to the manufacturer's instructions (Helena Laboratories, U.K. Ltd., England). Following electrophoresis, plates were incubated with the appropriate enzyme staining solution (**Table 4**). Samples of uninfected mouse blood were run in parallel with parasite samples as controls.

Table 4. Cellulose acetate electrophoresis running conditions and staining solutions.

Enzyme	Electrophoresis conditions		Assay solution	
	voltage	time		
ADA	200 V	15 min	0.1 M phosphate buffer, pH 7.5	2 ml
			Adenosine	10 mg
			MTT[a]	5 mg
			Xanthine oxidase	0.01 ml
			Nucleoside phosphorylase	0.01 ml
			PMS[b]	2 mg
LDH	150 V	25 min	0.05 M Tris-HCl buffer, pH 8.0	2 ml
			Lithium lactate	100 mg
			NAD[c]	5 mg
			MTT	5 mg
			PMS	2 mg

[a] MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide

[b] PMS : phenazine methosulfate

[c] NAD : nicotinamide adenine dinucleotide

2.8 Pulsed-Field Gradient Gel Electrophoresis (PFGE)

2.8.1 Preparation of PFG blocks and agarose gels

PFG blocks of *P. chabaudi* and *P. falciparum* blood forms were prepared following the method of Kemp *et al* (1985). Freshly saponin-lysed parasites were diluted with 1 x PBS to a final concentration of 4 x 10^9 parasites/ml. An equal volume of 2% (w/v) low melting temperature (LMT) agarose (Sigma), made in 1 x PBS and cooled to

42°C, was added to the parasites, the solution mixed, pipetted into a pre-warmed mould (BioRad), and allowed to set at 4°C for 20 minutes. Solidified blocks were removed into PFG lysis solution (**Appendix 1**) containing 2 mg/ml proteinase K (Boehringer Mannheim) and incubated at 42°C for 48 hours with one change of lysis solution and proteinase K. Blocks were stored at 4°C in lysis solution only.

0.8-1.0% (w/v) Molecular Biology Grade (IBI) or Chromosomal Grade (BioRad) agarose gels were made in 0.5 x TBE buffer in a BioRad mould. Blocks were cut to the required size, loaded into the wells and sealed with 0.8% (w/v) LMT agarose in 0.5 x TBE buffer.

2.8.2 PFG electrophoresis conditions

All PFG gels were run using a CHEF (Contour-clamped Homogeneous Electric Field) system (Chu *et al.*, 1986), in which the electric field is generated from multiple electrodes that are arranged in a hexagonal contour around a horizontal gel and clamped to predetermined potentials. The gels were run in 0.5 x TBE buffer (**Appendix 1**), cooled to 10-14°C. Two sets of conditions were used:

(a) separation of low molecular weight chromosomes

First setting : voltage 140 V; pulse-time 120 seconds; for 24 hours;
Second setting : voltage 130 V; pulse-time 300 seconds; for 24 hours;
Final setting : voltage 140 V; pulse-time 180 seconds; for 24 hours.

(b) separation of high molecular weight chromosomes

voltage 80 V; pulse-time 180-900 seconds (ramped); for 7 days.
(A 'ramp' is a decrease in the ratio of run time to pulse time, which allows enhanced resolution of chromosomes in a certain size class.)

S. cerevisiae and *Schizosaccharomyces pombe* DNA size standards (BioRad) were run on all gels. After electrophoresis, gels were stained with ethidium bromide at a final concentration of 0.5 µg/ml for 1/2 hour and visualized under ultra-violet light.

P. falciparum chromosomes were numbered according to convention, with 3D7 taken as the standard clone (Triglia *et al.*, 1992). *P. chabaudi* chromosomes were numbered using AS(sens) as the standard clone, as shown in **Chapter 3**.

2.9 Extraction of genomic DNA

P. falciparum and *P. chabaudi* parasites, freed from their host red blood cells by saponin lysis, were incubated in an equal volume of lysis solution containing 0.2 mg/ml proteinase K, at 37°C overnight. DNA was extracted once with TE buffer (**Appendix 1**)-equilibrated phenol containing 0.1% (w/v) 8-hydroxyquinoline, twice with 1:1 (v/v) phenol/chloroform and finally with 24:1 (v/v) chloroform/isoamyl-

alcohol (Sambrook *et al.*, 1989), precipitated in 0.3 M ammonium acetate and one volume of isopropanol overnight at -20°C, pelleted at 1300 **g** for 10 minutes, washed in 70% ethanol and resuspended in TE, pH 7.4.

2.10 Genomic DNA digests

P. chabaudi genomic DNA was digested following normal procedures (Sambrook *et al.*, 1989). Briefly, 2.5-3 µg of DNA was incubated over-night in 5-10 units of the appropriate restriction enzyme and the manufacturer's buffer, in a total volume of 20-30 µl. Restriction enzymes (Boehringer Mannheim and New England BioLabs) were chosen on the basis of their availability and recognition sequence. *Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A and *Rsa* I were routinely used for detecting RFLPs (restriction fragment length polymorphisms). However, these did not always reveal a polymorphism between the parasite lines under investigation and a series of other enzymes were used; *Ase* I, *Mse* I, *Ssp* I, *Swa* I, *Pac* I, *Pme* I, *Bam* HI, *Eco* RI, *Hind* III, *Asn*, *Dpn* II, *Mae* I, *Mae* II, *Sfu* I, *Taq* I. Certain restriction enzymes proved more likely to produce RFLPs than others; these quickly became apparent and took precedence over others during screening for RFLPs.

Genomic DNA digests were mixed with an appropriate volume of 1 x gel-loading buffer (**Appendix 1**) and electrophoresed on conventional TAE buffer (**Appendix 1**) agarose gels (Sambrook *et al.* 1989). DNA size markers (bacteriophage λ digested with *Hind* III) were run alongside samples on all gels. After electrophoresis, gels were stained with ethidium bromide for 15 minutes and visualised, as described previously.

2.11 Southern blotting

Agarose gels were Southern blotted (Southern, 1975) onto Hybond-N+ (Amersham International) by alkaline transfer following the manufacturer's guide-lines. RFLP gels were depurinated in 0.25 M HCl for 10 minutes, and DNA transfer allowed to proceed for 4-6 hours. PFG gels were depurinated for 1/2 hour, and the DNA allowed to transfer for 24 hours. After blotting, membranes were carefully marked to allow the identification of tracks, and stored in Saran Wrap (Dow Chemical Company) at 4°C.

2.12 Manipulation of recombinant DNA molecules

Many *Plasmodium* genome markers were obtained as cloned inserts in the polylinker sites of plasmid DNA. In order to produce abundant insert and to store the

markers for future use, all recombinant plasmids were transformed into *E. coli* and preparations of plasmid DNA made.

2.12.1 Transformation

Competent *E. coli* strains INVαF' (Invitrogen) or XL1Blue (Stratagene), and *E. coli* strain NM522 made competent by a modified calcium chloride procedure (Mandel and Higa, 1970) were transformed with plasmid DNA, following Sambrook *et al* (1989). A control sample of competent cells to which 2 µl of TE had been added was treated in parallel to detect contamination of the cells by foreign DNA.

2.12.2 Long-term storage of transformed cells

E. coli transformed with recombinant plasmid DNA were preserved for long-term storage as glycerol stocks, following the protocol in Sambrook *et al.* (1989). Glycerol stocks were stored at -70°C. Viable bacteria were recovered by scratching the surface of the frozen stock and streaking the loosened cells onto LB agar plates containing the appropriate antibiotic.

2.12.3 Extraction and digestion of plasmid DNA

Plasmid DNA was prepared using the Wizard™ Minipreps and Wizard™ Maxipreps DNA purification systems (Promega) following the manufacturer's instructions. Inserts were released by digestion of plasmid DNA with the appropriate restriction enzyme, following normal procedures (Sambrook *et al.*, 1989), and using Boehringer Mannheim enzymes and buffers. Digests were electrophoresed on 0.8-2% (w/v) LMT agarose (Gibco BRL) mini gels with Boehringer Mannheim DNA size markers VI and VII. After electrophoresis, gels were stained and visualised as described previously.

2.12.4 Insert purification

LMT agarose containing the required insert was excised, melted at 70°C and the DNA extracted using the Wizard™ PCR Preps DNA purification system (Promega) as described in the manufacturer's instructions. Inserts were stored in TE pH 8.0 at -20°C.

2.13 Polymerase Chain Reaction (PCR)

2.13.1 PCR reagents, reaction conditions and electrophoresis

PCR reactions were carried out using a TRIO-Thermoblock PCR machine (Biometra) in 50 µl volumes containing 100 nM of each appropriate oligonucleotide

(Oswel DNA Service, Edinburgh, Scotland) in 1 x PCR reaction buffer (**Appendix 1**), 1 x PCR dNTP solution (**Appendix 1**), 1 unit *Taq* DNA polymerase (Boehringer Mannheim) and 100-400 ng of *P. falciparum* or *P. chabaudi* genomic DNA. Reactions were overlaid with 100 µl light mineral oil (Sigma). Negative controls for each primer pair contained all the above components except for 2 µl of TE in place of genomic DNA.

PCR was performed using standard conditions of amplification (Saiki *et al.*, 1993). An initial DNA denaturation step was followed by a primer annealing stage and finally elongation of the synthesised strand. Specific temperatures were dependent upon the template DNA being amplified and are listed where necessary.

5 µl of the total 50 µl PCR reaction was electrophoresed on mini TAE agarose gels and stained and visualised as described.

2.13.3 Purification of PCR-amplified fragments

The remainder of the PCR reaction was purified to remove unincorporated deoxynucleotides and primers using the Wizard™ PCR Preps DNA purification system (Promega) following the manufacturer's protocol.

2.14 Cloning PCR products

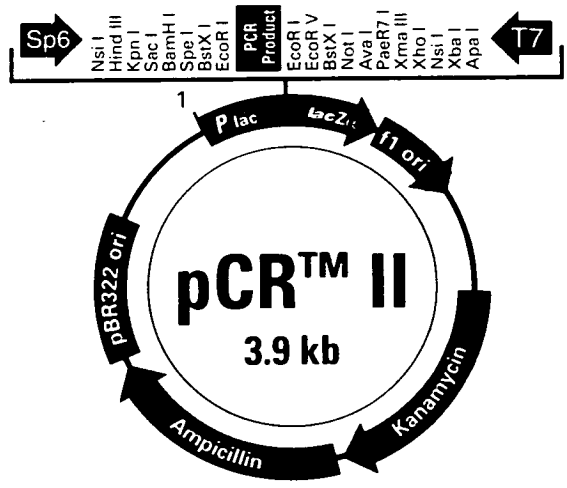
PCR products were cloned into the vector pCR™II using the TA Cloning™ System (Invitrogen) and following the manufacturer's instructions. **Figure 5** is a map of the pCR™II vector.

INVαF' (Invitrogen) competent cells were transformed with the plasmids and plated out on fresh LB plates containing 50 µg/ml kanamycin and 25 µl X-gal (40 mg/ml stock solution). Colonies were picked 15-18 hours later, grown in overnight cultures and plasmid mini-preps made. Plasmid inserts were amplified by PCR using Sp6 and T7 promoter primers (Promega) to verify the insert size. PCR cycling conditions were:

Step 1. 95°C, for 1 minute
Step 2. 47°C, for 1 minute
Step 3. 70°C, for 2 minutes 30 seconds

for 35 cycles. Important PCR products were also sequenced. Once identified, clones were preserved as glycerol stocks at -70°C (**section 2.12.2**).

Figure 5. Map of vector pCR™II (Invitrogen) used to clone PCR products.



2.15 Dideoxy chain termination sequencing

Dideoxy sequencing (Sanger *et al.*, 1977) was performed on cloned PCR products using the Sequenase® Version 2.0 DNA Sequencing Kit (United States Biochemical Corp.). 30 µl of a plasmid mini-prep was alkaline-denatured in 0.4 M NaOH and 0.1 mM EDTA at 37°C for 30 minutes, precipitated in 0.3 M sodium acetate and 2 volumes of ethanol, washed with 70% (v/v) ethanol, and resuspended in 12 µl of TE, pH 8. 0.6 µl of this was used for each seqencing reaction.

Sequencing was carried out following the manufacturer's suggestions and including the modified procedure of Winship (1989). Sp6 and T7 oligonucleotides (Promega) or the original PCR oligonucleotides were used as sequencing primers. Sequencing reactions were electrophoresed using standard conditions on 6% denaturing polyacrylamide gels made using either a Baserunner apparatus (International Biotechnologies, Inc.) or a Sequi-Gen Nucleic Acid Sequencing Cell (BioRad).

Gels were dried to 3 MM chromatography paper (Whatman) on a vacuum dryer (Hoeffer Scientific Instruments) for 1.5 hours at 75°C and exposed to autoradiography film (Kodak XAR-5) at room temperature in an autoradiography cassette. Film was developed in an automatic autoradiographer developer (Exograph). DNA sequences were entered into the University of Wisconsin Genetics Computer Group DNA sequence analysis software for comparison with other known sequences on databases (EMBL™, Genbank™, *etc.*).

2.16 DNA radiolabelling

A Random Primed DNA Labeling kit from Boehringer Mannheim was used to label probes with [α ^{32}P] dATP (Amersham International), following the manufacturer's protocol. 1 µg of carrier DNA was added to the random prime mixture, the sample loaded onto a Chroma Spin™-30 column (Clontech), and centrifuged for 5 minutes at 700 **g**. The purified probe was collected in an Eppendorf tube and stored at 4°C until used.

2.17 Hybridisation of labelled DNA to Southern blots

2.17.1 Hybridisation

Southern blots were incubated in pre-hybridisation solution (**Appendix 1**) for at least one hour before the addition of the probe. Pre-hybridisation incubation temperatures were the same as hybridisation temperatures, which depended upon the probe being used. *P. chabaudi* 'homologous' probes were incubated at 65°C; all other *Plasmodium* 'heterologous' probes were incubated at 40-60°C. Blots were incubated inside a Maxi-Oven (Hybaid) using screw-capped glass hybridisation bottles (Hybaid).

0.1 mg sheared salmon sperm carrier DNA was added to the labelled probe, the volume made up to 1 ml with water and the solution boiled for 3 minutes. After snap-cooling on ice for 15 minutes, the probe was added to the pre-hybridising blots and allowed to hybridise overnight.

2.17 2 Washing Southern blots

Hybridisation solutions were collected and stored at -20°C for further use, if required. Unless a very low stringency was necessary, all blots were washed once at room temperature in 2 x SSC, 0.1% SDS for 5 minutes. Blots hybridised with homologous probes were then washed at 0.5 x SSC, 0.1% SDS, 65°C for one hour with one change of wash solution. Blots hybridised with heterologous probes were also washed for one hour with one change of wash solution, at a stringency appropriate to the degree of conservation between the probe and *P. chabaudi*.

Washed blots were sandwiched in Saran Wrap and autoradiographed with intensifying screens at -70°C. After film development, blots were re-exposed or stripped.

2.17.3 Stripping Southern blots

Southern blots were stripped in 100 mM NaOH for 30 minutes at room temperature, with one change of solution.

2.18 Chromosome markers

Three types of probe were used as chromosome markers for the *P. chabaudi* genome:- (i) genes which had been cloned and characterised in other laboratories; (ii) anonymous markers from a *P. chabaudi* genomic DNA library; and (iii) DNA fragments developed from the RAPD-PCR technique (Random Amplified Polymorphic DNA-PCR).

2.18.1 Markers from other laboratories

A large number of antigen genes of malaria parasites have been cloned and sequenced. However, genes coding for antigens are more likely to have diverged in sequence than are house-keeping genes, due to immune selection by the host. For this reason, most of the markers chosen for this study were house-keeping genes from other *Plasmodium* species, which were more likely to cross-hybridise with *P. chabaudi.*

Table 5 lists all the probes which were obtained from other laboratories and investigated for their suitability as chromosome markers for the *P. chabaudi* genome. Each probe has been given a Probe Number which is referred to throughout the text. The markers were excised from their vectors by enzyme digestion, or amplified by PCR. The DNA was then purified, radiolabelled, and used to probe blots of PFG gels of *P. falciparum* and *P. chabaudi* chromosomes, and of restricted *P. chabaudi* DNA.

Table 5. DNA probes investigated for their suitability as chromosome markers for the *P. chabaudi* genome.

Probe No.	Marker	Reference	Insert	Information
1	*Ca^{2+}-ATPase*	Murakami *et al.* (1990)	3.3 kb in *Hind* III site of pUC19; clone yH4	Contains 70% of *P. yoelii* Ca^{2+}-ATPase gene
2	235 kDa	Holder *et al.* (1991)	5.5 kb in *Eco* RI site of pUC9; clone E8	Clone of *P. yoelii* gene coding for 235 kDa virulence antigen
3	pBS 110	W. Deleersnijder, Institute for Molecular Biology, Brussels, Belgium	1.2 kb cDNA in *Eco* RI site of pBluescript	*P. chabaudi* schizont-specific gene

4	*MSP-1*	Deleersnijder *et al.* (1990)	5.4 kb in *Eco* RI of pBluescript, clone pBS RX4	*P. chabaudi* genomic clone of major merozoite surface protein, MSP-1
5	*PcEMA 1*	Deleersnijder *et al.* (1992)	2.3 kb in *Eco* RI of pBluescript; clone pBS X2A/1	*PcEMA1*, *P. chabaudi* acidic phosphoprotein gene
6	*DHFR*	Cowman and Lew (1989)	2.0 kb PCR fragment[a]	*P. chabaudi* dihydrofolate reductase gene (*DHFR*), bifunctional enzyme with thymidylate synthase (TS)
7	*pfmdr-1*	Foote *et al.* (1989)	4.8 kb in *Xho* I/*Sph* I site of pIC20H	*P. falciparum* multi-drug resistance gene
8	*TBP*	McAndrew *et al.* (1993)	690 bp in *Eco* RI/*Xba* I site of pGEM-4Z	*P. falciparum* TATA-binding protein gene
9	*Enolase*	Read *et al.* (1994)	1.3 kb in *Eco* RI site of M13.	Clone of two-thirds of *P. falciparum* enolase gene
10	*DHPS*	Brooks *et al.* (1994)	980 bp in *Eco* RI/*Nco* I site in pET22b.	*P. falciparum* dihydropteroate synthetase gene
11	*H2A*	Creedon *et al.* (1992)	360 bp PCR fragment[b]	*P. falciparum* histone 2A gene
12	9.2	Ponzi *et al.* (1990)	4.2 kb in *Eco* RI site of pUC 8	*P. berghei* anonymous probe
13	*RPIII*	Li *et al.* (1991)	7 kb in *Eco* RI site of pBluescript; clone E1	*P. falciparum* RNA polymerase III gene
14	*5S rRNA*	Shippen-Lentz *et al.* (1988); Shippen-Lentz and Vezza (1988)	450 bp in *Sma* I site of pGEM1	*P. falciparum* 5S rRNA gene

15	*EF1-α*	D. Williamson, National Institute for Medical Research, Mill Hill, London, Genbank accession no. X60488	4.3 kb in *Hind* III site of EMBL8, clone pPF-1; cut out 451 bp EF1-α gene with *Pvu* II	*P. falciparum* clone containing linked pfPK5 (yeast cdc-2 gene homologue) and EF1-α (elongation factor) genes
16	*CDC2*	Janse *et al.* (1994)	1 kb in *Bam* HI/*Eco* RI site of pBluescript KS	*P. berghei* yeast CDC-2 gene homologue (homologue of *P. falciparum* pfPK5)
17	*VAP A*	Karcz *et al.* (1993b)	1.5 kb in *Eco* RI site of pGEM-4Z	*P. falciparum* vacuolar ATPase A subunit homologue
18	*VAP B*	Karcz *et al.* (1994)	2.5 kb in *Eco* RI site of pGEM-4Z	*P. falciparum* vacuolar ATPase B subunit homologue
19	*G6PD*	O'Brien *et al.* (1994)	588 bp in *Eco* RI site of pGEM	*P. falciparum* glucose 6 phospate dehydro-genase (G6PD) gene.
20	*hsp-70*	Sheppard *et al.* (1989a)	1.1 kb in *Eco* RI site of pUC13; clone BTA505 HS	3' half of *P. chabaudi* gene coding for a heat shock protein
21	*Pfcrk-1*	Doerig *et al.* (1995)	2.4 kb in *Xho* I site of pJFE	*P. falciparum* cdc2-related protein kinase-1 gene
22	*Pfmap-1*	C. Doerig, Division of Biological Sciences, Edinburgh University, Edinburgh	1.8 kb in *Xho* I site of pJFE-DAF	*P. falciparum* mitogen-activated protein kinase gene
23	*Pfcrk-3*	"	500 bp in *Eco* RI site of pCR II™	*P. falciparum* cdc2-related protein kinase-3 gene

24	*DNA pol α*	Ridley *et al.* (1991)	1 kb in *Eco* RI of pUBS; clone pUBS 0/5	Conserved region from *P. falciparum* DNA polymerase a gene
25	*DNA pol δ*	Ridley *et al.* (1991)	1.7 kb in *Eco* RI site of pUBS; clone pUBS 2a	Conserved region of *P. falciparum* DNA polymerase δ gene
26	*RAP-1*	Ridley *et al.* (1990)	2.4 kb in *Pst I/Hind* III site of pUBS	*P. falciparum* rhoptry antigen gene
27	*Topo I*	K. Tosh, Division of Biological Sciences, Edinburgh University, Genbank accession no. X83758	1 kb PCR product; primer sequences not known	*P. falciparum* Topoisomerase I gene
28	*Topo II*	Cheesman *et al.* (1991)	2.6 kb in *Hind* III site of pUC19; 0.8 kb fragment excised with Hind III/*Bam* HI	*P. falciparum* topoisomerase II gene
29	*PCNA*	Kilbey *et al.* (1993)	800 bp in *Hind* III/*Eco* RI site of pBluescript	*P. falciparum* proliferating cell nuclear antigen (PCNA) gene
30	*GPI*	Kaslow and Hill (1990)	2.2 kb in *Eco* RI/*Hind* III site of pUC13	*P. falciparum* glucose phosphate isomerase (GPI) gene
31	*HPRT*	King and Melton (1987)	1059 bp in *Eco* RI site of pUC8; clone 7-3G	*P. falciparum* hypoxanthine-guanine phosphoribosyl-transferase (HPRT) gene
32	*Calmod-ulin*	Robson and Jennings (1991)	2.2 kb in *Eco* RI site of pBR328; clone PfCM 211	*P. falciparum* genomic clone containing most of the calmodulin gene

33	Pcsv4	Viriyakosol *et al.* (1989)	1.4 kb in *Eco* RI site of mp13	*P. chabaudi* fingerprinting probe isolated after screening λgt11 library with polyclonal sera raised against MSP-1 antigen
34	*RESA*	Snounou *et al.* (1988)	1.5 kb in *Eco* RI site of mp13	Codes for an epitope of the *P. chabaudi* 105 kDa antigen[c]; clone pPC105e
35	*αI tubulin*	Holloway *et al.* (1989; 1990)	1.9 kb in *Eco* RI site of pBR322	*P. falciparum* α I tubulin gene, 5' terminus
36	*αII tubulin*	"	1.75 kb in *Eco* RI site of pBR322	*P. falciparum* α II tubulin gene, 3' terminus
37	*aldo-1*	Meier *et al.* (1992)	900 bp *Nco* I fragment	*P. berghei* aldolase-1 gene, less 150 bp
38	*β tubulin*	Belkum *et al.* (1991)	1 kb in *Sst* I site of pGEM-3Zf(-)	*P. berghei* β tubulin gene
39	*AMA-1*	Marshall *et al.* (1989)	2.6 kb in *Bam* HI/*Eco* RI site of pBluescript	Complete *P. chabaudi* apical membrane antigen gene
40	*pfran*	Sultan *et al.* (1994)	1.4 kb in *Bst* XI site of pJFE14	Complete *P. falciparum* Ran/TC4 GTPase homologue
41	*RNA pol II*	Giesecke *et al.* (1991)	300 bp *Eco* RI fragment in pUC18	*P. berghei* RNA polymerase II gene
42	cDNA 167	Silveira *et al.* (1984); Sharkey *et al.* (1988)	190 bp in *Pst* I site of pBR322	Uncharacterised cDNA clone
43	cDNA 121	"	455 bp in *Pst* I site of pBR322	"
44	cDNA 365	"	180 bp in *Pst* I site of pBR322	"
45	cDNA 148	"	330 bp in *Pst* I site of pBR322	*P. chabaudi* 37 kDa antigen gene

46	Ag3008	Favaloro (1993); Favaloro *et al.* (1993)	0.8 kb in *Eco* RI site of pGEX-2T	*P. chabaudi* cDNAclone, encodes a 24 kDa protein located in parasitophorous vacuole membrane[d]
47	Ag3003 A	Favaloro (1993); Favaloro *et al.* (1993)	1.5 kb in *Eco* RI site of pGEX-2T	*P. chabaudi* cDNA that expresses a protein that reacts with hyperimmune serum.
48	Ag3003 B	"	850 bp in *Eco* RI site of pGEX-2T	*P. chabaudi* cDNA that expresses a protein that does not react with hyperimmune serum
49	Ag3020	"	1.3 kb in *Eco* RI site of pGEX-2T	Uncharacterised *P. chabaudi* cDNA clone
50	Ag3024	"	Insert size not determined; in *Eco* RI site of pGEX-2T	"
51	Ag3015	"	"	"
52	Ag3027	"	2 kb in *Eco* RI site of pGEX-2T	"
53	Ag3042 A	"	1.5 kb in *Eco* RI site of pGEX-2T	Probably non-coding *P. chabaudi* cDNA clone
54	Ag3042 B	"	700 bp in *Eco* RI site of pGEX-2T	*P. chabaudi* cDNA clone which cross-hybridises with a cDNA clone encoding an exported dense-granule antigen (EDGA)
55	Ag3010	"	1.8 kb in *Eco* RI site of pGEX-2T	Uncharacterised *P. chabaudi* cDNA clone
56	Ag3012	"	900 bp in *Eco* RI site of pGEX-2T	"

57	Ag3035	"	1.1 kb in *Eco* RI site of pGEX-2T	*P. chabaudi* cDNA clone that expresses a protein which reacts strongly with hyperimmune serum
58	Ag3037	"	450 bp in *Eco* RI site of pGEX-2T	Uncharacterised *P. chabaudi* cDNA clone
59	Ag3040	"	240 bp in *Eco* RI site of pGEX-2T	"
60	Ag3062	"	300 bp in *Eco* RI site of pGEX-2T	"
61	Ag3057	"	650 bp in *Eco* RI site of pGEX-2T	"

[a] *DHFR-TS* PCR primers designed from Cowman and Lew (1989) 5'-CCC CTG CAG TTA AGC TGC CAT ATC CAT ACT G-3'. PCR conditions: 94°C, 60 seconds; 45°C, 60 seconds; 68°C, 150 seconds; for 34 cycles; then 94°C, 60 seconds; 45°C, 60 seconds; 68°C, 10 minutes; 1 cycle.

[b] *H2A* primers designed from Creedon *et al.* (1992) by P. Meaney (Division of Biological Sciences, Edinburgh University). H2A/1: 33 bp from start of gene 5'-GCC TCA AAG GGA ACT TCA AAT TC-3'; H2A/2: 6 bp from end of gene 5'-ATC TTG ATT GGC AGT ACC AGC TT-3'. PCR conditions: 94°C, 60 seconds; 55°C, 60 seconds; 72°C, 60 seconds; for 30 cycles.

[c] Equivalent to the Pf 155 antigen (*RESA*) of *P. falciparum.* (Gabriel *et al.* 1986).

[d] May be the homologue of a *P. falciparum* circumsporozoite protein-related antigen exp-1 (Coppel *et al.* 1985).

2.18.2 Construction of a *P. chabaudi* genomic DNA library

(i) DNA digestion, ligation and transformation

2 µg of *P. chabaudi* genomic AJ DNA was digested with *Sau* 3A and 2 µg of pBluescript®II KS (Stratagene; **Figure 6**) digested with *Sal* I. The recessed 3' ends of the genomic DNA were filled with dATP and dGTP, and plasmid DNA with dATP and dTTP, using Klenow enzyme following normal procedures (Sambrook *et al.*, 1989). Digested genomic DNA was ligated into the plasmid using T4 DNA ligase (Sambrook *et al.*, 1989). *E. coli* NM522 competent cells were transformed, and recombinant colonies identified by blue/white colour selection.

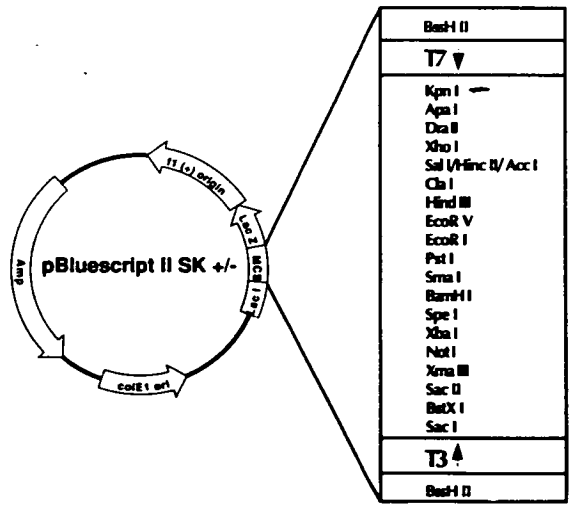
(ii) Detection of plasmids containing repetitive DNA

Plasmids containing repetitive DNA are of limited use as markers for linkage analysis because such DNA often occurs at multiple loci in the genome. For this reason, recombinant plasmids were screened and those containing repetitive DNA excluded from RFLP analysis, in the following way: recombinant colonies were picked, grown in culture overnight, and plasmid mini-preps made using a modified boiling method of Holmes and Quigley (1981). An aliquot of each mini-prep was electrophoresed on a 1% agarose gel and the gels Southern blotted. Blots were probed with random-primed AJ total genomic DNA and washed in 1 x SSC, 0.1% SDS, at 45°C for 1.5 hours with three changes of wash solution. Blots were then exposed to autoradiographic film. Plasmids to which the labelled DNA hybridised were classified as containing repetitive DNA and those which did not as containing unique DNA sequences.

(iii) Storage of recombinant plasmids

Recombinant plasmids were stored as glycerol stocks in duplicate wells of a 96-well microtitre plate (Falcon Microtest II, Becton Dickinson & C°), as described in **Section 2.12.2.**

Figure 6. Map of vector pBluescript® II (Stratagene) used in the construction of a *P. chabaudi* library.



2.19 RAPD-PCR (Random Amplified Polymorphic DNA-PCR)

RAPD-PCR is a novel technique which has the potential to identify large numbers of polymorphisms in the DNA of different parasite clones and species. The technique

involves amplifying fragments of genomic DNA with short, single primers of arbitrary sequence (Welsh and McClelland, 1990; Williams *et al.*, 1990). Unlike conventional PCR of known loci (Saiki *et al.*, 1993), no prior sequence information is required. The reaction takes place under conditions of low stringency that encourage the simultaneous amplification of DNA at a number of loci. The amplified products are separated by agarose gel electrophoresis and visualised by ethidium bromide staining.

The majority of RAPD-PCR products are expected to be identical in different individuals of a single species. A small proportion are likely to be polymorphic, variant forms being shown by: (i) the presence or absence of amplified bands in different parasite strains; or (ii) by variations in the size of amplified bands; or (iii) by variations in the intensity of amplified bands. Such polymorphisms are probably due to differences in primer binding sites caused by the mutation, deletion or insertion of DNA sequences.

2.19.1 RAPD-PCR reagents

RAPD-PCR reactions were performed using AJ and AS(3CQ) DNA, extracted as described previously, using each of 80 decamer primers (Operon Technologies, Kits E, L, O and R, each kit containing 20 decamers with a G+C content of at least 60%). The primer sequences are given in **Appendix 2**. Each reaction was carried out in 15 µl containing 0.4 µM of primer in 1 x reaction buffer (10 mM Tris-Cl pH 8.8., 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100; Clontech), 200 µM each of dATP, dCTP, dTTP, dGTP, 2.5 mM $MgCl_2$, 0.6 units *Taq* DNA polymerase (Clontech) and 200 ng of *P. chabaudi* DNA. Reactions were overlaid with 100 µl light mineral oil. Negative controls for each primer contained all the above components except for 2 µl of TE in place of *P. chabaudi* DNA. Each RAPD-PCR reaction was repeated at least three times, using the same DNA and reagents in most cases.

2.19.2 Reaction conditions and electrophoresis

Cycling conditions were as follows:

Step 1. 1 cycle of 92°C for 3 minutes, 36°C for 1 minute 45 seconds, 72°C for 2 minutes

Step 2. 35 cycles of 92°C for 1 minute, 36°C for 1 minute 45 seconds, 72°C for 2 minutes

Step 3. 1 cycle of 92°C for 1 minute, 36°C for 1 minute 45 seconds, 72°C for 9 minutes

Amplification products were electrophoresed on 1.5% agarose gels, and visualised by ethidium bromide staining.

2.19.3 Screening RAPD-PCR polymorphisms for RFLPs

Many polymorphic bands obtained by this technique could not be amplified reproducibly in repeated experiments. This problem was circumvented by screening polymorphic bands for RFLPs in Southern blots of restricted *P. chabaudi* DNA. To do this, polymorphic bands were excised from agarose gels and the DNA purified using the method of Vaux (1992). This DNA was radiolabelled by random priming and used to probe Southern blots of restricted *P. chabaudi* DNA and PFG gels, as descibed in **Sections 2.16 and 2.17**.

Polymorphic bands which produced clear and reproducible RFLPs and which appeared to exist as single copies in the genome of each *P. chabaudi* clone, were re-amplified using the same primers and reagents as described above, but with 2 µl of the purified DNA of the excised band in place of *P. chabaudi* DNA. Amplification conditions were:

Step 1. 92°C for 30 seconds
Step 2. 50°C for 30 seconds
Step 3. 72°C for 1 minute 30 seconds

for 25 cycles. Re-amplified PCR products were then cloned using the TA Cloning™ System and stored as glycerol stocks for future use as probes.

2.20 *P. chabaudi* MDR gene homologue

PCR primers recognising the nucleotide binding sites (nbs1 and nbs2) of the *P. falciparum* MDR gene (*pfmdr1*) were designed by A. Sultan and M. Foley (Division of Biological Science, Edinburgh University), using the sequence published by Foote *et al.* (1989). Degeneracy was included in such a way as to enable amplification of either ATP-binding cassette, and possibly the sequence between them. Sequences of the primers, referred to as 857S and 858S, are given in **Figure 7.**

P. chabaudi and *P. falciparum* genomic DNA was amplified using these primers, and using the PCR reagents as described in **Section 2.13**. Cycling conditions were as follows:

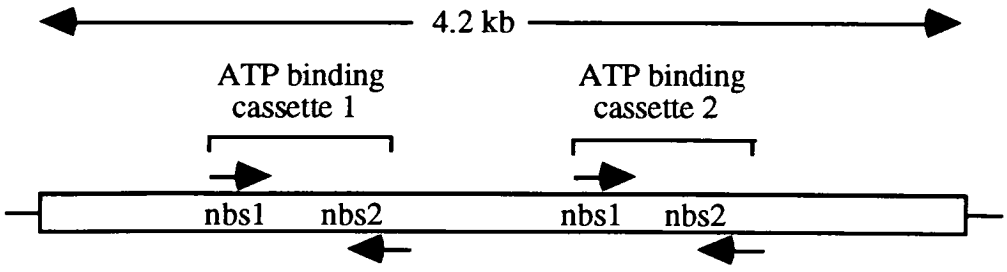
Step 1. 94°C, 30 seconds
Step 2. 45°C, 1 minute
Step 3. 70°C, 1 minute 30 seconds

for 35 cycles. Amplification products were electrophoresed on 1.4% agarose gels and visualized by ethidium bromide staining.

The *P. falciparum* amplified product was sequenced by A. Sultan to confirm its identity as part of the *pfmdr1* gene. The *P. chabaudi* amplified product, called pcATP-PCR, was cloned using the TA Cloning™ System and sequenced. The cloned insert

was radiolabelled by random priming and hybridised to Southern blots of *P. chabaudi* and *P. falciparum* PFG gels, and RFLP blots.

Figure 7. Diagram of *pfmdr1* showing the position of the oligonucleotide primers used to amplify the *P. chabaudi* homologue.



Oligonucleotide primer sequence for amplification of nbs1 (primer 858S)

5'-GGG GGA TTC GGT GAG TCT GGA TGT GGG AAA TC -3'
A A A A T A A

Oligonucleotide primer sequence for amplification of nbs2 (primer 857S)

5'-GGG GCA TGC CCA AAG AAG ATG TAG CTT C-3'
T GG T G G C
T

2.21 *P. falciparum* chromosome 7-specific markers

2.21.1 *P. chabaudi* pS590.7 marker 'homologue'

PCR primers for the *P. falciparum* chromosome 7-specific marker, pS590.7, were made using the published sequence (Wellems *et al.*, 1991). The primers are referred to as 421K and 422K and their sequences are given in **Figure 8**. *P. chabaudi* and *P. falciparum* genomic DNA was amplified using these primers, and PCR reagents as described in **Section 2.13**. Cycling conditions were as follows:

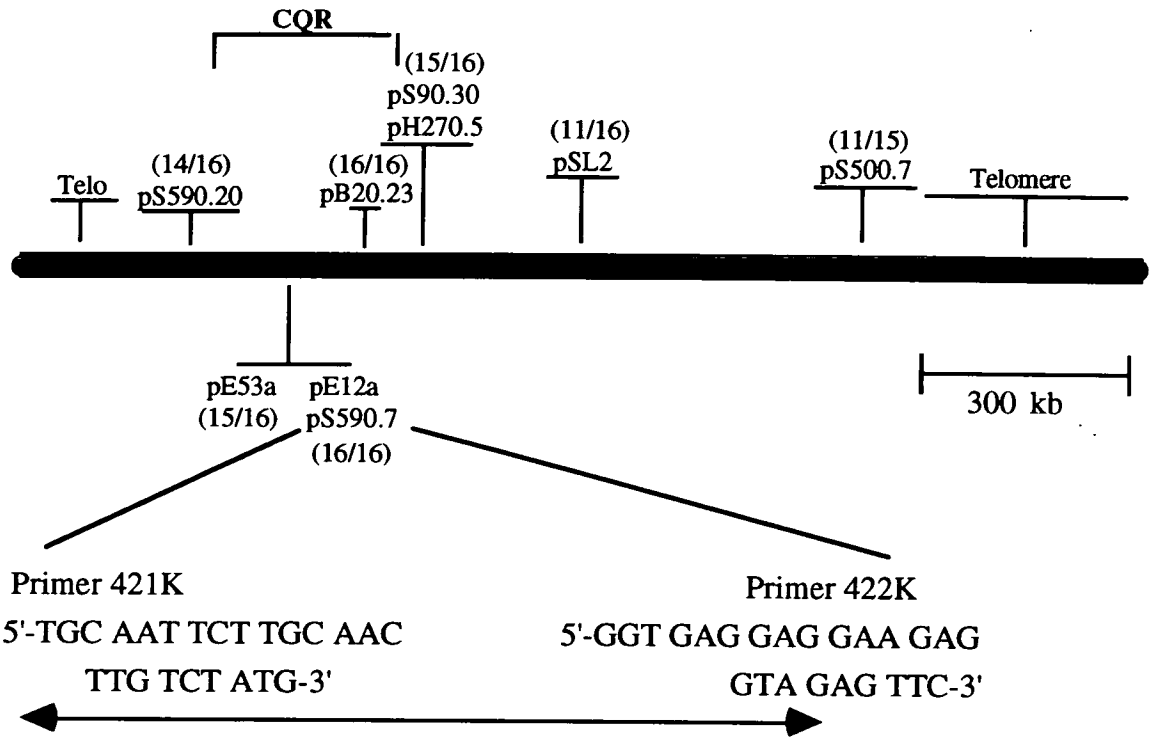
Step 1. 93°C, 30 seconds
Step 2. 52°C, 1 minute
Step 3. 65°C, 2 minutes

for 35 cycles. Amplification products were electrophoresed on 1.4% agarose gels and visualized by ethidium bromide staining.

The *P. falciparum* amplified product, called pfpS590.7, was sequenced directly from the PCR product, to confirm its identity. The *P. chabaudi* amplified product, called pcpS590.7, was cloned using the TA Cloning™ System and sequenced. The

cloned insert was radiolabelled by random priming and hybridised to Southern blots of *P. chabaudi* and *P. falciparum* PFG gels, and RFLP blots.

Figure 8. Diagram of *P. falciparum* chromosome 7 showing the relative position of nine markers, and the oligonucleotide sequences used to amplify the putative *P. chabaudi* homologue of pS590.7.



CQR : area encompassing Wellems *et al* (1991) chloroquine resistance locus
Numbers in brackets refer to the linkage ratio among the sixteen progeny of Wellems *et al* HB3 x Dd2 cross
Telo : telomere
Taken from Wellems *et al.* (1991) with additional information supplied by T. Wellems (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethseda, Maryland, U.S.A.).

2.21.2 Synteny analysis

Several similarities between the PCR products pfpS590.7 and pcpS590.7 suggested that they might be homologous. To substantiate this one way or the other, a further eight chromosome 7-specific *P. falciparum* markers, some of which were known to be close to the marker pS590.7, were chosen. These are shown in **Table 6** and were kindly provided by T. Wellems. Their names and relative positions on *P.*

falciparum chromosome 7 are shown in **Figure 7.** Competent cells were transformed with the markers, plasmid DNA was prepared and the inserts cut out from the vector using *Bam* HI and *Xho* I. The inserts were radiolabelled and used to probe Southern blots of PFG gels and RFLP gels.

Table 6. Eight chromosome 7-specific markers used to examine synteny between *P. chabaudi* chromosome 13 and *P. falciparum* chromosome 7

Marker	Reference	Insert Size (kb)
pE12a	Walker-Jonah *et al.* (1992)	0.7
pE53a	Wellems *et al.* (1991)	1.0
pB20.23	Wellems *et al.* (1991); T.Wellems, personal communication	1.9
pH270.5	Walker-Jonah *et al.* (1992)	0.9
pS90.30	Wellems *et al.* (1991)	1.55
pS590.20	Wellems *et al.* (1991)	1.6
pSL2	Wellems *et al.* (1991)	0.8
pS500.7	Wellems *et al.* (1991)	1.5

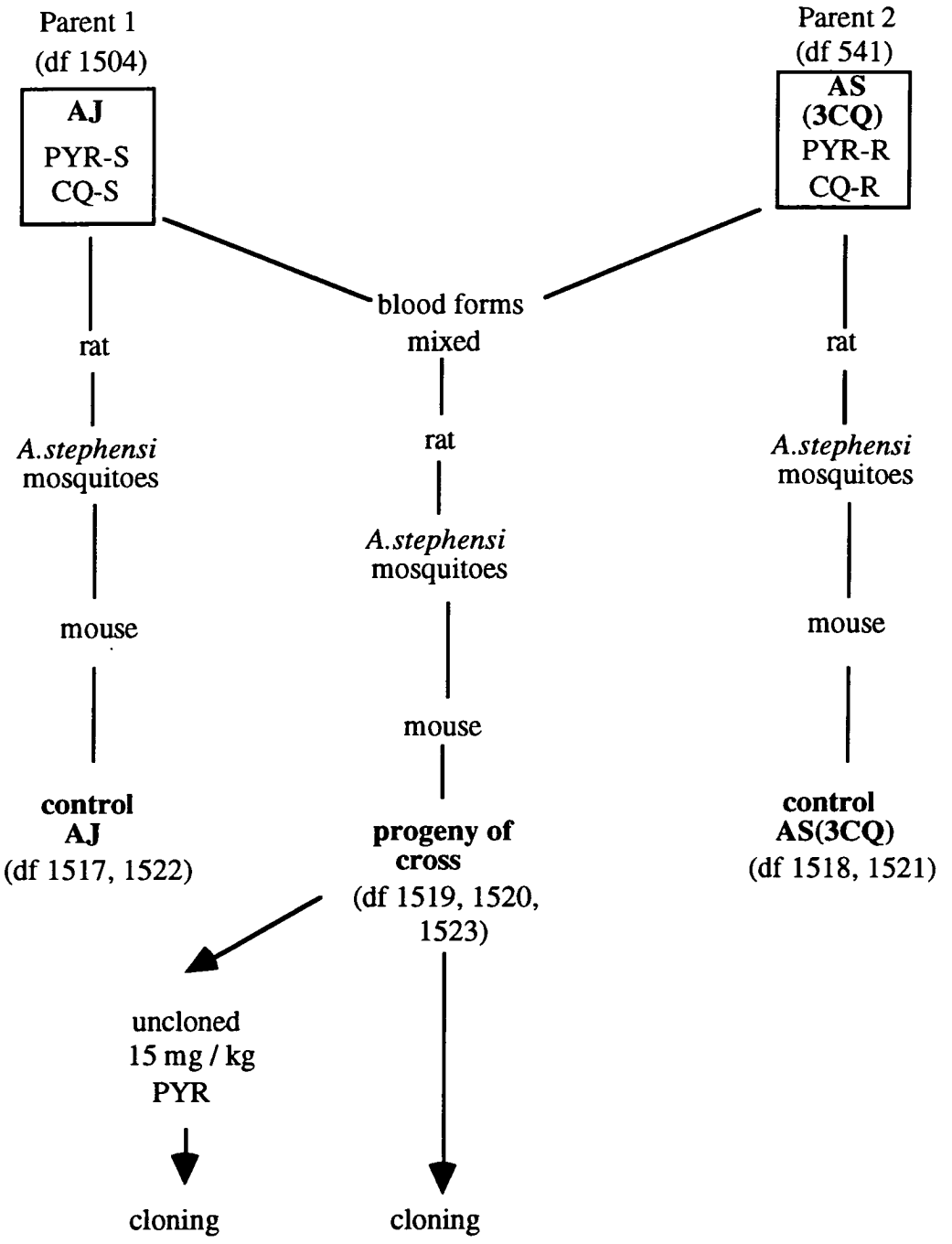
2.22 Crossing technique and analysis of the uncloned progeny

2.22.1 Conducting the cross and recovering the progeny

P. chabaudi clones AJ and AS(3CQ) were crossed by transmission through *A. stephensi* mosquitoes. The crossing procedure followed that of Walliker *et al.* (1975), with minor modifications, and is illustrated in **Figure 9**. AJ and AS(3CQ) parasites were inoculated into separate groups of 4 mice and allowed to attain a parasitaemia of 20-30% (day 4-5). 0.5 ml of blood from each line were then taken together, mixed and injected intraperitoneally into a splenectomised rat (Cornelissen and Walliker, 1985). Control studies on each parent line were conducted in parallel. When a large number of mature gametocytes were present in the blood (day 3-4), approximately 500 4-6 day-old *A. stephensi* which had been starved of glucose for 2 days, were fed on the rats to enable crossing between gametes of each clone to occur within the mosquito mid-gut.

Ten days later, a sample of mosquito midguts were examined for mature oocysts. The mosquitoes were allowed to feed on uninfected mice 6 and 8 days subsequently

Figure 9. Schematic diagram of the cross between AS(3CQ) and AJ.



Each clone was examined for chloroquine susceptibility and parent clone markers.

PYR-S/R : sensitive/resistant to 15 mg/kg pyrimethamine for 4 days
CQ-S/R : sensitive/resistant to 3 mg/kg chloroquine for 6 days
df : deep-freeze stabilate (see **Figure 4**)

in order to establish a new infection with sporozoites. The blood forms which developed in these animals, termed the progeny of the cross, became patent 5-6 days after feeding and were stored deep-frozen in glass capillaries under liquid nitrogen.

2.22.2 Testing for recombinant forms among the progeny

The uncloned progeny of the cross were examined for their alloenzyme forms of LDH and ADA after treatment with pyrimethamine in a standard drug test (**Section 2.5.3**). This gave an indication of whether cross-fertilization had occurred between AS(3CQ) and AJ. If the parental lines were transmitted without cross-fertilization, only the enzyme combinations of the drug resistant parent AS(3CQ) would be observed after drug treatment.

2.22.3 Linkage analysis of the uncloned progeny

An experiment was carried out to investigate the possibility of analysing the progeny of the cross without producing clones. Progeny were treated with pyrimethamine and chloroquine in separate standard drug tests, and DNA was made from the surviving resistant parasites. The DNA was cut using *Hind* II and *Sau* 3A restriction enzymes, and Southern blots made. Genome markers pBS 110, *MSP-1*, *PcEMA1* and *DHFR* (**Table 5**, Probe. Nos. 3, 4, 5 and 6 respectively), P.9 and P.12 (**Tables 8** and **9**) and pcpS590.7, were radiolabelled and used to probe the relevant Southern blot.

The results from this experiment (discussed in **Chapter 5**) indicated that it was necessary to produce clones from the progeny mixture and analyse each of them individually.

2.23 Characterization of the cloned progeny of the cross

2.23.1 Cloning the progeny

Progeny clones were obtained from the products of the cross by limiting dilution, as described in **Section 2.4.3**. In some experiments, the uncloned progeny were treated with pyrimethamine in a standard drug test, and clones made from the surviving resistant parasites.

2.23.2 RFLP analysis of progeny clones

All progeny clones were tested for the inheritance of AJ- or AS(3CQ)-alleles of markers. Southern blots of restricted genomic DNA from progeny clones and parental clones were made, and hybridised with radiolabelled genome markers. Each progeny

clone was marked as having inherited one or other of the parental-type RFLPs associated with the marker.

2.23.3 Phenotyping the progeny for chloroquine resistance

Those progeny clones which were identified as recombinant through linkage analysis were phenotyped for susceptibility to chloroquine using the standard test for chloroquine resistance. Parental clones AS(3CQ) and AJ were included as controls. Each test was repeated at least twice. Clones were typed as chloroquine resistant if a parasite infection appeared by D_{13} post-inoculation, and chloroquine sensitive if parasites appeared on or after D_{15}.

2.23.4 Parental growth tests

During the progeny phenotyping it became apparent that parental clone AJ was growing faster than parental clone AS(3CQ). It was decided to monitor the parasitaemia of each parental clone from a group of control mice during the progression of an infection. Groups of two mice for each clone were infected with 10^6 parasites and blood smears taken every 24 hours. Each smear was subsequently stained and inspected.

2.23.5 Linkage analysis

The inheritance of all markers by the recombinant progeny clones and their chloroquine phenotype was analysed for linkage. Any marker showing linkage with 10 or more progeny clones was investigated using the binomial coefficient. This enabled a probability value to be assigned to each candidate marker, as an indication of the likelihood of linkage between the marker and a gene determining chloroquine resistance.

3. Results: Analysis of the *P. chabaudi* genome

3.1 Karyotype analysis of *P. chabaudi*

Previous work has shown that *P. chabaudi* appears to contain up to 14 chromosomes (Langsley *et al.*, 1987; Sharkey *et al.*, 1988; Sheppard *et al.*, 1989b), although this was speculative because of the quality of PFGs at the time the work was carried out. During this project, PFGE analysis (as described herein), and restriction enzyme digestion of chromosomal bands and hybridisation with a telomeric probe (Janse *et al.*, 1994) has established beyond doubt that *P. chabaudi* has 14 chromosomes.

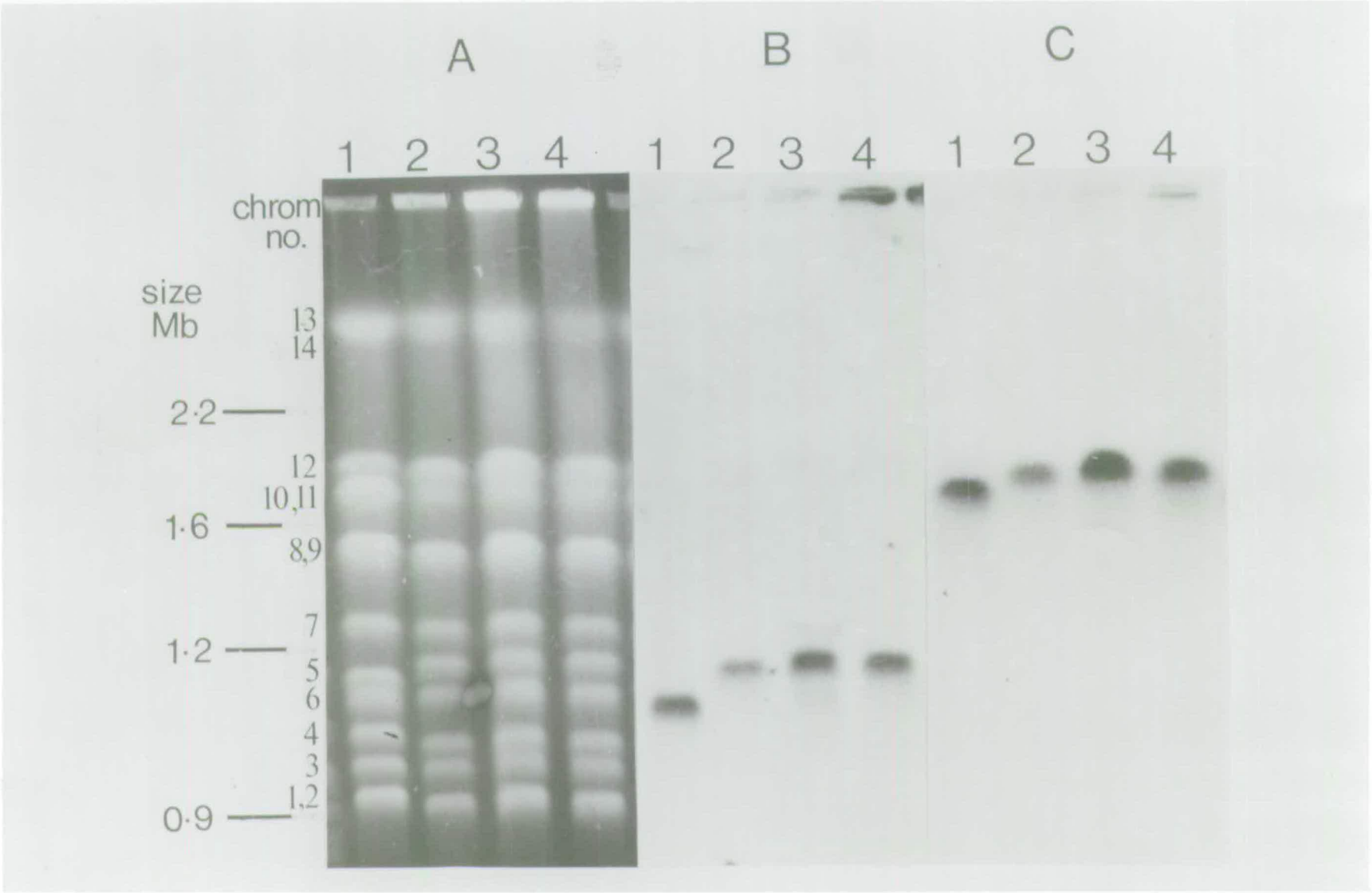
Figure 10A is a photograph of an ethidium bromide-stained PFG. The fourteen chromosomes range in size from 0.9 Mb (chromosomes 1 and 2) to 3.2 Mb (chromosomes 13 and 14). Chromosomes 1 and 2 migrate as a single band, which stains with ethidium bromide at a greater intensity than other bands which contain only single chromosomes. AJ chromosomes 5 and 6 migrate in reverse order compared to the equivalent AS(3CQ) chromosomes, and they are polymorphic in size; AS(3CQ) chromosomes 5 and 6 are approximately 1.14 Mb and 1.20 Mb respectively, whereas the same chromosomes in AJ are approximately 1.18 Mb and 1.10 Mb respectively. **Figure 10B**, a PFG probed with the chromosome 6-specific marker, DNA polymerase α, illustrates this polymorphism clearly. Chromosome 11 is also polymorphic between the two clones, migrating with chromosome 12 in AS(3CQ), but with chromosome 10 in AJ. This is shown in **Figure 10C**. Finally, the two largest chromosomes 13 and 14 are numbered in reverse order, in keeping with the nomenclature devised by Janse *et al.* (1994) for rodent malaria chromosomes. In the other rodent malaria species *P .berghei*, *P. yoelii* and *P. vinckei*, chromosome 13 is smaller and migrates further than chromosome 14; in *P. chabaudi* however, chromosome 13 is larger than chromosome 14. This was shown using markers known to be on each of these chromosomes in *P. berghei*, *P. yoelii* and *P. vinckei* (Janse *et al.*, 1994).

No obvious chromosomal rearrangements were found in the karyotypes of the AS clone selected for pyrimethamine resistance, or of the clone selected for chloroquine resistance, compared with the original AS(sens) clone from which they were selected (**Figure 10A**). This was not unexpected for the pyrimethamine resistant line AS(0CQ) as there is evidence that the resistance is due to a point mutation in the gene encoding DHFR (Cheng and Saul, 1994), which would not alter the size of chromosome 7 on which the gene is located. The absence of large chromosomal

Figure 10. Karyotype and chromosome number of *P. chabaudi* chromosomes of clones AJ, AS(sens), AS(0CQ) and AS(3CQ).

P. chabaudi chromosomes were separated by PFGE (**A**), and probed with DNA markers *DNA polymerase* α (**B**) and *PCNA* (**C**). Chromosomes in Lane 1 (clone AJ) are numbered according to the karyotype shown by clone AS, which is taken as the standard for *P. chabaudi*. As a consequence, chromosome numbers 5 and 6 are shown in reverse order, and chromosome 11 is shown next to chromosome 10 (discussed in the text).

Lane 1 AJ
Lane 2 AS(sens)
Lane 3 AS(0CQ)
Lane 4 AS(3CQ)


A
B
C
1 2 3 4
1 2 3 4
1 2 3 4
chrom no.
size Mb
13
14
2·2
12
10,11
1·6
8,9
7
1·2
5
6
4
3
1,2
0·9

rearrangements in AS(3CQ) also suggested that the mechanism behind this type of resistance did not involve gene amplification.

The PFGE conditions used here produced poor resolution of the high molecular weight chromosomes 10, 11, 12, 13 and 14. Small scale chromosomal rearrangements could have occured in these chromosomes which were not detectable under the conditions used here.

3.2 Chromosome locations and RFLPs of markers

Sixty-one characterised markers were analysed for their suitability as polymorphic markers for genetic crossing work with *P. chabaudi*. Each of these probes was radiolabelled and hybridised to Southern blots of *P. chabaudi* PFGs, as well as to blots of AJ and AS(3CQ) genomic DNA which had been cut with a variety of restriction enzymes. The chromosomal locations and RFLPs detected for each probe are listed in **Table 7**. For cross-reference, each marker is given the same Probe Number as in **Table 5**. Various washing conditions were employed to optimise the hybridisation, but only those conditions which produced the best results are given in the table.

Table 7. Chromosome location and RFLPs of *Plasmodium* markers analysed for their suitability as polymorphic DNA markers

Probe No.	Genome Marker	Washing Stringency	*P.chabaudi* Chromosome Location	Enzymes Tested for RFLPs	RFLP(s)
1	*Ca*$^{2+}$-*ATPase*	1 x SSC, 0.1% SDS, 60°C	2[a]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Bam* HI, *Eco* RI, *Hind* III, *Ase* I, *Pac* I, *Pme* I, *Asn* I, *Dpn* I, *Mae* I, *Mae* II, *Sfu* I, *Taq* I, *Ase* I, *Ssp* I	*Ase* I
2	235 kDa	1 x SSC, 0.1% SDS, 47°C	1/2[b], 3, 5, 8/9[b], 13/14[b]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I
3	pBS 110	Homologous probe	3	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Hind* II[c], *Rsa* I

4	*MSP-1*	"	8[a]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Dra* I, *Sau* 3A, *Rsa* I
5	*PcEMA-1*	"	10	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Hinf* I (faint)
6	*DHFR*	"	7	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Hind* II
7	*pfmdr1*	PFG blots: 1 x SSC, 0.1% SDS, 50°C; RFLP blots: 2 x SSC, 0.1% SDS, 42°C	12	*Hind* II, *Hinf* I, *Sau* 3A, *Ase* I, *Mse* I	None
8	*TBP*	1 x SSC, 0.1% SDS, 50°C	11	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Dra* I, *Hinf* I, *Rsa* I
9	*Enolase*	1 x SSC, 0.1% SDS, 50°C	12	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	None
10	*DHPS*	1 x SSC, 0.1% SDS, 50°C	13/14	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	None
11	*H2A*	1 x SSC, 0.1% SDS, 55°C	11	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Hind* III, *Ase* I	*Hind* III
12	9.2	1 x SSC, 0.1% SDS, 50°C	5, 11	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Hind* II, possibly *Rsa* I,
13	*RPIII*	1 x SSC, 0.1% SDS, 55°C	13	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I
14	*5S rRNA*	1 x SSC, 0.1% SDS, 50°C	10	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Hind* II, *Rsa* I

15	*EF1-α*	1 x SSC, 0.1% SDS, 50°C	11	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Hind* II
16	*CDC2*	1 x SSC, 0.1% SDS, 60°C	11	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Hind* II, *Rsa* I
17	*VAP A*	1 x SSC, 0.1% SDS, 50°C	14	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	None
18	*VAP B*	1 x SSC, 0.1% SDS, 50°C	10	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Bam* HI, *Eco* RI, *Hind* III, *Ase* I, *Pac* I, *Pme* I	*Hind* III
19	*G6PD*	1 x SSC, 0.1% SDS, 50°C	13[a]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Hind* II
20	*hsp-70*	Homologous probe	7, 12	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	None
21	*Pfcrk-1*	1 x SSC, 0.1% SDS, 45°C	7	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Bam* HI, *Eco* RI, *Hind* III, *Ase* I, *Pac* I, *Pme* I	None
22	*Pfmap-1*	1 x SSC, 0.1% SDS, 50°C	10	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	possibly *Hind* II
23	*Pfcrk3*	1 x SSC, 0.1% SDS, 50°C	13	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Sau* 3A, possibly *Hind* II
24	*DNA pol α*	1 x SSC, 0.1% SDS, 50°C	6	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Hinf* I
25	*DNA pol δ*	1 x SSC, 0.1% SDS, 50°C	5	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Rsa* I

26	*RAP-1*	6 x SSC, 0.1% SDS, 42°C to 4 x SSC, 0.1% SDS, 65°C, in increments of 5°C and decrements of 1 x SSC, produced either much background or no significant hybridisation to both PFGs and RFLP blots.			
27	*Topo I*	2 x SSC, 0.1% SDS, 50°C	11	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Rsa I*
28	*Topo II*	1 x SSC, 0.1% SDS, 55°C	10	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Hinf* I
29	*PCNA*	1 x SSC, 0.1% SDS, 45°C	11	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Hind* II
30	*GPI*	1 x SSC, 0.1% SDS, 50°C	10	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I possibly but faint
31	*HPRT*	1 x SSC, 0.1% SDS, 50°C and 0.5 x SSC, 0.1% SDS, 60°C, produced either much background or no significant hybridisation to both PFGs and RFLP blots.			
32	*Calmod-ulin*	PFG blots: 0.5 x SSC, 0.1% SDS, 65°C; RFLP blots: 2 x SSC, 0.1% SDS, 55°C	10	*Alu* I, *Dra* I, *Hinf* I, *Sau* 3A, *Rsa* I	Bands faint; no RFLPs
33	Pcsv4	0.5 x SSC, 0.1% SDS, 65°C	13/14	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Bam* HI, *Eco* RI, *Hind* III, *Ase* I, *Pac* I, *Pme* I	None
34	*RESA*	Homologous probe	6	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Hinf* I, *Rsa* I

35	*α1 tubulin*	1 x SSC, 0.1% SDS, 50°C	4 or 5	*Dra* I, *Hind* II, *Hinf* I, *Rsa* I	*Hind* II
36	*α11 tubulin*	0.5 x SSC, 0.1% SDS, 55°C	4 or 5	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Hind* II, *Rsa* I
37	*aldo-1*	1 x SSC, 0.1% SDS, 60°C	13[a]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Hind* II
38	*β tubulin*	1 x SSC, 0.1% SDS, 50°C	12	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Rsa* I	None
39	*AMA-1*	Homologous probe	9[a]	*Dra* I, *Hind* II, *Hinf* I, *Ase* I, *Ssp* I, *Swa* I	*Ssp* I, *Hinf* I
40	*pfran*	0.5 x SSC, 0.1% SDS, 50°C	9[a]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Hind* II
41	*RNA pol II*	1 X SSC, 0.1% SDS, 65°C	9[a]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Ase* I, *Mse* I, *Ssp* I, *Swa* I	None
42	cDNA 167	"	5[d]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Ase* I, *Mse* I, *Ssp* I	None
43	cDNA 121	"	10[e]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Hind* II
44	cDNA 365	"	12[e]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I
45	cDNA 148	"	13[e,f]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Dra* I
46	Ag3008	"	1/2	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	None
47	Ag3003 A	"	3	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Rsa* I
48	Ag3003 B	"	3	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	None

49	Ag3020	"	1/2	*Dra* I, *Hind* II, *Sau* 3A, *Rsa* I	*Dra* I, *Hind* II, *Sau* 3A
50	Ag3024	"	5	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Rsa* I	*Dra* I
51	Ag3015	"	5 (faint), 8/9, 13/14 (faint)	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Bam* HI, *Eco* RI, *Hind* III, *Ase* I, *Pac* I, *Pme* I	*Hinf* I, *Eco* RI, both 'blurred'
52	Ag3027	"	9[f]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Hinf* I
53	Ag3042 A	"	5, 6, 8/9, 12	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	None
54	Ag3042 B	"	1/2, 4, 5, 7, 8/9, 11; 13 AJ only	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Hind* II, *Sau* 3A, *Rsa* I
55	Ag3010	"	8/9	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Bam* HI, *Eco* RI, *Hind* III, *Ase* I, *Mse* I, *Ssp* I	*Dra* I
56	Ag3012	"	7	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Bam* HI, *Eco* RI, *Hind* III, *Ase* I, *Pac* I, *Pme* I	*Hinf* I
57	Ag3035	"	5	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I
58	Ag3037	"	5, 6, 8/9, 12	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	None
59	Ag3040	"	12, 14[f] (weak signal)	*Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Dra* I, *Hinf* I, *Sau* 3A, *Rsa* I
60	Ag3062	"	1/2	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	None

61	Ag3057	"	5, 6, 8/9, 12	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	None

[a] As determined by Janse *et al.* (1994).

[b] Incomplete separation of chromosomes 1 and 2, 8 and 9 and 13 and 14 during PFGE made it difficult to determine the precise chromosomal location of some probes. In such cases, the chromosome location is referred to as 1/2, 8/9 and 13/14.

[c] Restriction enzymes underlined represent those used to analyse the inheritance of RFLPs in the progeny of the AJ x AS(3CQ) cross.

[d] As determined by Sharkey *et al.* (1988)

[e] Previous chromosomal location as determined by Sharkey *et al.* (1988) was inaccurate due to the quality of PFGs at the time the work was carried out

[f] Chromosomal location determined through inheritance of alleles in the progeny of the AJ/AS(3CQ) cross. See **Table 14**.

3.3 Markers obtained from *P. chabaudi* genomic DNA library

3.3.1 Identification of recombinant DNA plasmids

A genomic DNA library of clone AJ was constructed in order to produce random markers within the *P. chabaudi* genome. 72 recombinant colonies chosen by blue/white colour selection were collected and screened for the presence of repetitive and low copy number DNA sequences. **Table 8** presents the results.

Table 8. Recombinant plasmids from a *P. chabaudi* genomic DNA library

recombinant plasmids containing low copy number DNA sequences	**P1**, **P9**, **P10**, **P11**, **P12**, **P13**, P14, P18, **P20**, **P21**, **P22**, **P23**, P25, **P29**, **P31**, **P36**, **P38**, P53, P55, P57, P61, P67, P71, P72, P73, P81, P82, P84, P88, P90, P97, P109, P110, P113, P116, P117, P119, P122, P128, P131, P138, P139, P141
recombinant plasmids containing repetitive DNA sequences	R33, R77, R102, R104, R106, R107, R118, R124, R129, R132, R133, R134, R136
recombinant plasmids containing partially repetitive DNA sequences	3, 7, 17, 19, 59, 68, 69, 70, 78, 79, 80, 94, 95, 99, 112, 142

Plasmids in bold face were analysed for chromosome location and RFLPs, as indicated in **Table 9**.

Forty-three plasmids appeared to contain low copy number DNA, and the remaining 29 to contain repetitive or partially repetitive DNA. This represents approximately 60% of the recombinants which contained low copy number DNA sequences. This can be compared with the anonymous *P. falciparum* library screened for polymorphic loci (Walker-Jonah *et al.*, 1992), in which 50% of the recombinant plasmids contained unique sequences. However, in the current work, the small number of *P. chabaudi* recombinants obtained indicates that the library was not representative of the whole *P. chabaudi* genome; low copy number DNA may have been preferentially cloned over repetitive DNA.

3.3.2 Chromosome location and RFLPs of unique sequence DNA

Plasmid inserts which appeared to be of unique sequence were radiolabelled and used to probe Southern blots of *P. chabaudi* PFGs and restricted AJ and AS(3CQ) genomic DNA. **Table 9** gives the chromosomal location and RFLPs of these markers. In addition, each marker has been given a Probe Number.

Table 9. Insert size, chromosome location and RFLPs of markers analysed from an anonymous *P.chabaudi* library.

Plasmid No.	Insert Size (kb)	*P.chabaudi* Chromosome Location	Restriction Enzymes Tested for RFLPs	RFLP(s)	Probe No.
P.1	0.35	4	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Ase* I, *Mse* I, *Ssp* I, *Swa* I	*Ssp* I	62
P.9	0.8	4	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I, *Hind* II[c]	63
P.10	1.0	13/14[b]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	none	64
P.11	0.5	no hybridisation	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	no hybridisation	65
P.12	0.55	6	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Sau* 3A	66
P.13	0.52	13/14	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	no hybridisation to RFLP blots	67

P.20	0.5	no hybridisation	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Ase* I, *Mse* I, *Ssp* I	no conclusive hybridisation to any RFLP blot	68
P.21	0.45	unable to radiolabel marker			69
P.22	1.4	11	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Ase* I, *Mse* I, *Ssp* I, *Bam* HI, *Eco* RI, *Hind* III, *Pac* I, *Pme* I, *Asn* I, *Dpn* I, *Mae* I, *Mae* II, *Sfu* I, *Taq* I	*Asn* I, *Mae* II	70
P.23	1.2	7	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I, *Ase* I, *Mse* I, *Ssp* I, *Pac* I, *Swa* I, *Pme* I	*Ase* I	71
P.29	0.7	5	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Alu* I	72
P.31	0.6	no hybridisation	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	no hybridisation	73
P.36	1.1	13/14	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	none	74
P.38	1.3	8/9[b]	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	none	75

Legend as for **Table 7**.

Thus, of the 14 recombinants containing unique sequence DNA which were analysed, 6 (approximately 43%) produced RFLPs. This is in contrast to the *P. falciparum* library of Wellems *et al.* in which only 5-10% of the anonymous probes produced useful RFLPs (Wellems *et al.*, 1991; Walker-Jonah *et al.*, 1992). However only a small number of unique sequence recombinants were screened, which may have resulted in bias.

3.4 Chromosome locations and RFLPs of RAPD-PCR markers

The technique of RAPD-PCR was explored in an endeavour to reveal DNA polymorphisms more quickly and efficiently than was possible by screening libraries for RFLPs. Preliminary experiments showed that primers less than 10 bp in length, and less than 60-70% G + C, did not produce as many amplified products as primers with those characteristics (data not shown). Accordingly, 80 decamer primers of 60-70% G + C were chosen at random (**Appendix 2**) and used to amplify DNA from both parasite lines.

3.4.1 Initial RAPD-PCR results

In an initial screening, 51 out of the 80 primers produced one or more amplified DNA bands which were polymorphic between the two clones. Each of the three different polymorphisms expected (**Section 2.19**) was observed, and examples of these are given in **Figure 11**.

As can be seen, most of the bands were present in both AJ and AS. **Figure 11A** shows the products amplified using primer OPR-12. A 0.5 kb band is present in AJ but absent from AS(3CQ). **Figure 11B** shows the products amplified using primer OPR-10. A 1.3 kb band has been amplified from AS(3CQ) DNA that stains more intensely with ethidium bromide than its conterpart in clone AJ. Presumably there has been greater amplification of the AS(3CQ) fragment compared to the AJ fragment. **Figure 11C** shows the products amplified using primer OPL-08. A 2.1 kb band is present in clone AJ but absent from AS(3CQ), and a second band of 1.9 kb is present in clone AS(3CQ) but absent from AJ. This may represent sequence differences between the primer binding sites at a single locus, or alternatively, such bands could be presence and absence types of polymorphism at two loci.

3.4.2 Amplified products within the negative control lanes

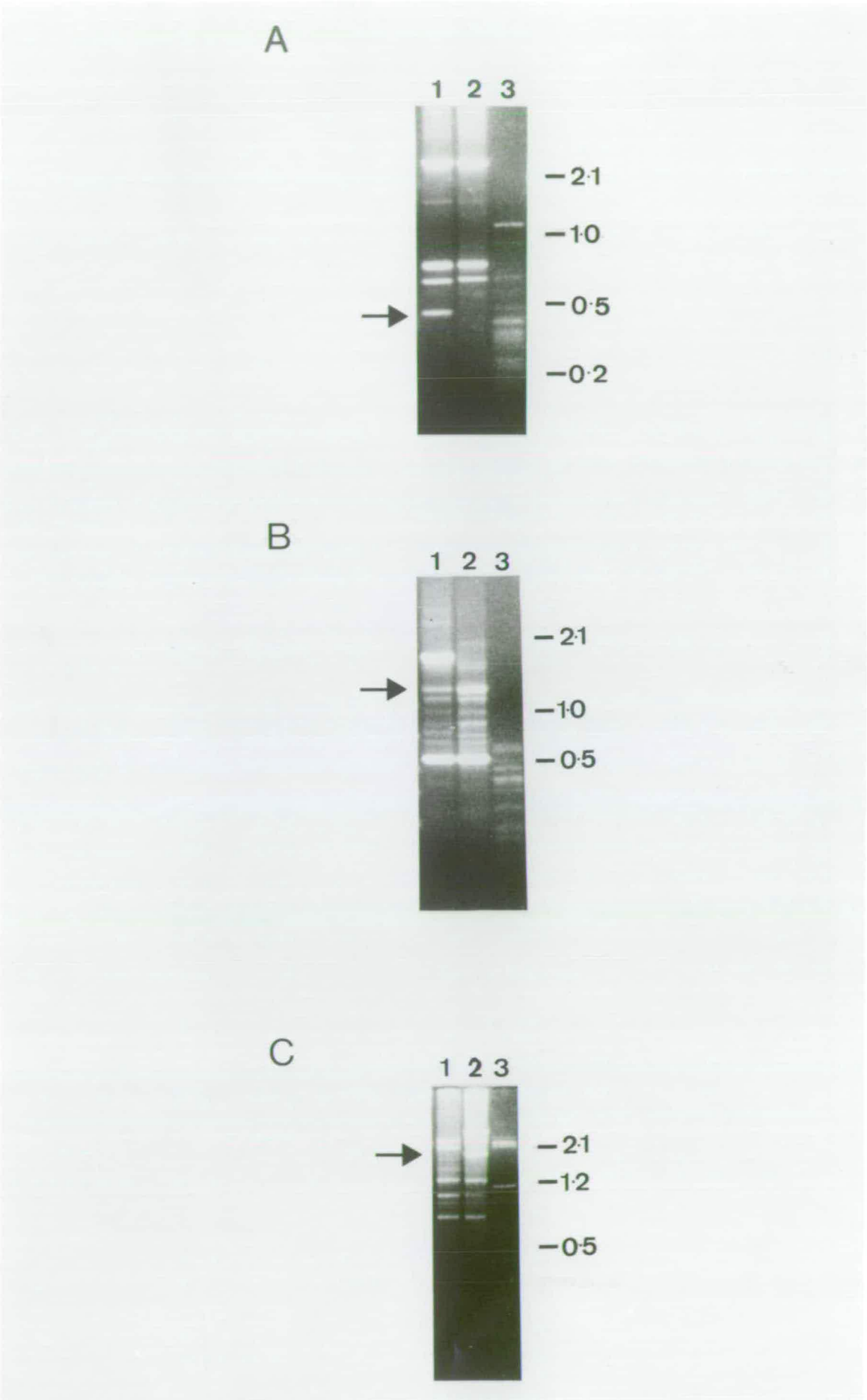
Figure 11 also shows the presence of amplified bands within the control lanes of all three primers. The majority of the other RAPD primers tested also produced bands in their negative controls. The control reactions contained all the components of an average RAPD-PCR reaction, except for 2 µl of TE in place of *P. chabaudi* DNA. In an attempt to identify the contaminating DNA, the amplified products of two negative controls were radiolabelled and used to probe Southern blots of restricted parasite DNA. No hybridisation to the blots was seen (data not shown), a result which eliminated the possibility of contamination of the controls by *P. chabaudi* DNA.

Recently it has been shown that contamination of preparations of *Taq* polymerase with *T.aquaticus* DNA is a common occurrence (Bottger, 1990). The most likely

Figure 11. Bands amplified from *P. chabaudi* genomic DNA using the RAPD-PCR technique.

RAPD-PCR reactions were carried out using primers (**A**) OPR-12, (**B**) OPR-10 and (**C**) OPL-08 . Arrows mark the site of polymorphic bands mentioned in the main text. Size markers to the right of each plate are in kb.

Lane 1 AJ DNA
Lane 2 AS(3CQ) DNA
Lane 3 control without DNA


A
1 2 3
2·1
1·0
0·5
0·2
B
1 2 3
2·1
1·0
0·5
C
1 2 3
2·1
1·2
0·5

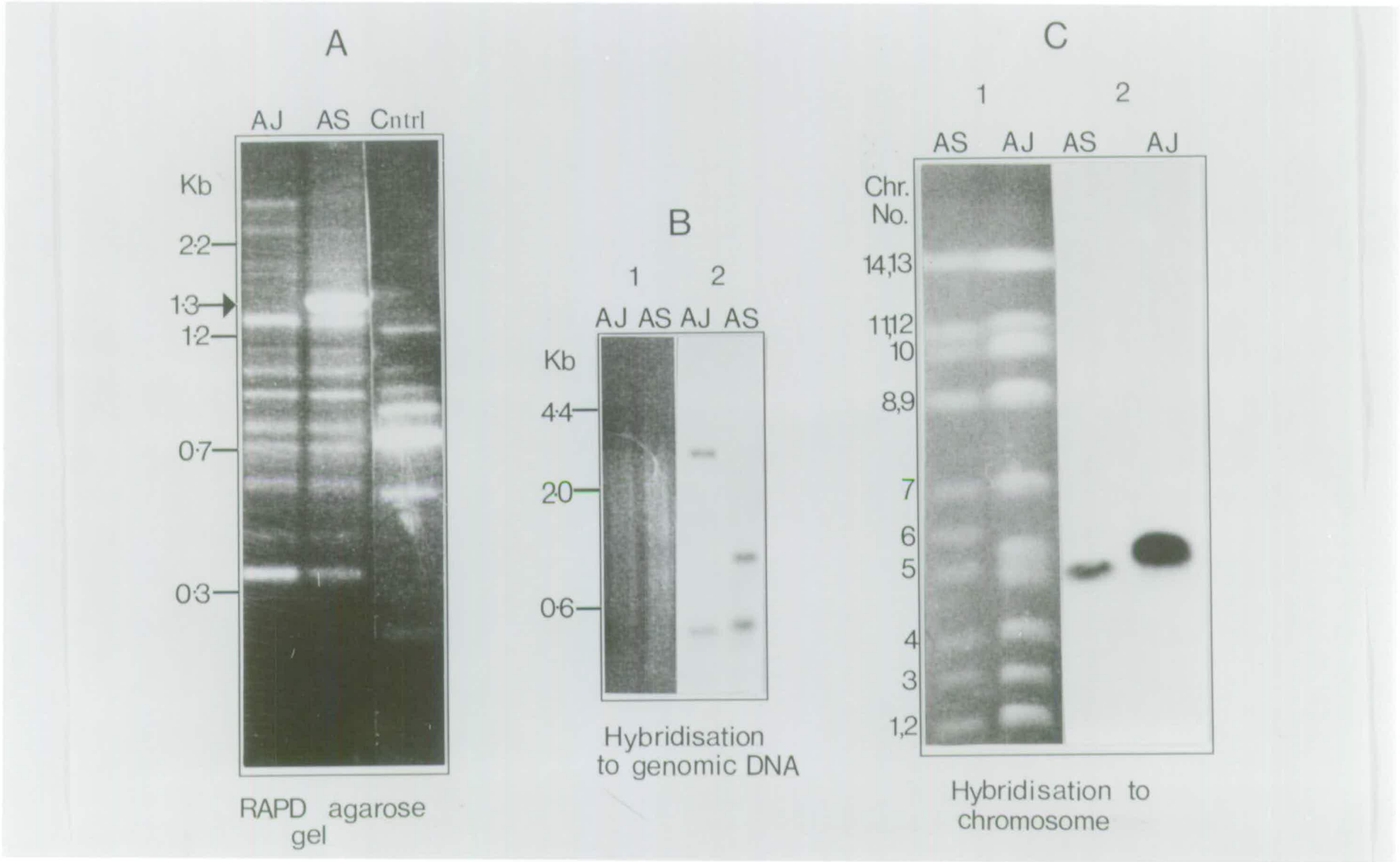
explanation for the presence of the amplified bands in the negative controls is amplification of small amounts of such contaminating DNA. In the presence of an excess of *P. chabaudi* DNA, it is possible that the primers are able to anneal to this DNA at more sites, and so to 'compete-out' the contaminating material.

3.4.3 Reproducibility of the RAPD-PCR technique

RAPD-PCR reactions were repeated at least three times for each primer. It soon became apparent that the reproducibility of the technique was poor. Although the majority of the primers produced at least one polymorphic band, these could not be reproduced every time. Some polymorphic bands were amplified on all three occasions, but there was no guarantee that they would be reproducible on subsequent occasions. This problem with reproducibility has been noted in many studies with RAPD-PCR on other organisms e.g. (Riedy *et al.*, 1992; Ellsworth *et al.*, 1993; Kernodle *et al.*, 1993; Meunier and Grimont, 1993; Schierwater and Ender, 1993). Some laboratories have optimised the PCR reagents and conditions for the organism under study (Dias Neto *et al.*, 1993; Tighe *et al.*, 1993; Waitumbi and Murphy, 1993) to give reproducible results.

In this work it was decided to circumvent the problem of reproducibility by first excising amplified polymorphic bands from the agarose gels and screening them for RFLPs in restriction enzyme digests of genomic DNA. An example is shown in **Figure 12.** The amplification products of the primer used, OPL-16, are shown first after gel electrophoresis (**A**). As can be seen, several DNA fragments were amplified, ranging in size from 0.3 to 2.4 kb. Two polymorphisms are evident, an amplified band of approximately 1.3 kb in AS which is absent from AJ, and a difference in the intensity of a band of approximately 0.35 kb, the AJ 0.35 kb band being more intense than the AS(3CQ) band. **Figure 12B** and **C** shows the OPL-16 1.3 kb polymorphic band, which has been excised and radiolabelled, hybridised to Southern blots of *Alu* I-restricted DNA and to *P. chabaudi* chromosomes in a PFG. An RFLP of this marker is clearly seen in digests of AS(3CQ) and AJ genomic DNA, and it appears to reside on chromosome 5.

In addition to the RFLP obtained with RAPD primer OPL-16, a further six polymorphic bands obtained from six different amplifications were screened for RFLPs. **Table 10** presents the type of RFLP and chromosome location for all seven bands. Five of the bands produced clear and reproducible RFLPs consistent with being present as single copies in the genome of each clone. The remaining two, OPL-08 and OPL-13, produced amplified bands which were present on more than one chromosome, and thus may contain repetitive sequences or be members of a multigene


A
AJ AS Cntrl
Kb
2·2
1·3
1·2
0·7
0·3
RAPD agarose gel
B
1 2
AJ AS AJ AS
Kb
4·4
2·0
0·6
Hybridisation to genomic DNA
C
1 2
AS AJ AS AJ
Chr. No.
14,13
11,12
10
8,9
7
6
5
4
3
1,2
Hybridisation to chromosome

family. Their precise chromosomal location was unclear due to incomplete separation of the chromosomes during PFG electrophoresis. Each RFLP was also given a Probe Number.

Table 10. RAPD primers which produced polymorphic bands subsequently used to detect RFLPs in *P.chabaudi*..

Primer	Sequence	RFLP	Chromosome Location	Probe No.
OPL-04	5'-GACTGCACAC-3'	*Eco* RI	11	76
OPL-12	5'-GGGCGGTACT-3'	*Hind* II	13	77
OPL-16	5'-AGGTTGCAGG-3'	*Alu* I, *Rsa* I	5	78
OPR-02	5'-CACAGCTGCC-3'	*Alu* I, *Hind* II, *Hinf*I,*Rsa* I	14	79
OPR-14	5'-CAGGATTCCC-3'	*Hind* II	12	80
OPL-08	5'-AGCAGGTGGA-3'	*Alu* I, *Dra* I, *Hind* II,*Hinf* I, *Sau* 3A, *Rsa* I, *Eco* RI	1/2, 7, 8/9, 11, 13/14	81
OPL-13	5'-ACCGCCTGCT-3'	*Alu* I, *Dra* I, *Hind* II,*Hinf* I, *Sau* 3A, *Rsa* I, *Eco* RI	1/2, 3, 4, 5 (AJ only), 14	82

3.5 The multiple drug resistance gene of *P. chabaudi*

3.5.1 Isolation and chromosome location

The multiple drug resistance (MDR) gene in *P. falciparum*, *pfmdr-1*, has been implicated in the mechanism of chloroquine resistance (Foote *et al.*, 1989; Wilson *et al.*, 1989; Foote *et al.*, 1990b). Accordingly it was decided to examine the rôle the homologous MDR gene in *P. chabaudi* might have in chloroquine resistance in this species.

Cross-hybridising the *pfmdr1* gene to Southern blots of *P. chabaudi* proved problematical; bad background and weak hybridisation signals meant that some restriction enzymes could not be tested for RFLPs, and those which were did not reveal an RFLP (**Table 7**, Probe Number 7). As a consequence, it was decided to isolate the *P. chabaudi* homologue of *pfmdr1*.

PCR oligonucleotides 857S and 858S, which recognise highly conserved nucleotide binding sites within the two ATP binding cassettes of *pfmdr1*, were used in an attempt to amplify *P. chabaudi* DNA. These primers amplify a 550 bp fragment from *P. falciparum*, which has been found to code for the amino terminal ATP binding cassette of *pfmdr1* (**Figure 13A**; A. Sultan, personal communication). Using the same primers, a 600bp fragment, called here pcATP-PCR, was amplified from both AJ and AS(3CQ) DNA, as shown in **Figure 13A**. The marker was given the Probe Number 83. Occasionally, a few faint, larger bands were amplified from both *P. falciparum* and *P. chabaudi* DNA. It is possible that these were the result of amplification of a fragment recognised by primer 858S of the ATP-binding cassette 1 and primer 857S of cassette 2.

Figure 13B shows the chromosome location of pcATP-PCR in *P. chabaudi* to be chromosome 12. *pfmdr1* is found on chromosome 5 in *P. falciparum* (Foote *et al.*, 1989; Wilson *et al.*, 1989). pcATP-PCR cross-hybridises to chromosome 5 of *P. falciparum* and *pfmdr1* cross-hybridises to chromosome 12 of *P. chabaudi* at a stringency of 50°C, 1 x SSC, 0.1% SDS, when hybridised to PFG blots of each respective species (data not shown). These results suggested that a part of the *P. chabaudi* MDR gene had been isolated; it was decided to clone and sequence the PCR amplified product of *P. chabaudi*.

3.5.2 Sequence analysis

The pcATP-PCR fragment was cloned and partially sequenced. The nucleic acid sequence and deduced amino acid sequence are shown in **Figure 14**. The DNA sequence was translated in all three reading frames and common restriction sites mapped using the MAP programme on the UWGCG (University of Wisconsin Genetics Computer Group) package (version 7), on the VAX computer, University of Edinburgh Computing Service. Alignment between the amino acid sequence of the *P. chabaudi* fragment and the two ATP binding cassettes of *pfmdr1* was made using the PILEUP programme (also from UWGCG), and this is shown in **Figure 15**. The PCR product shows more homology at the amino acid level with the carboxyl ATP-binding cassette of *pfmdr1* (C.*pfmdr1*) than with the amino ATP-binding cassette (N.*pfmdr1*). 86.7% of the residues are conserved between C.*pfmdr1* and pcATP-PCR, and 43.6% between N.*pfmdr1* and pcATP-PCR.

The high homology between *pfmdr1* and the *P. chabaudi* PCR fragment suggested that the MDR gene homologue of *P. chabaudi* had been isolated. The PCR fragment was renamed *pcmdr1*.

Figure 13. Amplification and chromosomal location of pcATP-PCR.

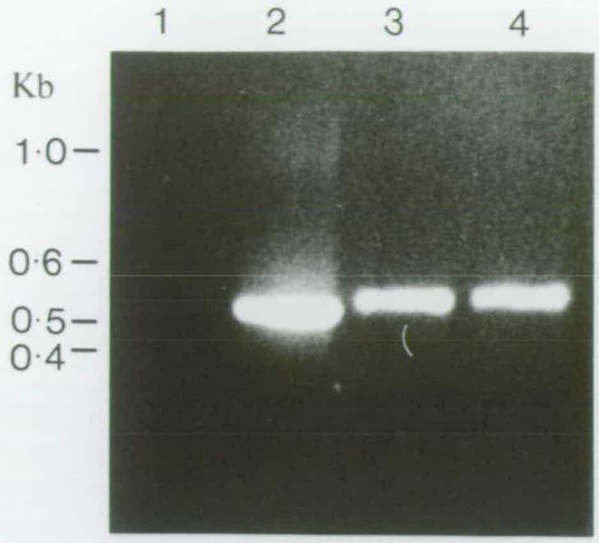
A. PCR products resulting from the amplification of *Plasmodium* DNA using primers which recognise the nucleotide binding sites of *pfmdr1*.

Lane 1 negative control
Lane 2 *P. falciparum* clone HB3
Lane 3 *P. chabaudi* clone AJ
Lane 4 *P. chabaudi* clone AS(3CQ)

B. Hybridisation of pcATP-PCR to chromosome separations of *P. chabaudi*. Chromosome numbering and sizes are as for **Figure 10**. The arrow points to chromosome 12.

Lane 1 AJ
Lane 2 AJ, after transmission through mosquitoes
Lane 3 AS(3CQ)
Lane 4 AS(3CQ), after transmission through mosquitoes

A



B

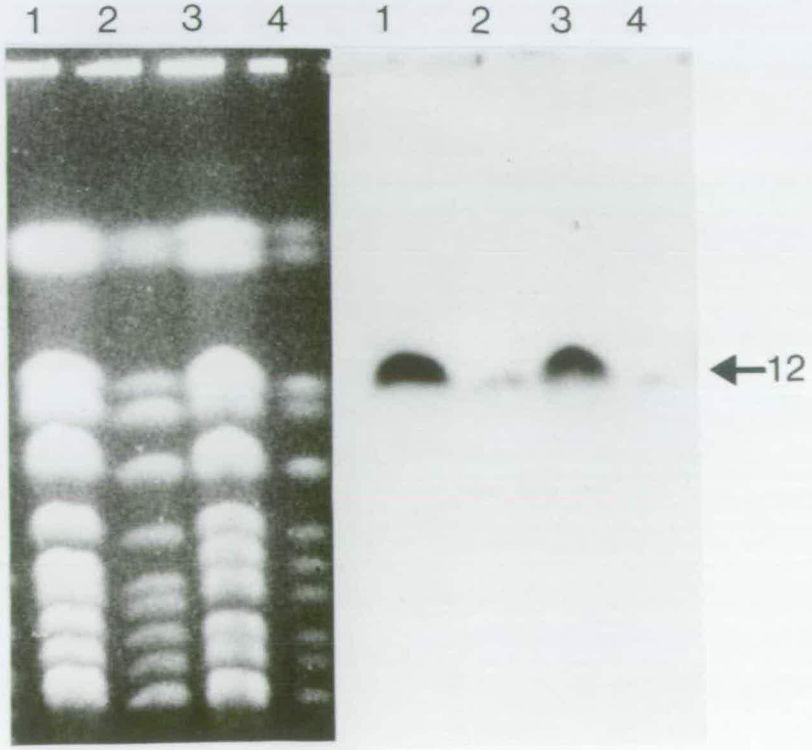


Figure 14. Nucleotide and deduced amino acid sequence of 301 bp of *P. chabaudi* marker pcATP-PCR.

```
1                                  Hinc II
AAGTCTCTAAGAAAATTATTTGCGATAGTTAACCAAGAACCAATGTTGTTTAATATGTCT
---------+---------+---------+---------+---------+---------+
K  S  L  R  K  L  F  A  I  V  N  Q  E  P  M  L  F  N  M  S
 S  L  *  E  N  Y  L  R  *  L  T  K  N  Q  C  C  L  I  C  L
  V  S  K  K  I  I  C  D  S  *  P  R  T  N  V  V  *  Y  V  Y

61
ATTTATGAAAATATAAAATTCGGTAAAGAAGATGCAACATAGAAGATGGTAAAAAGGGCT
---------+---------+---------+---------+---------+---------+
I  Y  E  N  I  K  F  G  K  E  D  A  T  L  E  D  V  K  R  A
 F  M  K  I  *  N  S  V  K  K  M  Q  H  *  K  M  *  K  G  L
  L  *  K  Y  K  I  R  *  R  R  C  N  I  R  R  C  K  K  G  L

121
TGTAGATTGGCTGCTATTGACGAATTTATTGAACCATTACCAAATAAATATGATACTAAT
---------+---------+---------+---------+---------+---------+
C  R  L  A  A  I  D  E  F  I  E  P  L  P  N  K  Y  D  T  N
 V  D  W  L  L  L  T  N  L  L  N  H  Y  Q  I  N  M  I  L  M
  *  I  G  C  Y  *  R  I  Y  *  T  I  T  K  *  I  *  Y  *  C

181        Hind III Alu I
GTAGGACCTTATGGAAAAAGCTTATCAGGTGGTCAAAAACAACGAGTTGCTATTGCTAGA
---------+---------+---------+---------+---------+---------+
V  G  P  Y  G  K  S  L  S  G  G  Q  K  Q  R  V  A  I  A  R
 *  D  L  M  E  K  A  Y  Q  V  V  K  N  N  E  L  L  L  L  E
  R  T  L  W  K  K  L  I  R  W  S  K  T  T  S  C  Y  C  *  S

241                                          <-----primer 857S----
GCCCTATTAAGAGAACCTAAAATATTGTTGTTAGACGAGGCCACATCATCTCTGGCATGC
---------+---------+---------+---------+---------+---------+
A  L  L  R  E  P  K  I  L  L  L  D  E  A  T  S  S  L  A  C
 P  Y  *  E  N  L  K  Y  C  C  *  T  R  P  H  H  L  W  H  A
  P  I  K  R  T  *  N  I  V  V  R  R  G  H  I  I  S  G  M  P

301
-->
CCC
---
P
```

Amino acid abbreviations are given in **Abbreviations**.

The section of sequence marked '**primer 857S**' refers to the oligonucleotide sequence incorporated during amplification of parasite DNA.

Restriction sites are underlined or are outlined in bold if two or more enzymes recognise the same sequence.

Figure 15. Comparison of pcATP-PCR with the two ATP binding cassettes of *pfmdr1*, at the amino acid level.

The amino (N.*pfmdr1*) and carboxyl (C.*pfmdr1*) ATP binding cassettes of *pfmdr1* are marked in blue, pcATP-PCR is marked in red, and matching residues between the *P. chabaudi* and *P. falciparum* sequences are marked in green. Dots indicate gaps in the sequence, which enable maximum alignment between the sequences.

```
                1                                  30
N.pfmdr1        TYENKNFSLI SN.....SMT SNELLEMKKE
C.pfmdr1        RDLRNLFSIV SQEPMLFNMS IYENIKFGRE
pcATP-PCR       KSLRKLFAIV NQEPMLFNMS IYENIKFGKE

               31                                  60
N.pfmdr1        YQTIKDSDVV DVSKKVLIHD FVSSLPDKYD
C.pfmdr1        DATLE..DVK RVSKFAAIDE FIESLPNKYD
pcATP-PCR       DATLE..DVK RACRLAAIDE FIEPLPNKYD

               61                                  90
N.pfmdr1        TLVGSNASKL SGGQKQRISI ARAIMRNPKI
C.pfmdr1        TNVGPYAKSL SGGQKQRIAI ARALLREPKI
pcATP-PCR       TNVGPYGKSL SGGQKQRVAI ARALLREPKI

               91          103
N.pfmdr1        LILDEATSSL DNK
C.pfmdr1        LLLDEATSSL DSN
pcATP-PCR       LLLDEATSSL ACG
```

3.5.3 RFLPs and copy number of *pcmdr1*

Twelve restriction enzymes were screened for RFLPs within *pcmdr1* to give an indication of the copy number of the gene. The copy number was important for two reasons; firstly, to exclude the possibility that the cloned fragment was part of a pseudogene, and secondly to search for amplification of the gene in parasite lines which had been selected for drug resistance.

Figure 16 shows the results of two Southern blots of restricted *P. chabaudi* DNA hybridised with radiolabelled *pcmdr1*. The number of restriction sites determined by

Figure 16. Hybridisation of *pcmdr1* to Southern blots of *P. chabaudi* restricted DNA.

Restricted DNA samples are to the left of the plate, and autoradiographs from hybridisations to the right.

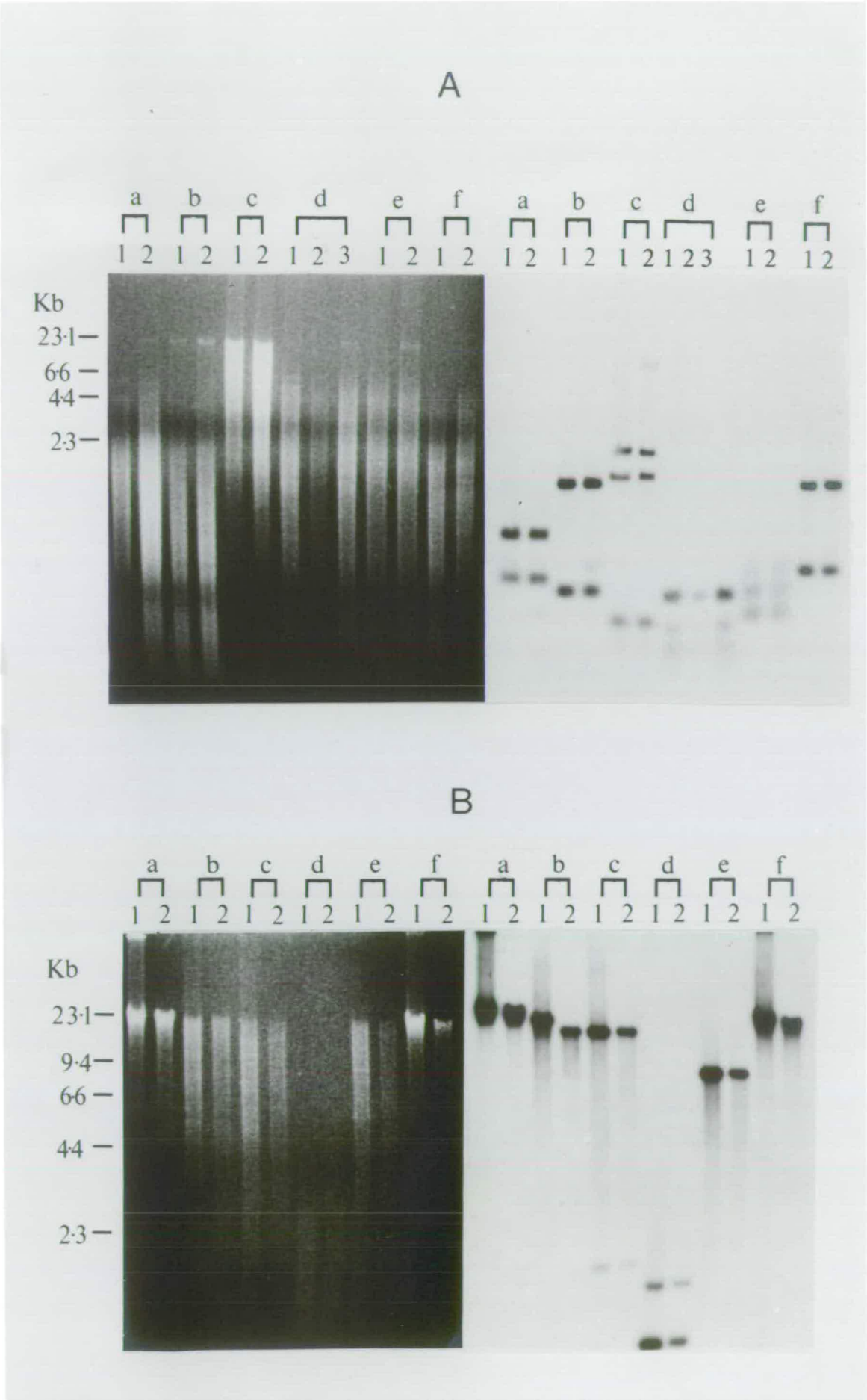
Lane 1 AJ genomic DNA

Lane 2 AS(3CQ) genomic DNA

Lane 3 Second sample of AS(3CQ) genomic DNA

A. DNA restricted with:
- a *Alu* I
- b *Dra* I
- c *Hind* II
- d *Hinf* I
- e *Sau* 3A
- f *Rsa* I

B. DNA restricted with:
- a *Bam* HI
- b *Eco* RI
- c *Hind* III
- d *Ase* I
- e *Pac* I
- f *Pme* I


A
a b c d e f a b c d e f
1 2 1 2 1 2 1 2 3 1 2 1 2 1 2 1 2 1 2 1 2 3 1 2 1 2
Kb
23·1
6·6
4·4
2·3
B
a b c d e f a b c d e f
1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2
Kb
23·1
9·4
6·6
4·4
2·3

sequencing the PCR fragment correlate with the number of bands seen on the autoradiographs, with the exception of *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I and *Ase* I which produced bands that had not been predicted from the restriction map. However, digestion of the *pcmdr1* fragment with each enzyme revealed the presence of these restriction sites (data not shown). The sites probably exist in the section of the fragment that was not sequenced, or alternatively, the sequence data may be slightly inaccurate. Problems were encountered during sequencing of *pcmdr1*, resulting in poor quality autorads which may account for this.

Only *Eco* RI produced a size polymorphism between AJ and AS(3CQ) (**Figure 16B**), and this was subsequently used to follow the inheritance of *pcmdr1* among the progeny of the cross.

Hybridisation with *pcmdr1* and washing of PFG blots under conditions of low stringency (1 x SSC, 0.1% SDS, 50°C), revealed possible homologous genes to be located on *P. chabaudi* chromosomes 3 and 6 (data not shown). This is discussed further in **Chapter 6.**

3.6 The *P. chabaudi* genome marker pcpS590.7

3.6.1 Isolation and chromosome location

In an attempt to isolate the *P. chabaudi* homologue of pS590.7, the *P. falciparum* chromosome 7 marker linked to a chloroquine resistance gene as postulated by Wellems *et al.* (1991), oligonucleotide primers 421K and 422K which recognised pS590.7 were made. These amplify a 456bp fragment of pS590.7 from DNA of *P. falciparum* clones Dd2 and HB3 (Wellems *et al.*, 1991), referred to here as pfpS590.7. This fragment recognises an RFLP in Southern blots of *Rsa* I restricted Dd2 and HB3 DNA, which segregates with chloroquine resistance in the progeny of the HB3 x Dd2 cross of Wellems *et al.*.

The oligonucleotides were used in a PCR reaction to amplify possible homologous sequences from *P. chabaudi*. A high annealing temperature of 52.5°C was used to prevent non-specific amplification. The results are shown in **Figure 17A**. A 400bp PCR fragment referred to as pcpS590.7 was amplified from both AJ and AS(3CQ) DNA. The AJ pcpS590.7 fragment was cloned and subsequently used to probe Southern blots of *P. chabaudi* chromosomes. **Figure 17B** shows that pcpS590.7 is found on chromosome 13 in *P. chabaudi*. The marker was given the Probe Number 84.

To obtain further evidence that pcpS590.7 could be the *P. chabaudi* homologue of pfpS590.7, the AJ PCR fragment was used to probe a Southern blot of *P. falciparum* separated chromosomes, as shown in **Figure 18A**. This shows that pcpS590.7

Figure 17. Amplification of *P. chabaudi* marker pcpS590.7 by PCR, and hybridisation to chromosomes of *P. chabaudi*.

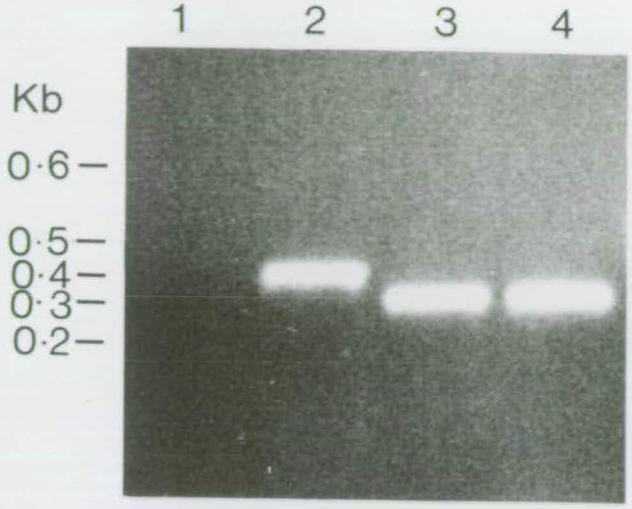
A. PCR products resulting from the amplification of *Plasmodium* DNA using primers which recognise marker pS590.7.

Lane 1 negative contol
Lane 2 *P. falciparum* clone HB3
Lane 3 *P. chabaudi* clone AJ
Lane 4 *P. chabaudi* clone AS(3CQ)

B. Hybridisation of pcpS590.7 to chromosome separations of *P. chabaudi*. Chromosome numbering and sizes are as for **Figure 10**. Although the figure is not clear, careful measurement of the distance between the wells and the signal showed the marker to be located on chromosome 13 (arrow).

Lane 1 AJ
Lane 2 AJ, after transmission through mosquitoes
Lane 3 AS(3CQ)
Lane 4 AS(3CQ), after transmission through mosquitoes

A



B

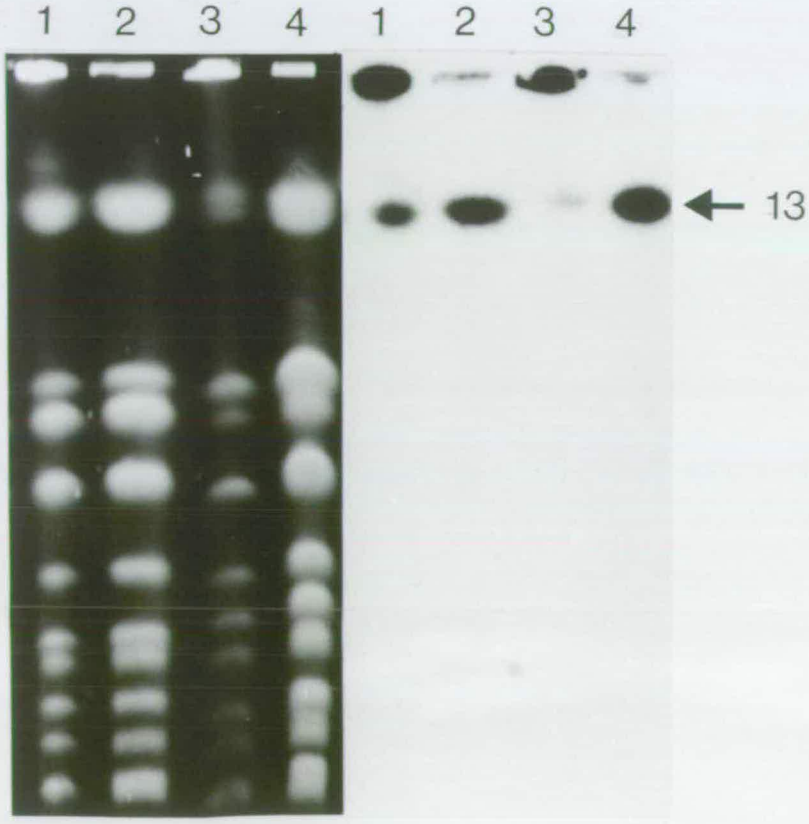


Figure 18. Hybridisation of *P. chabaudi* marker pcpS590.7 to *P. falciparum* chromosome separations and to Southern blots of *P. chabaudi* restricted genomic DNA.

A. Hybridisation of pcpS590.7 to chromosome separations of *P. falciparum.*

Lane 1 *P. falciparum* clone 3D7

Lane 2 *P. falciparum* clone Dd2

(i) Chromosome separations of *P. falciparum* clones 3D7 and Dd2. Chromosome numbering and sizes are shown for clone 3D7 only.

(ii) Autoradiograph of pcpS590.7 hybridised to a blot of the gel.

(iii) Autoradiograph of a *P. falciparum* chromosome 7-specific probe, pS500.7 (see **Table 6** and **Figure 8**), hybridised to the blot to confirm the identity of the chromosome number. The blot was stripped prior to probing.

B. Hybridisation of pcpS590.7 to Southern blots of restricted *P. chabaudi* DNA.

Lane 1 AJ

Lane 2 AS(3CQ)

DNA restricted with:

a *Alu* I

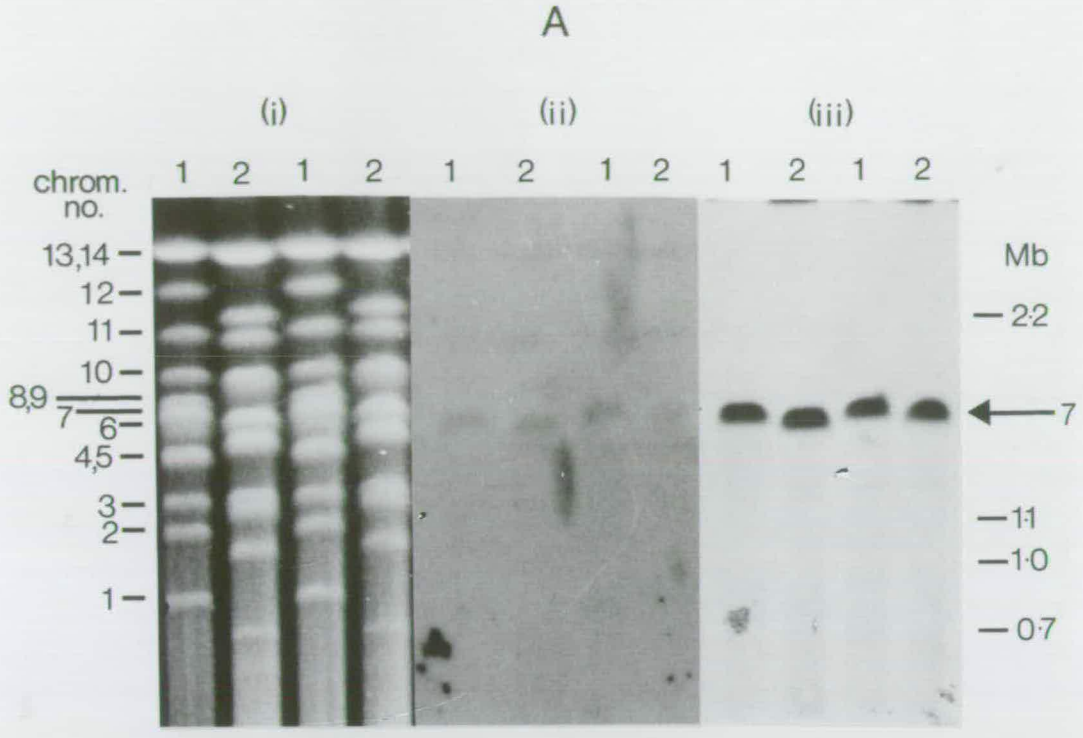
b *Dra* I

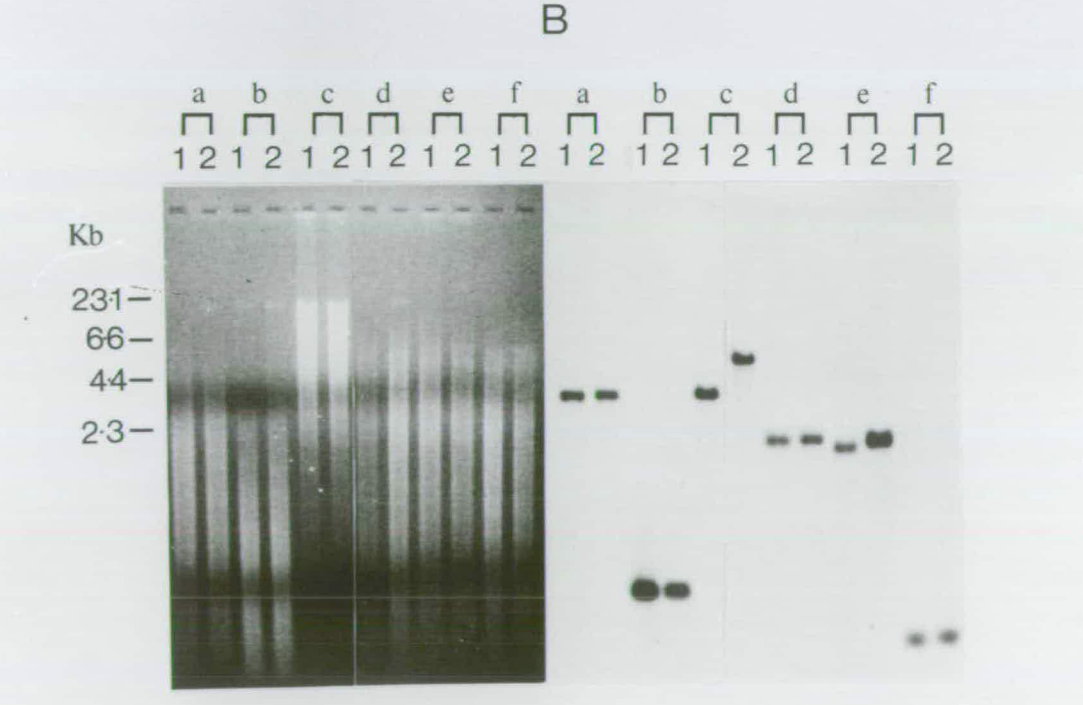
c *Hind* II

d *Hinf* I

e *Sau* 3A

f *Rsa* I


A
(i)
(ii)
(iii)
chrom. no.
1 2 1 2 1 2 1 2 1 2 1 2
13,14
12
11
10
8,9
7
6
4,5
3
2
1
Mb
2·2
7
1·1
1·0
0·7


B
a b c d e f a b c d e f
1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2 1 2
Kb
23·1
6·6
4·4
2·3

hybridises to *P. falciparum* chromosome 7, the same chromosome on which marker pS590.7 is located.

3.6.2 Copy number and RFLPs of pcpS590.7

Six restriction enzymes were screened for RFLPs within pcpS590.7 and to determine whether the marker exists as a single copy on chromosome 13. **Figure 18B** shows that *Hind* II and *Sau* 3A produced polymorphisms between AJ and AS(3CQ). Both RFLPs were subsequently used to follow the inheritance of pcpS590.7 among the progeny of the AJ x AS(3CQ) cross. Evidence suggests that the marker may occur as a single copy on chromosome 13, because no unexpected bands, as deduced from the restriction map (**Section 3.6.3**), appeared on the autoradiographs. The detection of a single *Sau* 3A band as opposed to the two predicted by the restiction map is expected because of the position of the *Sau* 3A site 14 bp from one end of the probe (**Section 3.6.3**); such a small region of homology is unlikely to have been sufficient for hybridisation of the probe to the second genomic DNA fragment.

3.6.3 Sequence analysis

Although the similar PCR product sizes, and the cross-hybridisation to *P. falciparum* chromosome 7 suggested that pcpS590.7 might be the homologue of pfpS590.7, it was decided to sequence pcpS590.7 and compare it to the sequence of pfpS590.7. **Figure 19** gives the nucleotide sequence of pcpS590.7 as deduced from dideoxy chain termination sequencing. The 400bp fragment is 92.8% A + T rich and does not appear to be within a coding region of DNA; each reading frame of the amino acid sequence (deduced using MAP, UWGCG) produced a stop codon (**Figure 19**).

Analysis of the nucleotide sequence of pfpS590.7, kindly provided by T. Wellems (National Institutes of Health, Maryland, U.S.A), revealed this DNA segment to be 77.5% A/T rich. It also does not contain an open reading frame as determined using MAP (data not shown).

A comparison of the two sequences was made using PILEUP (UWGCG) and this is shown also in **Figure 20**. There are many small regions of homology between the two sequences, for example from 54 bp to 72 bp, 16 out of 19 nucleotides are conserved. This is not surprising considering the level of A + T richness that exists in the two sequences. Thus the homology between the sequences could have occurred purely by chance.

Figure 19. Nucleotide and deduced amino acid sequence in all three frames, of *P. chabaudi* marker pcpS590.7.

```
1
ATTTGAAAATGGAGTTCGAAAATTTAATAATAATTATGATAAAAATGTTAGACGAAAAAT
---------+---------+---------+---------+---------+---------+
I  *  K  W  S  S  K  I  *  *  *  L  *  *  K  C  *  T  K  N
 F  E  N  G  V  R  K  F  N  N  N  Y  D  K  N  V  R  R  K  I
  L  K  M  E  F  E  N  L  I  I  I  M  I  K  M  L  D  E  K  F

61
TTTTAATTTACCAAAGTAAAAAGGAAATGGGATGGTAGTTTATGATGAAGAAGATGACT
A
---------+---------+---------+---------+---------+---------+--------+
F  *  F  T  K  D  K  R  K  W  D  G  S  L  *  *  R  R  *  L
 F  N  L  P  K  I  K  G  N  G  M  V  V  Y  D  E  E  D  D  Y
  L  I  Y  Q  R  *  K  E  M  G  W  *  F  M  M  K  K  M  T  I

121
TAATGATAGTCAAGAAAAAATCAGACAAAAAAAGAAAAGTAGGAAAGAGGAAAAACGAGA
---------+---------+---------+---------+---------+---------+---------+
*  *  *  S  R  K  N  Q  T  K  K  E  K  *  E  R  G  K  T  R
 N  D  S  Q  E  K  I  R  Q  K  K  K  S  R  K  E  E  K  R  E
  M  I  V  K  K  K  S  D  K  K  R  K  V  G  K  R  K  N  E  N

181
ACAAAAATTAAAAAGTATATTACAAGATATGAATAAAAAAAAAAACAAATATAACTAAAA
---------+---------+---------+---------+---------+---------+---------+
T  K  I  K  K  Y  I  T  R  Y  E  *  K  K  K  Q  I  *  L  K
 Q  K  L  K  S  I  L  Q  D  M  N  K  K  K  N  K  Y  N  *  K
  K  N  *  K  V  Y  Y  K  I  *  I  K  K  K  T  N  I  T  K  N

241
ATTCGATTGAAGATGAAATGGGTAATATATCAAATAAAATGAATAATTATAAAGACATT
A
---------+---------+---------+---------+---------+---------+--------+
I  R  L  K  M  K  W  V  I  Y  Q  I  K  *  I  I  I  K  T  L
 F  D  *  R  *  N  G  *  Y  I  K  *  N  E  *  L  *  R  H  *
  S  I  E  D  E  M  G  N  I  S  N  K  M  N  N  Y  K  D  I  K

301                                          Sau 3A        352
AAAATGTTATTGCTGATATATGTAATAATATTATGGGTGATCAAACTATGTA
---------+---------+---------+---------+---------+---------+--
K  M  L  L  L  I  Y  V  I  I  L  W  V  I  K  L  C
 K  C  Y  C  *  Y  M  *  *  Y  Y  G  *  S  N  Y  V
  N  V  I  A  D  I  C  N  N  I  M  G  D  Q  T  M
```

Amino acid abbreviations are listed in **Abbreviations**.

The single *Sau* 3A site is underlined.

Figure 20. Comparison of pcpS590.7 with pfpS590.7 at the nucleotide level.

pcpS590.7 is marked in blue, pfpS590.7 is marked in red, and matching bases are marked in green. Dots indicate gaps in the sequence, which enable maximum alignment between the sequences.

```
1                                                                   50
.......... .......... ..........A TTTGAAAATG GAGTTCGAAA
AATTCATCAA ACGTTTTGTT ACCCTTTGTT GAGGAGCATG AACATGTAAA
51                                                                 100
ATTTAATAAT AATTATGATA AAAATGTTAG ACGAAAAATT TTTAATTTAC
AGATAAAAAT GATAATGATA AATGTATAAA ATATGAATAT GTTAATGATA
101                                                                150
CAAAGTAAAA AGGAAATGGG ATGGTAGTTT ATGATGAAGA AGATGACTAT
AATGTATAAA AGATGAACAT GGTGAATTCG AAAGAGTAGA AGAGAATATA
151                                                                200
AATGATAGTC AAGAAAAAAT CAGACAAAAA AAGAAAAGTA GGAAAGAGGA
AAATTAAGTG AAGATATAAT AAATATTATT GAAAATATTT TAAAAAAATA
201                                                                250
AAA........ .........AC GAGAACAAAA ATTAAAAAGT ATATTACAAG
TAATGTTGTT TTATTTATGA AAGGCACAGC TTTAAATCCT TATTGTAAAT
251                                                                300
ATATGAATAA AAAAAAAAAC AAATATAACT AAAAATTCGA TTGAAGATGA
ATAGTAAACA AGCTATTCAC ATTTTAAAAT TAAATAAAGT AAAACAAATT
301                                                                350
AATGGGTAAT ATATCAAATA AAATGAATAA TTATAAAGAC ATTAAAAATG
CATACAGTCA ATATTTTAGA TAATCAAGAA TTAAGAAACG CTTTAAAAAT
351                                                                400
TTATTGCTGA TATATGTAAT AAT.....AT TATGGGTGAT CAAACTATGT
TTATTCGAAA CTGGCCTACA TTTCCTCAAT TATATGTTAA TCAAAAATTT
401
A........
ATAGGTGG
```

It was decided to look for further evidence that pcpS590.7 and pfpS590.7 might be related by hybridising other chromosome 7-specific *P. falciparum* markers to Southern blots of separated *P. chabaudi* chromosomes, and looking for regions of synteny. This is discussed in **Chapter 4**.

4. Results: Regions of synteny between *P. chabaudi* and *P. falciparum*

The criteria used for defining homologies between genes in different species have been proposed by the Committee on Comparative Mapping (Lalley *et al.*, 1987). The classification depends upon the number and order of the homologous genes, thus:
(i) homology segments refer to regions of DNA homology, for example within a single gene. This is the fundamental unit of comparative gene mapping, because it represents the first evidence concerning the location of a homologous chromosomal segment between species.
(ii) conserved syntenies are homology segments composed of two or more pairs of homologous genes located on the same chromosome, and regardless of gene order. This provides the first evidence of conservation of DNA sequences. Finally;
(iii) conserved linkages are groups of genes conserved not only in synteny, but also in gene order.

This chapter deals with regions of synteny conservation between the two species *P. chabaudi* and *P. falciparum* .

4.1 Synteny between the genomes of *P. falciparum* and *P. chabaudi*

During the search for RFLPs in the genome of *P. chabaudi*, many *P. falciparum* genes were cross-hybridised to *P. chabaudi* chromosomes to test their suitability as genome markers for the latter. It soon became apparent that certain pairs of homologous genes were conserved in their chromosomal location between the genomes of the two species.

The homologous genes identified as being present in such conserved syntenies are shown grouped together on their respective chromosomes in **Table 11**. The significance of these regions of synteny is discussed in **Chapter 6**.

Table 11. Conserved syntenies between the genomes of *P. falciparum* and *P. chabaudi.*

Chrom. No.	*P. falciparum* (3D7)	*P. chabaudi* (AS)
1	RESA[1] Ca^{2+} ATPase[2]	Ag3008(46)
2		Ca^{2+} ATPase(1)
3	RNA pol II[3] **PfTRAP-2[4]*, CSP[5].**	
4	**DHFR-TS[6], Pfcrk-1(21)*.** DNA pol α(24) αII tubulin(36) VAP ß(18)	**PfTRAP-2*, CSP[17].** α? tubulin[18]*
5	pfmdr-1(7) **TBP(8), Topo I(27)*.**	DNA pol δ* α? tubulin[18]*
6	Histone 2A(11)	RESA(34) DNA pol α*
7		**DHFR-TS(6), Pfcrk-1*.** hsp-70[19]
8	hsp-70-1[7] DHPS(10)	**PGK, MSP-1(4).** RNA pol II(41)*
9	**PGK[8], MSP-1[9].** αI tubulin(35)	**AMA-1(39), pfran*.**
10	**β tubulin[10], enolase(9).** DNA pol δ(25)	**5s rRNA(14)*, Topo II(28), Calmodulin(32), GPI(30), PfMAP-1(22)*.** VAP B*
11	exp-1[11] **AMA-1[12], pfran(40).**	**PCNA*, CDC2(16), EF1α*.** Histone 2A* **TBP*, Topo I*.**
12		pcmdr-1* hsp-70[19] **β tubulin(38)*, enolase*.**

13	PCNA(29), PfPK5[13], EF1α(15). VAP A(17) RNA pol III(13), pfcrk3(23)*, TRAP[14].	RNA pol III*, pfcrk-3*, SSP II[20]. Aldolase I(37) + II[21]*, G6PD*.
14	5s rRNA(14)*, Topo II(28), Calmodulin(32), GPI(30), PfMAP-1(22)*. Actin I[15] Aldolase [16], G6PD(19).	VAP A* Actin I

The table shows the chromosome location of 40 homologous genes in the reference clones 3D7 of *P. falciparum*, and AS of *P. chabaudi*. An asterisk marks those genes whose location was determined during this project. Groups of genes in bold colour represent synteny groups conserved between the two species; blue groups contain two genes, green groups contain three genes, and red groups contain five genes. Numbers in parenthesis refer to the Probe Number as given in **Tables 5** and **7**. Superscript numbers refer to notes for genes not previously mentioned, as follows:-

1 *P. falciparum* ring-infected erythrocyte surface antigen (Kemp *et al.*, 1987)

2 *P. falciparum* Ca^{2+}-ATPase gene (Kimura *et al.*, 1993); localised by Trottein and Cowman (1995)

3 *P. falciparum* RNA polymerase II gene (Li *et al.*, 1989)

4 *P. falciparum* TRAP-2 gene, 0.5 kb in *Eco* RI site of pCRII™; C. Doerig 1994

5 *P. falciparum* circumsporozoite protein gene (Kemp *et al.*, 1987); see Janse *et al.* (1994)

6 *P. falciparum* dihydrofolate reductase-thymidylate synthase gene (Cowman *et al.*, 1988; Peterson *et al.*, 1988)

7 *P. falciparum* heat shock protein 70-1(Sharma, 1992)

8 *P. falciparum* phosphoglycerate kinase gene (Hicks *et al.*, 1991); see Janse *et al.* (1994)

9 *P. falciparum* MSP-1 gene (Kemp *et al.*, 1987)

10 *P. falciparum* β tubulin gene (Holloway *et al.*, 1990)

11 *P. falciparum* exp-1(circumsporozoite protein-related antigen gene) (Coppel *et al.*, 1985)

12 *P. falciparum* apical membrane antigen gene (Kemp *et al.*, 1987)

13 *P. falciparum* CDC2-like protein kinase gene (Ross-Macdonald *et al.*, 1994)

14 *P. falciparum* thrombospondin-related anonymous protein gene (Robson *et al.*, 1990)

[15] *P. falciparum* actin I gene (Wesseling *et al.*, 1988); see Janse *et al.* (1994)

[16] *P. falciparum* aldolase gene (Knapp *et al.*, 1990; Triglia *et al.*, 1992)

[17] *P. berghei* circumsporozoite protein gene (Lockyer *et al.*, 1990); see (Janse *et al.*, 1994)

[18] αI and αII tubulin cross-hybridise to both chromosome 4 and 5 with equal intensities and it is not known which gene is located on which chromosome

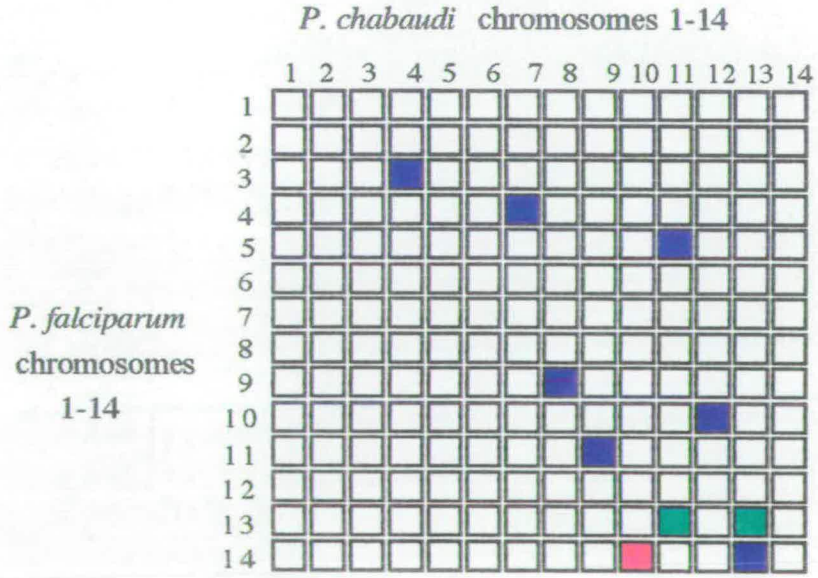
[19] *P. falciparum* hsp-70-1 cross-hybridises to two *P. chabaudi* chromosomes with equal intensities, but it is likely that one chromosome contains another hsp-like gene which is as yet uncharacterised

[20] *P. berghei* sporozoite surface protein-2 (Rogers *et al.*, 1992), homologue of *P. falciparum* TRAP (K. Robson, pers.comm.); see Janse *et al.* (1994)

[21] *P. berghei* aldolase II gene (Meier *et al.*, 1992); *P. falciparum* has only one aldolase gene

Another method of representing conserved syntenies is in the form of an Oxford grid (**Figure 21**). Conserved syntenies are shown as coloured blocks within the grid; this shows clearly that 10 blocks are conserved between the two genomes.

Figure 21. An Oxford grid showing the locations of homologous genes in the genomes of *P. falciparum* and *P. chabaudi*.



4.2 *P. falciparum* chromosome-7 conserved linkage groups

In **Chapter 1**, it was pointed out that the *P. falciparum* marker pS590.7 is closely linked to a gene determining chloroquine resistance on chromosome 7. The aim of this work was to determine whether a homologous region exists between chromosome 7 of *P. falciparum* and chromosome 13 of *P. chabaudi*. This would clarify the relationship between the pS590.7 markers in each species, and provide an indication of whether a chloroquine resistance locus on *P. falciparum* chromosome 7 might have a homologue on chromosome 13 of *P. chabaudi*.

Eight chromosome 7-specific *P. falciparum* markers were chosen for cross-hybridisation studies between the two species (**Table 6** and **Figure 8**.) No DNA sequence data existed for any of the markers, and it was not known whether they were parts of coding regions of DNA (Walker-Jonah *et al.*, 1992). Each marker was radiolabelled and used to probe Southern blots of PFG and RFLP gels. The results are given in **Table 12.** Various washing conditions were employed to optimise the hybridisation, but only those conditions which produced the best results are shown for each marker.

Initial results showed that 4 of the 8 markers hybridised to a band containing chromosomes 13 and 14 (referred to as 'chromosomes 13/14'). For example, marker pH270.5 which is approximately 40-240 kb from pS590.7 and shows partial linkage with chloroquine resistance in the *P. falciparum* HB3/Dd2 cross (T. Wellems, personal communication and **Figure 8**), hybridised weakly to *P. chabaudi* chromosomes 13/14.

Table 12. *P. chabaudi* chromosome location and RFLPs produced by cross-hybridisation of eight *P. falciparum* chromosome 7-specific markers.

Genome Marker	Approximate distance from pS590.7 (kb)[a]	Washing Stringency	*P.chabaudi* Chromosome Location[b]	Enzymes tested for RFLPs	RFLP(s)
pE12a	0 - 95	2 x SSC, 0.1% SDS, 40°C: bad background signal, no significant hybridisation		Not attempted	
pB20.23	0 - 130	1 x SSC, 0.1% SDS, 50°C: either bad background signal or no significant hybridisation		Not attempted	

pE53a	0 - 197	1 x SSC, 0.1% SDS, 50°C	8/9, 13/14	Not attempted	
pH270.5	45 - 235	1 x SSC, 0.1% SDS, 50°C	possibly 13/14	Not attempted	
pS90.30	45 - 235	1 x SSC, 0.1% SDS, 60°C: either bad background signal or no significant hybridisation to PFG or RFLP blots.			
pS590.20	125 - 500	5 x SSC, 0.1% SDS, 50°C	8/9, 13/14	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	None; poor hybrid-isation
pSL2	285 - 520	1 x SSC, 0.1% SDS, 50°C: 2 hour exposure, no hybridisation; 30 hour exposure, hybridised to all chromosomes		Not attempted	
pS500.7	490 - 710	PFG blots: 2 x SSC, 0.1% SDS, 60°C; RFLP blots: 3 x SSC, 0.1% SDS, 55°C	8/9, 12, 13/14 : not significant as poor repro-ducibility	*Alu* I, *Dra* I, *Hind* II, *Hinf* I, *Sau* 3A, *Rsa* I	*Rsa* I (repro-ducibility not good)

[a] An estimated range of distance between the marker and pS590.7, as deduced from **Figure 8**.

[b] It was not possible to distinguish between hybridisation to chromosome 13 or 14 in the majority of cases, due to the poor hybridisation signals emitted.

Interestingly, three markers hybridised to bands containing chromosomes 8 and 9 (referred to as 'chromosomes 8/9'), as well as to chromosomes 13/14. For example, marker pS590.20 which is approximately 125-500 kb from pS590.7 in *P. falciparum*, hybridised to chromosomes 8/9, and 13/14 in *P. chabaudi* (**Figure 22A**). The cross-hybridisation signals to these bands were weak.

Another example of a marker which hybridised to *P. chabaudi* chromosome 8/9 and and 13/14 is pS500.7, which is 490-710 kb from pS590.7 and is not linked to chloroquine resistance in the *P. falciparum* HB3/Dd2 cross (Wellems *et al.* (1991) and **Figure 8**). It also hybridised to *P. chabaudi* chromosome 12 which migrates as a doublet with chromosome 11 in AS(3CQ). Unfortunately, this result was not reproducible; an attempt to hybridise pS500.7 to a second PFG at the same stringency produced a hybridisation signal from all chromosomes, which subsequently washed off at an increased stringency (data not shown).

Markers pE12a, pB20.23 and pE53a which are 0-95 kb, 0-110 kb and 0-197 kb respectively from pS590.7 in *P. falciparum*, were the closest available markers to pS590.7. If a region of synteny were to exist between *P. falciparum* chromosome 7 and *P. chabaudi* chromosome 13, these were the most likely markers to indicate it. However, pE12a and pB20.23 did not cross-hybridise to any *P. chabaudi* chromosomes (**Table 12**). pE53a cross-hybridised weakly to chromosomes 8/9, and even more weakly to chromosomes 13/14 (**Figure 22B**).

Whether these results provide evidence for a region of synteny between the two chromosomes is discussed in **Chapter 7**.

4.2.1 Cross-hybridisation of *P. falciparum* marker pS590.7

A final experiment was carried out to determine whether pcpS590.7 was the genuine homologue of pfpS590.7. *P. falciparum* marker pS590.7 was hybridised to a blot of a PFG and the results are shown in **Figure 23**. Under stringent conditions (1 x SSC, 0.1% SDS, 60°C; **Figure 23B**) the marker hybridised to chromosome 12 only. Under conditions of lower stringency (2 x SSC, 0.1% SDS, 50°C), the marker hybridised to *P. chabaudi* chromosome 12 and weakly to chromosomes 1/2 and 8/9 (**Figure 23C**). pcpS590.7, the PCR product amplified from *P. chabaudi* DNA, hybridises to *P. chabaudi* chromosome 13. This suggests that pcpS590.7 is probably not the *P. chabaudi* homologue of the *P. falciparum* marker pS590.7, and is discussed further in **Chapter 7**.

Figure 22. Cross-hybridisation of two *P. falciparum* chromosome 7-specific markers to chromosome separations of *P. chabaudi.*

P. chabaudi chromosomes were separated by PFGE, and probed with (**A**) pS590.20 and (**B**) pE53a. Chromosome numbering and sizes are as for **Figure 10**. Arrows indicate chromosome numbers. In addition, PFG **B** was probed with marker *CDC2* (Probe Number 16) without prior stripping, for the purpose of identifying chromosome 11.

Lane 1 AJ
Lane 2 AJ after transmission through mosquitoes
Lane 3 AS(3CQ)
Lane 4 AS(3CQ) after transmission through mosquitoes

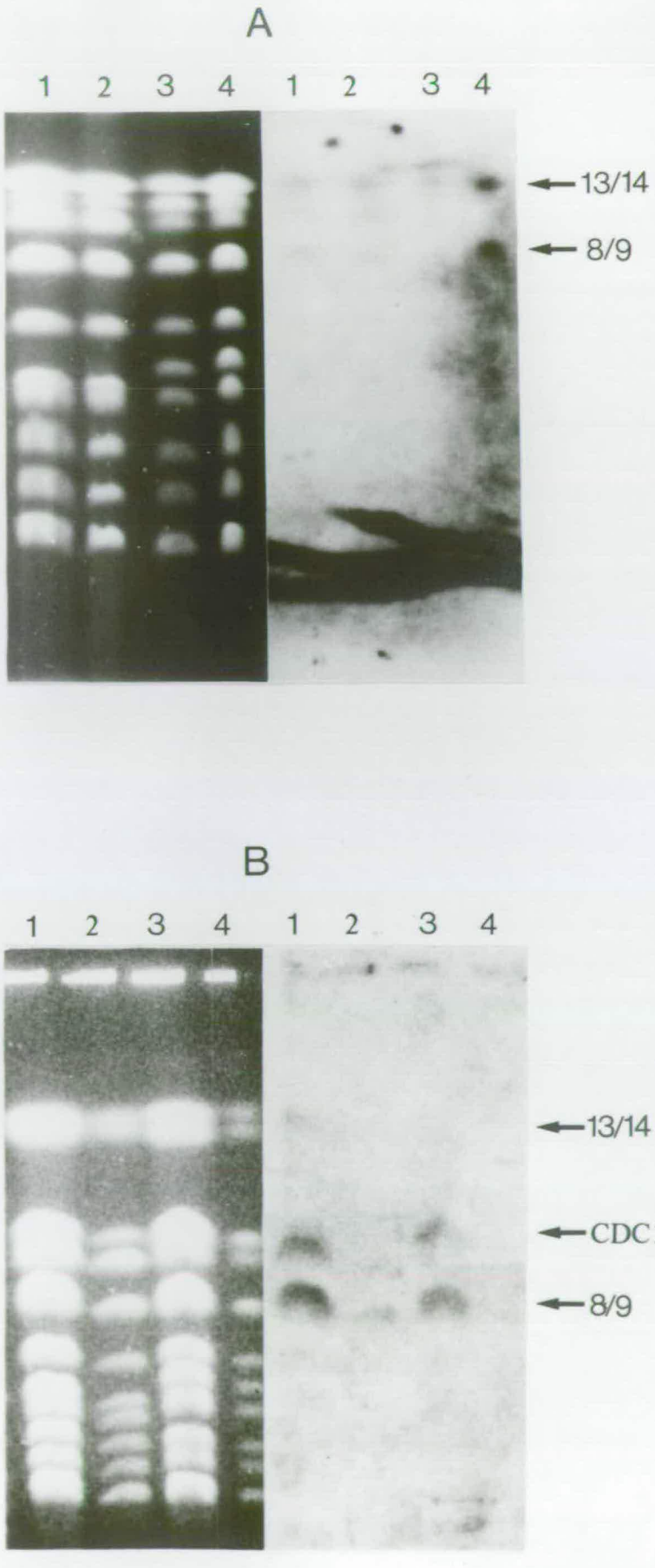
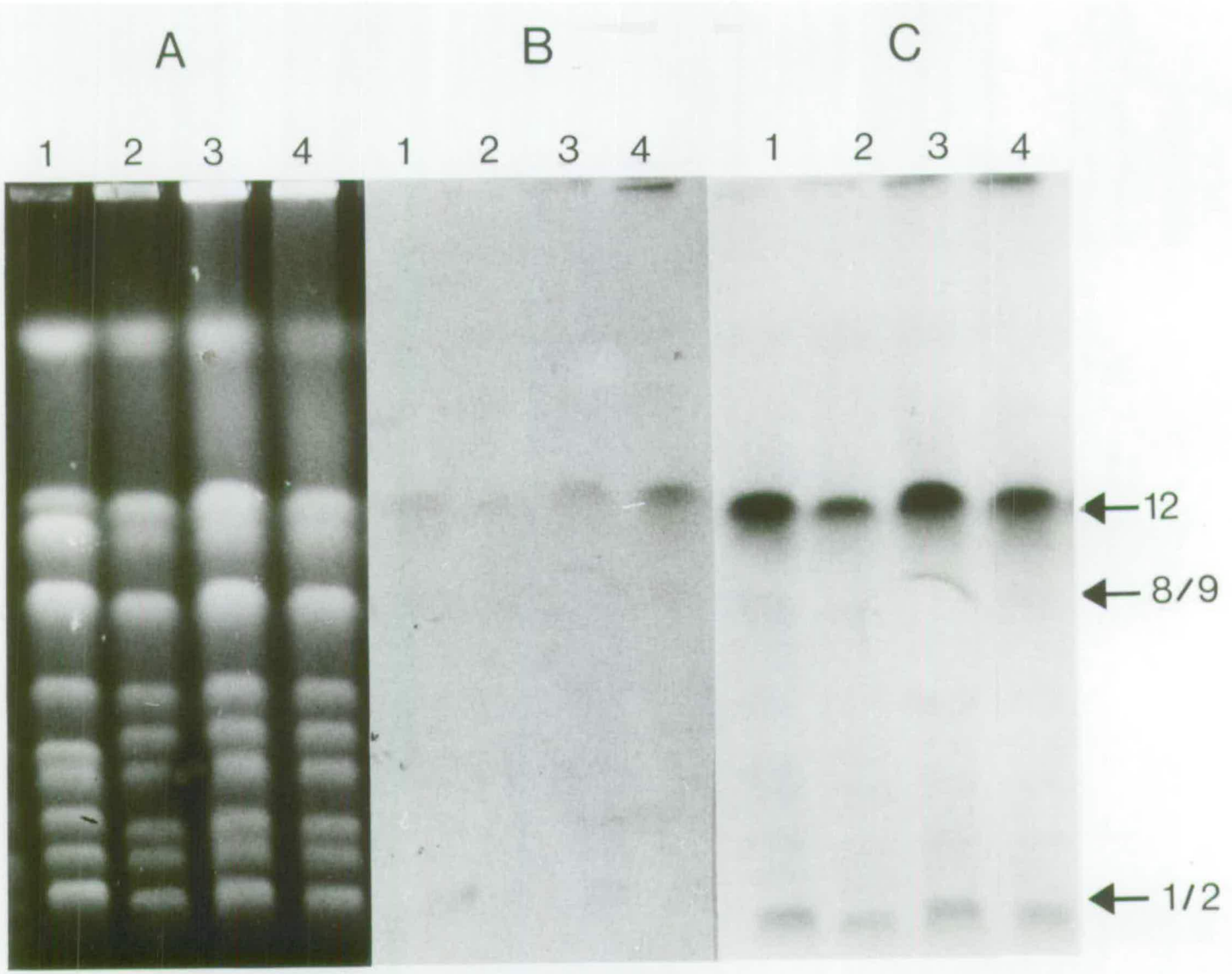

A
1 2 3 4 1 2 3 4
13/14
8/9
B
1 2 3 4 1 2 3 4
13/14
CDC2 (chr.11)
8/9

Figure 23. Hybridisation of *P. falciparum* marker pS590.7 to chromosome separations of *P. chabaudi.*

P. chabaudi chromosomes were separated by PFGE (**A**), and probed with marker pS590.7 under conditions of (**B**) high stringency and (**C**) low stringency. Chromosome numbering and sizes are as for **Figure 10**. Arrows indicate chromosome numbers.

Lane 1 AJ
Lane 2 AS(sens)
Lane 3 AS(0CQ)
Lane 4 AS(3CQ)


A
B
C
1 2 3 4
1 2 3 4
1 2 3 4
12
8/9
1/2

5. Results: The progeny of the cross

A cross was made between the chloroquine-sensitive clone AJ, and the chloroquine-resistant clone AS(3CQ), selected for resistance as described in **Chapter 2**.

5.1 Mosquito infections

Ten days after mosquitoes had been fed on the infected rats, a sample of mosquitoes from each cage was dissected and examined for oocysts. All of the cages were found to contain infected mosquitoes, as follows:

(i) six out of seven mosquitoes taken from the cage fed on the AS(3CQ)-infected rat had ten or more oocysts

(ii) two of three mosquitoes taken from the cage fed on the AJ-infected rat had three and six oocysts respectively

(iii) two of six mosquitoes taken from the cage fed on the rat infected with a mixture of AS(3CQ) and AJ parasites had one and eight oocysts respectively.

The presence of oocysts indicated that fertilisation and meiosis had occurred.

The mosquitoes were fed on separate, uninfected mice 16 and 18 days after their first blood meal, in order to recover the progeny of the cross. Seven days later, when the infections had become patent in the blood, each mouse was bled and the parasitised blood stored in liquid nitrogen, as stabilate numbers 1517 to 1523 inclusive.

5.2 The uncloned progeny of the cross

Experiments involving the uncloned progeny of the cross utilised parasites as close to the original source as possible, usually 1 passage through mice from the deep-frozen material. This was to prevent selection of more common genotypes over rare genotypes, which might have led to a bias in the results.

5.2.1 Karyotype of the uncloned progeny

Chromosomes of the uncloned progeny were separated by PFGE (**Figure 24**), and probed with markers P.12 and *PCNA* which recognised chromosomes 6 and 11 respectively (see **Tables 7** and **9**). These two chromosomes are different in the two parent clones, chromosome 6 being approximately 1.1 Mb in AJ and 1.2 Mb in AS(3CQ), and chromosome 11 being around 1.8 Mb and 1.9 Mb in AJ and AS(3CQ) respectively, as described previously in **Chapter 3** (**Figure 24B** and **C**).

The presence of both forms of both chromosomes among the uncloned progeny indicated that little selection involving these chromosomes had occurred between the

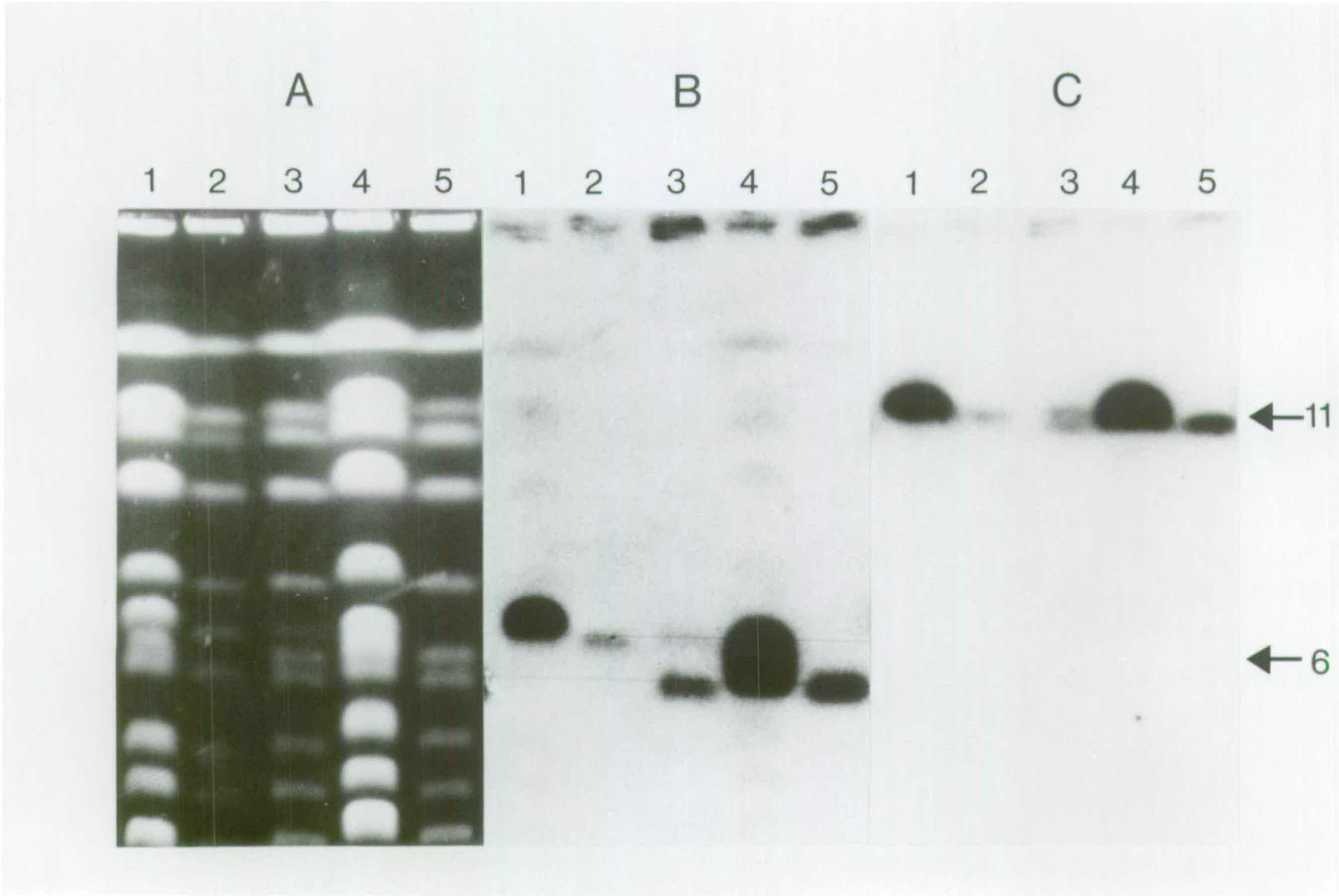
Figure 24. Karyotype and chromosome number of the uncloned progeny of the cross.

P. chabaudi chromosomes were separated by PFGE (**A**), and probed with chromosome markers P.12 (**B**) and *PCNA* (**C**). Chromosome numbering and sizes are as for **Figure 10**, except note the reverse order of the clones. Arrows indicate chromosome numbers.

Lane 1 AS(3CQ)
Lane 2 AS(3CQ) after transmission through mosquitoes
Lane 3 uncloned progeny of the cross
Lane 4 AJ
Lane 5 AJ after transmission through mosquitoes

B. Marker P.12 recognises chromosome 6, which is polymorphic between AJ and AS(3CQ) as shown by the presence of two bands in the uncloned progeny (Lane 3).

C. Marker *PCNA* recognises chromosome 11, also polymorphic between AJ and AS(3CQ) and which is also shown by the presence of two chromosome 11 bands in the uncloned progeny (Lane 3).


A
B
C
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
11
6

fertilisation events in the mosquitoes and the time when blood forms were examined. However, the AJ form of both chromosomes appeared to be present in greater amounts compared to the AS(3CQ) form. This may have been due to more AJ self-fertilisation occurring than AS(3CQ) self-fertilisation (see also **Discussion**).

The fact that both size polymorphisms of chromosomes 6 and 11 were present provided no information as to whether recombination had occurred between the two parent clones; this subject is discussed in the next section.

5.2.2 Testing for recombinant forms among the uncloned progeny

The uncloned progeny of the cross, both untreated and treated with either chloroquine or pyrimethamine, were examined for parent clone markers. The results were expected to be as follows:

(a) untreated progeny would be expected to exhibit both parental alleles, of each marker, in the absence of selection

(b) if recombination had not occurred between the parent clones, the drug-treated progeny would be expected to exhibit only the allelic forms of the resistant parent line AS(3CQ)

(c) if recombination had occurred, the drug-treated progeny would be expected to contain both parental alleles of all markers, with the exception of markers closely linked to gene(s) determining drug resistance; for these markers, only the alleles of the resistant parent clone would be expected to be present.

The uncloned progeny were examined first to detect whether crossing had occurred, and this was done using enzyme markers. They were then examined to determine whether any markers linked to chloroquine or pyrimethamine resistance could be detected.

(i) Enzyme markers

The uncloned progeny, both untreated and treated with pyrimethamine, were examined for alloenzyme forms of LDH and ADA; the chromosomal locations of these genes are not known in *P. chabaudi* (although see **Chapter 6**). Results are shown in **Table 13**. Both parental forms were found following drug treatment. This showed that recombination must have occurred during the cross. In the absence of recombination, only enzyme types LDH-3 and ADA-6 would have been present, as these are characteristic of the drug resistant parent.

Table 13. Analysis of the uncloned progeny with enzyme and DNA markers.

	Enzyme Markers		DNA Markers						
Parasite clone	LDH	ADA	*Pc-EMA1* (5)	P.9 (63)	*DHFR* (6)	*MSP-1* (4)	PBS 110 (3)	P.12 (63)	pc pS590.7 (84)
AJ	2	9	AJ	AJ	AJ	AJ	AJ	AJ	AJ
AS(3CQ)	3	6	AS	AS	AS	AS	AS	AS	AS
Uncloned progeny	2 + 3	9 + 6	AJ + AS	AJ + AS	AJ + AS	AJ + AS	AJ + AS	AJ	AJ + AS
Uncloned progeny treated with 15 mg/kg PYR for 4 days	2 + 3	9 + 6	AJ + AS	AJ + AS	AS	AJ + AS	AS	AJ	AJ
Uncloned progeny treated with 3 mg/kg CQ for 8 days	ND	ND	AJ + AS	AJ + AS	AJ + AS	AS	AJ + AS	AJ	AJ + AS
Chromosome Location	ND	ND	10	4	7	8	3	6	13

Numbers in parentheses refer to Probe Numbers as shown in **Tables 5** and **9**

2, 3 and 9,6 - alloenzyme types of LDH and ADA

CQ - chloroquine; PYR - pyrimethamine

ND - not determined

AJ - AJ-type allele inherited; AS - AS(3CQ)-type allele inherited

(ii) DNA markers

Seven DNA markers were analysed in the uncloned progeny, and in the uncloned progeny which had been treated with pyrimethamine or chloroquine (**Table 13**). Results for each marker were as follows:-

(i) *PcEMA-1* and P.9 : untreated progeny and progeny treated with both drugs exhibited both allelic forms of these markers. Thus they behaved as markers unlinked to drug resistance gene(s).

(ii) *DHFR* : pyrimethamine-treated progeny exhibited the AS(3CQ) allele of this marker only, which indicates that *DHFR* could be the gene, or linked to a gene, responsible for pyrimethamine resistance. Evidence has accumulated that a mutant *DHFR* plays a rôle in resistance to pyrimethamine in *P. chabaudi*, as it does in *P. falciparum* (see **Introduction 1.5.3**), and this result provides further evidence that this is the case. The chloroquine-treated progeny exhibited both parental alleles, showing that the DHFR gene was not involved in resistance to this drug.

(iii) *MSP-1*, pBS 110 : single allelic forms characteristic of AS(3CQ) were exhibited by the chloroquine-treated and pyrimethamine-treated progeny respectively, suggesting that these markers could play a rôle in, or be linked to genes involved in, each respective type of drug resistance.

(iv) P.12, pcpS590.7 : both these markers produced curious results. First, the AS(3CQ) allele of marker P.12 was not detectable in any of the uncloned progeny, treated or untreated; second, the AS(3CQ) allele of pcpS590.7 was not present among the pyrimethamine-resistant progeny.

A possible explanation for these results is as follows: during drug treatment, the parasites are kept at a sub-patent level by the drug. This is true even for resistant lines of parasite. As the parasites recover following removal of the drug, there may be competition between them, resulting in some genotypes being selected over others, for example due to their ability to grow faster. This produces bias in those genotypes which have survived the 'bottle-neck'. Thus it may be that the drug-selection procedure causes certain genotypes to be favoured over others.

The results of this work on the uncloned progeny showed that recombination between the parent clone markers had clearly occurred, but that individual progeny clones needed to be characterised in order to follow the segregation and recombination of each chromosome marker.

5.3 The cloned progeny of the cross

24 clones were isolated from the uncloned progeny (deep frozen stabilate no. 1519), taken from the first mouse fed upon by infected mosquitoes. Each clone was grown in mice and tested for its susceptibility to chloroquine, and subsequently analysed for the inheritance of 46 markers that distinguished the parent clones.

5.3.1 Inheritance of parental markers

Eighteen of the twenty-four clones examined were found to be recombinants. The remaining six clones (38 / 2, 38 / 3, 39 / 7, 40 / 4, 42 / 10 and 49 / 3) were AJ parental types. No AS(3CQ) parental type clones were isolated. One group of four clones (39 / 3, 73 / 3, 63 / 1 and 64 / 7), one group of three clones (103 / 3, 103 / 6 and 105 / 1), and two groups of two clones (39 / 8 and 72 / 2, and 38 / 9 and 64 / 3) were identical to each other as determined from inheritance data of approximately 35 markers. One clone from each group was selected as representative, and the rest were excluded from further analysis. **Table 14** shows the inheritance pattern of the markers among the resulting eleven independent recombinant clones.

The total number of polymorphic sites analysed during this study was 492. Of those, 241 (49%) were inherited from the AJ parent, and the remaining 251 (51%) from the AS(3CQ) parent. This is not significantly different from the expected ratio of 1:1. However, there does appear to be skewed inheritance of AS(3CQ) and AJ alleles by two chromosomes. The inheritance of chromosome 5 was examined at a total of 55 polymorphic sites, and 43 of these (approximately 78%) were found to be alleles of the AJ parent. Chromosome 11 was analysed at a total of 99 sites, and 76 (approximately 77%) were found to be alleles of the AS(3CQ) parent.

Two markers on chromosome 5 displayed skewed inheritance among the progeny clones. AJ alleles of markers Ag3035 and OPL-16 were inherited by 10 out of 11 progeny. This is of particular relevance to progeny clone 74 / 5, which inherited AS(3CQ) alleles for all markers studied except Ag3035.

The RFLP inheritance data also suggested possible chromosome locations for the genes coding for alloenzymes ADA and LDH. Neither of the genes determining these enzymes has been cloned in *P. chabaudi*. **Table 14** shows LDH to have the same RFLP pattern among the progeny clones as the chromosome 13 marker pcpS590.7, which may suggest that the LDH gene is on chromosome 13. The location of the gene coding for ADA is more difficult to determine. Markers P.9 and cDNA 121 are on chromosomes 4 and 10 respectively, and both show the same inheritance pattern as ADA in ten out of eleven progeny clones.

Table 14. Inheritance of marker alleles in 11 progeny from the AJ x AS(3CQ) *P. chabaudi* cross.

Probe Number	Probe Name[a]	Progeny Clones: 38 / 9	39 / 1	39 / 2	72 / 2	74 / 5	103 / 6	43 / 8	63 / 3	39 / 3	62 / 3	62 / 8	Linkage Ratio[b]
Chromosome 1													
49	Ag3020[c]	AS[d]	AJ[d]	AJ	AJ	AS	AJ	AJ	AS	AJ	AS	AJ	5/11
Chromosome 2													
1	Ca^{2+}-ATPase	AS	ND	AS	AS	AS	AJ	AS	AS	AJ	ND	AS	7/9
Chromosome 3													
3	pBS 110	AJ	AS	AS	AS	AS	AJ	AS	AJ	AS	AJ	AS	6/11
47	Ag3003 A	AJ	AS	AS	AS	AS	AJ	AS	AJ	AJ	AJ	AJ	8/11
Chromosome 4													
62	P.1	AS	AJ	AJ	AJ	ND	AJ	AJ	ND	AS	AJ	AS	2/9
63	P.9	AS	AJ	AJ	AJ	AS	AJ	AJ	AJ	AS	AJ	AS	3/11
Chromosome 4/5													
36	*α II tubulin*[e]	AJ	AJ	AS	AS	AS	AJ	AS	AJ	AS	AJ	AS	5/11

Table 14. continued.

Probe Number	Probe Name	38 / 9	39 / 1	39 / 2	72 / 2	74 / 5	103 / 6	43 / 8	63 / 3	39 / 3	62 / 3	62 / 8	Linkage Ratio
Chromosome 5													
57	Ag3035	AJ	AS	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	4/11
78	OPL-16	AJ	AJ	AJ	AJ	AS	AJ	AJ	AJ	AJ	AJ	AJ	4/11
25	*DNA pol δ*	AJ	AS	AJ	AJ	AS	AJ	AJ	AJ	AJ	AJ	AJ	5/11
72	P. 29	AJ	AS	AJ	AJ	AS	AJ	AJ	AJ	AJ	AJ	AS	4/11
50	Ag3024	AS	AS	AJ	AJ	AS	AJ	AJ	AS	AJ	AJ	AS	6/11
Chromosome 6													
66	P. 12	AJ	AS	AJ	AJ	AS	AJ	AJ	AS	AS	AJ	AS	4/11
24	*DNA pol α*	AJ	AS	AJ	AJ	AS	AJ	AJ	AJ	AJ	AJ	AS	4/11
34	*RESA*	AJ	AS	AJ	AJ	AS	AJ	AJ	AJ	AJ	AJ	AJ	5/11

Table 14. continued.

Probe Number	Probe Name	38 / 9	39 / 1	39 / 2	72 / 2	74 / 5	103 / 6	43 / 8	63 / 3	39 / 3	62 / 3	62 / 8	Linkage Ratio
Chromosome 7													
6	*DHFR*	AS	AJ	AJ	AJ	AS	AS	AJ	AS	AJ	AS	AJ	6/11
71	P. 23	AJ	AS	AJ	AS	AS	AS	AS	AJ	AJ	AJ	AJ	8/11
Chromosome 8													
4	*MSP-1*	AS	AJ	AS	AS	AS	AS	AS	AJ	AS	AJ	AJ	8/11
Chromosome 9													
51	Ag3027	AJ	AS	AJ	AJ	AS	AJ	AJ	AS	AS	AS	AS	3/11
39	*AMA-1*	AJ	AS	AJ	AJ	ND	AJ	AJ	ND	AS	AS	AJ	2/9
40	*pfran*	AJ	AS	AS	AS	AS	AJ	AS	AS	AS	AS	AJ	4/11
Chromosome 10													
14	*5s rRNA*	AJ	AJ	AJ	AJ	AS	AS	AJ	AS	AJ	AS	AS	4/11
43	cDNA 121	AS	AJ	AJ	AJ	AS	AJ	AJ	AJ	AS	AJ	AS	3/11
18	*VAP B*	AS	AS	AS	AJ	ND	AJ	AJ	ND	AS	AJ	AS	4/9

Table 14. continued.

Probe Number	Probe Name	38 / 9	39 / 1	39 / 2	72 / 2	74 / 5	103 / 6	43 / 8	63 / 3	39 / 3	62 / 3	62 / 8	Linkage Ratio
Chromosome 11													
16	*CDC2*	AS	AS	AS	AS	AS	AS	AS	AS	AJ	AS	AJ	10/11
15	*EF1α*	AS	AS	AS	AS	AS	AS	AS	AS	AJ	AS	AJ	10/11
29	*PCNA*	AS	AS	AS	AS	AS	AS	AS	AS	AJ	AS	AJ	10/11
76	OPL-04	AS	AS	AS	AS	AS	AS	AS	AS	AJ	AS	AJ	10/11
70	P. 22	AS	AS	AS	AS	AS	AJ	AS	AS	AJ	AS	AJ	9/11
11	*H2A*	AS	AS	AS	AS	AS	AJ	AS	AS	AJ	AS	AJ	9/11
12	**9.2**[f]	AS	AS	AS	AS	AS	AJ	AS	AS	AJ	AS	AJ	9/11
27	*Topo I*	AS	AS	AS	AS	AS	AJ	AS	AS	AJ	AS	AJ	9/11
8	*TBP*	AS	AS	AS	AS	AS	AJ	AS	AS	AJ	AS	AJ	9/11

Table 14. continued.

Probe Number	Probe Name	38 / 9	39 / 1	39 / 2	72 / 2	74 / 5	103 / 6	43 / 8	63 / 3	39 / 3	62 / 3	62 / 8	Linkage Ratio
Chromosome 12													
83	*pcmdr1*	AS	AS	AJ	AJ	AS	AS	AJ	AS	AJ	AS	AJ	7/11
80	OPR-14	AS	AS	AJ	AJ	AS	ND	AJ	AS	AJ	AS	AS	6/10
Chromosome 13													
37	*aldo-1*	AJ	AS	AS	AS	AS	AJ	AS	AJ	AS	AJ	AJ	7/11
54	**Ag-3042B**[f]	AJ	AS	AS	AS	AS	AJ	AS	AJ	AS	AJ	AJ	7/11
84	pc-pS590.7	AS	AJ	AS	AS	AS	AJ	AS	AJ	AS	AJ	AJ	7/11
13	*RNA pol III*	AS	AJ	AS	AS	AS	AJ	AS	ND	AS	AJ	ND	6/9
77	OPL-12	AS	AJ	AS	AS	AS	AJ	AS	AJ	AS	AJ	AJ	7/11
45	cDNA 148	AJ	ND	AS	AS	AS	AJ	AS	AJ	AJ	ND	AJ	6/9
19	*G6PD*	AJ	AJ	AS	AS	AS	AJ	AS	AJ	AJ	AJ	AJ	7/11

Table 14. continued.

Probe Number	Probe Name	38 / 9	39 / 1	39 / 2	72 / 2	74 / 5	103 / 6	43 / 8	63 / 3	39 / 3	62 / 3	62 / 8	Linkage Ratio
Chromosome 14													
79	OPR-02	AS	AJ	AJ	AJ	AS	AS	AJ	AJ	AJ	AJ	AS	5/11
59	**Ag-3040**[f]	AS	AS	AJ	AJ	AS	ND	AJ	AS	AJ	AS	AS	5/10

Alloenzyme markers. The chromosome location of the genes coding for these enzymes is not known.

LDH		AS	AJ	AS	AS	AS	AJ	AS	AJ	AS	AJ	AJ	7/11
ADA		AJ	AJ	AJ	AJ	AS	AJ	AJ	AJ	AS	AJ	AS	2/11

[a] The order of markers was determined in such a way as to minimise the number of cross-overs, according to the premise that double cross-overs are less frequent than single cross-overs, and that both of these occur less frequently than no cross-overs. However, as no long-range restriction mapping studies were made of the chromosomes during this project, the order may be imprecise.

[b] Ratio of the number of progeny showing linkage of chloroquine susceptibility with the marker, to the total number of progeny.

[c] Marker may be on chromosome 2, although RFLP pattern is markedly different from the only other marker on this chromosome.

[d] AS and AJ indicate alleles inherited from the chloroquine-resistant AS(3CQ) and chloroquine-sensitive AJ parents, respectively.

[e] α I and α II tubulin cross-hybridise to chromosomes 4 and 5, but it is not known which gene is present on which chromosome.

[f] Markers indicated in **bold** detected sequences on more than one chromosome.

ND - not determined

N.B. Progeny clones typed as chloroquine-resistant are marked in red and those typed as chloroquine-sensitive are marked in blue.

5.3.2 Distribution of cross-overs among the chromosomes

The data in **Table 14** revealed the number of cross-overs which had occurred within each chromosome during meiosis, and these are given in **Table 15**. It is apparent that the number of cross-overs increases as the number of markers for each chromosome increases. The one exception to this is chromosome 11, which was analysed using 9 markers, but only 1 cross-over event was identified. It was not possible to determine if any crossing-over events had occurred within chromosomes 1, 2 and 8 because of the insufficient number of markers examined.

It was not possible to calculate a meaningful recombination frequency from this data because the position of the chromosome markers and the distance between them is not known.

Table 15. Number of cross-overs for each chromosome in the AJ x AS(3CQ) cross.

Chromosome number	Approximate chromosome size (kb)[a]	Number of markers	Number of cross-overs
1	900	1	-
2	900	1	-
3	940	2	2
4	1000	3[b]	4
5	1140	5	6
6	1200	3	3
7	1320	2	6
8	1580	1	-
9	1580	3	4
10	1800	3	7
11	2100	9	1
12	2100	2	1
13	3200	7	4
14	3100	2	3

[a] Determined from chromosome separations of *P. chabaudi* clone AS

[b] Includes marker *αII tubulin*

5.3.3 Chloroquine susceptibility

Each clone was tested for its susceptibility to chloroquine at least twice using a standard 8-day drug test (**Materials and methods 2.5.3**). Tail smears were taken on D_{11}, D_{13} and D_{15}. Each smear was examined for the presence of parasites in 20-25 fields (each field containing approximately 500 rbcs) and scored using a simple system based on that of Padua (1981). This form of scoring takes into account any changes in parasite morphology, for example the presence of gametocytes which indicate that the parasites are under duress, as well as estimating the number of parasites present. Parasites appearing on or before D_{13} were typed as chloroquine-resistant and those appearing on or after D_{15} as chloroquine-sensitive.

Table 16 shows the results of the chloroquine susceptibility tests carried out on all 11 recombinant progeny. The clones were tested in two batches of six and five, (**Tables 16A** and **16B** respectively), and separate control tests were carried out on AS(3CQ) and AJ for each test. The parent controls in the second drug test appeared to take 48 hours longer for their development, as compared with previous drug test results. Consequently the results for clones 38 / 9, 39 / 2, 72 / 2, 103 / 6, 39 / 3 and 62 / 8 (**Table 16A**) were also 48 hours later. The results for this drug test have been adjusted to take this into account.

Clones 38 / 9, 39 / 1, 39 / 2, 72 / 2, 74 / 5 and 103 / 6 were typed as chloroquine-resistant, and 39 / 3, 62 / 3 and 62 / 8 as chloroquine-sensitive. Two clones showed a susceptibility between that of the sensitive and resistant phenotypes; clones 43 / 8 and 63 / 3 showed patent parasitaemias on D_{11}, but the parasitaemia remained low and did not develop as high as the AS(3CQ) control. These were typed as low-level chloroquine resistance clones. The importance of detecting two types of resistant phenotype is discussed in **Chapter 6**.

Table 16A. Chloroquine susceptibility results of six recombinant progeny from the AJ x AS(3CQ) cross.

	Day 5 control not drugged	Day 11 drugged	Day 13 drugged	Day 15 drugged	Conclusion
AJ test 1	[a]1 ++++++ 2 ++++++	1 - 2 - 3 -	1 - 2 - 3 -	1 +- 2 - 3 -	sensitive parent
AJ test 2[b]	1 ++++ 2 ++++	1 - 2 - 3 -	1 - 2 - 3 -	1 - 2 - 3 -	
AS(3CQ) test 1	[a]1 +++++ 2 +++++	1 - 2 +- 3 +-	1 +- 2 ++ 3 ++	ND	resistant parent
AS(3CQ) test 2[b]	1 ++++ 2 ++	1 +- 2 +- 3 +-	1 ++ 2 ++ 3 ++	1 +++++ 2 ++retics 3 ++++	
38 / 9 test 1	1 ++++ 2 ++++	1 - 2 - 3 -	1 +- 2 +- 3 +-	[c]1 +++ 2 ++ 3 +++	resistant
38 / 9 test 2[b]	[a]1 +++ 2 +++	1 +- 2 - 3 +--	1 +- 2 - 3 +	1 ++ 2 + 3 +++	
39 / 2 test 1	[a]1 +++++ 2 +++++	1 +- 2 +- 3 -	1 ++ 2 ++ 3 -	1 ND 2 ND 3 +-	resistant
39 / 2 test 2[b]	1 +++++ 2 +++++	1 +- 2 - 3 +	1 ++ 2 - 3 +++	1 ++++++ 2 ++ 3 +++retics	

Table 16A. continued

	Day 5 control not drugged	Day 11 drugged	Day 13 drugged	Day 15 drugged	Conclusion
72 / 2 test 1	[a]1 +++++ 2 +++++	1 +- 2 +- 3 +--	1 +- 2 + 3 +	1 ++ 2 + 3 +	resistant
72 / 2 test 2[b]	1 ++++ 2 +++	1 - 2 + 3 +--	1 + 2 +- 3 +-	1 +++ 2 ++retics 3 ++	
103 / 6 test 1	[a]1 +++++ 2 +++++	1 - 2 - 3 -	1 +- 2 +- 3 -	1 +++ 2 +++ 3 +--	resistant
103 / 6 test 2[b]	1 +++++ 2 +++	1 - 2 + 3 +	1 - 2 ++ 3 ++	1 ++ 2 ++retics 3 +retics	
39 / 3 test 1	[a]1 +++++ 2 +++++	1 - 2 - 3 -	1 - 2 - 3 -	1 - 2 - 3 -	sensitive
39 / 3 test 2[b]	1 +++++ 2 +++++	1 - 2 - 3 -	1 - 2 - 3 -	1 - 2 +-- 3 -	
62 / 8 test 1	[a]1 ++++ 2 +++++	1 - 2 - 3 -	1 - 2 - 3 -	[c]1 - 2 - 3 -	sensitive
62 / 8 test 2[b]	1 +++++ 2 +++++	1 - 2 - 3 -	1 - 2 - 3 -	1 +-- 2 - 3 -	

[a] Blood smears taken on Day 6; [b] See **Results 5.3.3**; [c] Blood smears taken on Day 16; retics - reticulocytes present; ND - not determined

Parasitaemia scoring: +-- 1 parasite/500 rbcs; +- <0.5>0.1 parasite/500 rbcs; + >0.5 parasite/500 rbcs: ++ >2 parasites/500 rbcs; +++ >5 parasites/500 rbcs; ++++ >10 parasites/500 rbcs; +++++ >20 parasites/500 rbcs; ++++++>50 parasites/500 rbcs

Table 16B. Chloroquine susceptibility results of five recombinant progeny from the AJ x AS(3CQ) cross.

	Day 5 control not drugged	Day 11 drugged	Day 13 drugged	Day 15 drugged	Conclusion
AJ test 1	1 ++++ 2 +++++	1 - 2 - 3 -	1 - 2 - 3 -	1 - 2 +-- 3 -	sensitive parent
AJ test 2	1 ++++ 2 +++++	1 - 2 - 3 -	1 - 2 - 3 -	1 - 2 - 3 +-	
AS(3CQ) test 1	1 ++++ 2 ++++	1 +- 2 + 3 +-	1 ++++ 2 ++ 3 +++	1 ++++pp 2 +++pp 3 +++pp	resistant parent
AS(3CQ) test 2	1 +++++ 2 +++++	1 +- 2 +- 3 d	1 +++ 2 +++	1 +++++ 2 +++++	
39 / 1 test 1	1 +++++ 2 ++++	1 + 2 +- 3 ++	1 ++ 2 +++ 3 ++	1 ++pp 2 ++ 3 +	resistant
39 / 1 test 2	1 +++++ 2 +++++	1 +-- 2 - 3 +-	1 + 2 +- 3 +	1 +++ 2 + 3 +++	
74 / 5 test 1	1 ++++ 2 +++++	1 +-- 2 +- 3 +-	1 + 2 ++ 3 ++	1 +++ 2 +++++ 3 ++++	resistant
74 / 5 test 2	1 +++++ 2 ++++++	1 +- 2 - 3 +--	1 ++gams 2 +- 3 ++retics	1 ++++ 2 ++++ 3 +++++	

Table 16B. continued.

	Day 5 control not drugged	Day 11 drugged	Day 13 drugged	Day 15 drugged	Conclusion
43 / 8 test 1	1 ++++++ 2 ++++	1 - 2 - 3 +-	1 +- 2 +-- 3 +	1 +retics 2 + 3 ++	low level resistance
43 / 8 test 2	1 +++++ 2 ++++	1 +-- 2 - 3 -	1 +- 2 +-- 3 -	1 +- 2 +- 3 -	
63 / 3 test 1	1 +++++ 2 +++++	1 +-- 2 +- 3 +--	1 +- 2 + 3 +-	1 ++ 2 + 3 +-retics	low level resistance
63 / 3 test 2	1 +++++ 2 +++++	1 +-- 2 - 3 +--	1 - 2 +-- 3 +--	1 +- 2 +- 3 +-	
62 / 3 test 1	1 ++++ 2 +++++	1 - 2 - 3 d	1 - 2 -	1 - 2 +-	sensitive
62 / 3 test 2	1 +++ 2 +++++	1 - 2 - 3 -	1 - 2 - 3 -	1 - 2 - 3 -	

gams - gametocytes present; pp - post-peak of infection; d - died from unknown cause

Parasitaemia scoring as for **Table 16A**.

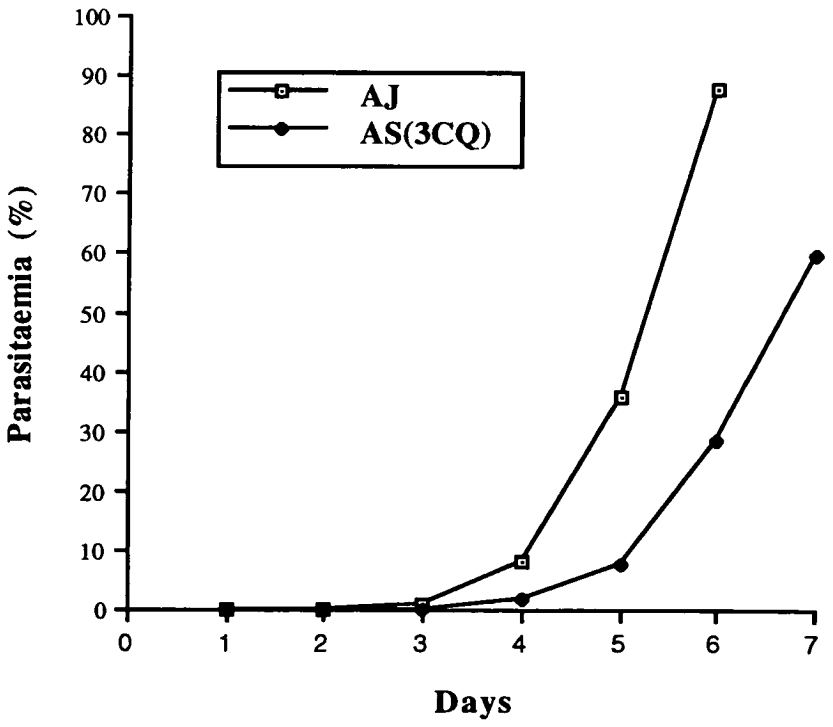
5.3.4 Course of infection of parent clones

During the progeny phenotyping it became obvious that parental clone AJ grew faster than parental clone AS(3CQ) in undrugged control mice, although equal numbers of parasites had initially been inoculated. **Figure 25** is a graph plotting the course of AS(3CQ) and AJ infections. Each point on the graph represents the average parasitaemia of two mice, determined from two counts of 350-500 red blood cells for each mouse. Standard errors (s.e; data not shown) around these parasitaemia measurements were calculated using the formula:

$$s.e = \sqrt{\frac{p(1-p)}{n}}$$ where: p is the measured proportion of parasitised rbcs
n is the total number of cells counted in the two mice

A significant difference in growth rates could be seen from D_2 onwards ($P < 0.01$). For example, AJ reached a parasitaemia of 40% by D_5, whereas AS(3CQ) did not reach this level until D_6. The importance of this observation, as regards the progeny of the cross, is discussed in **Chapter 6**.

Figure 25. The course of infection of parental clones AJ and AS(3CQ).



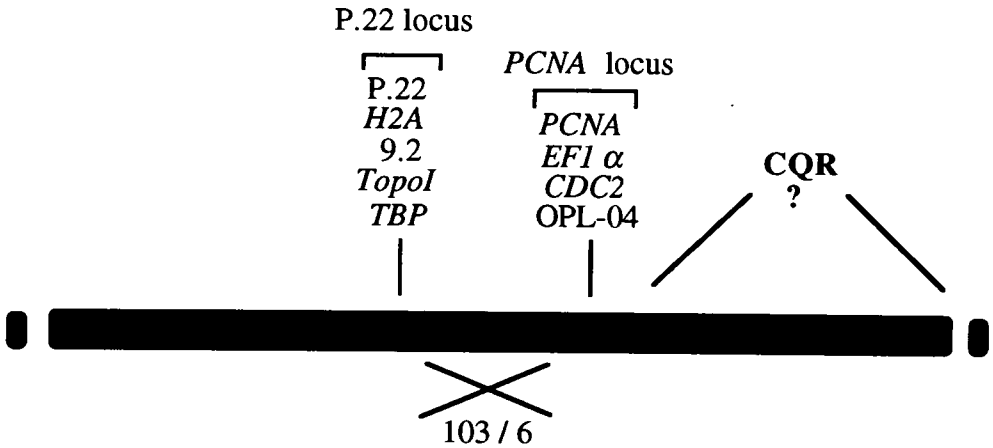
5.4 Linkage analysis

Analysis of the inheritance data of the 46 markers among the progeny clones immediately suggested a possible chloroquine resistance locus on chromosome 11 (**Table 14**, column labelled 'Linkage Ratio').

In all the clones examined, with the exception of clone 62 / 3, four markers (*PCNA*, *CDC2*, *EF1α* and OPL-04) co-segregated with the chloroquine resistance phenotype. In nine of the eleven clones, another five markers also segregated with chloroquine resistance (P.22, *H2A*, 9.2, *Topo I* and *TBP*). No such co-segregation with resistance was seen for markers on any of the other chromosomes.

From this information it was possible to produce a provisional linkage map of the markers along chromosome 11 (**Figure 26**). This shows a division of the nine

Figure 26. Genetic linkage map of *P. chabaudi* chromosome 11.



Schematic linkage map of a section of chromosome 11 showing the positions of nine markers relative to each other and to a possible chloroquine resistance locus (**CQR?**), as determined from the inheritance data in **Table 14**. The single crossing-over event which gave rise to recombinant clone 103 / 6 is marked. The map is not to scale.

markers into two 'loci', termed here the '*PCNA* locus' and the 'P.22 locus'. A cross-over event between these loci would account for the genotype of clone 103 / 6. A cross-over between the *PCNA* locus and the putative locus determining chloroquine resistance could also account for the genotype of clone 62 / 3. It was not possible to order the markers at the *PCNA* and P.22 loci in any more detail, because of the lack of cross-overs between them among the progeny clones.

An alternative explanation for the chromosome 11 results was that the chloroquine resistance locus was not on this chromosome, and that the co-segregation of the markers had occurred purely by chance. This possibility was investigated by

calculating the probability that association of the *PCNA* locus with chloroquine resistance could have occurred simply by chance, using the binomial probability, as follows:

The probability that association of the *PCNA* locus with chloroquine resistance in 10 of the 11 progeny could have occurred simply by chance:

Probability of obtaining observed result or better,

$$Pr = \sum_{x=r}^{n} \left[\frac{n!}{x!(n-x)!}\right] (P)^x (1-P)^{n-x}$$

where n = number of independent recombinant progeny

and r = number of recombinant progeny showing association with *PCNA* locus

and P = the probability of an association between chloroquine susceptibility and the *PCNA* locus, assuming no linkage

and 1-P = the probability of no association between chloroquine susceptibility and the *PCNA* locus

For n = 11 and r = 10, then

$$Pr = \left[\frac{11!}{10!(11-10)!}\right] (0.5)^{10} (0.5)^1 + \left[\frac{11!}{11!(11-11)!}*\right] (0.5)^{11} (0.5)^0$$

$$= 11\ (0.5)^{10}\ (0.5) + 1\ (0.5)^{11}$$

$$= 0.0054 \text{ (to 4 d.p.)} + 0.0005$$

$$= 0.0059 \text{ (to 4 d.p.)} < 0.05, \text{ i.e. significant at the 5\% level}$$

* Note that by definition, 0! is 1

Thus there is a 1 in 169 chance of the association being spurious. If the result had shown a chance of 1 in 20 or less, with the confidence interval set at 95%, there would have been a significant possibility that the association was spurious.

However, the probability of a false association between marker and phenotypic trait increases as the number of markers examined increases. At one extreme, the total number of markers used to obtain the inheritance data can be included in the statistical analysis to take account of this, although the result is likely to be highly conservative because markers representing the same locus are not excluded. For the data presented here, 46 markers in total were analysed. Assuming that the markers are independent of each other, the probability of obtaining the observed result or better, after testing 46 markers (i.e 46 different tests), is:-

$$Pr' = 1 - (\text{probability of obtaining a worse result})^{46}$$

$$= 1 - (1-Pr)^{46}$$

$$= 1 - (1 - 0.0059)^{46}$$

= 0.24 (to 2 d.p.) > 0.05, i.e. not significant at the 5% significance level

Therefore, there is a 1 in 4 chance of having obtained spurious linkage.

At the other extreme, a limited number of markers can be included in the analysis, because markers present on the same chromosome do not act independently of each other due to linkage. Therefore, the number of independent tests is not equal to the number of markers tested, but rather to the number of linkage groups within the genome. In all organisms which have been intensively studied genetically, the number of linkage groups is expected to be equal to n, the haploid number of chromosomes. In *P. chabaudi*, n = 14 (**Results 3.1**; Janse *et al.*, 1994). If the number of independent tests is taken to be 14, then:-

Probability of obtaining observed result or better, after 14 independent tests

$Pr'' = 1 - (1-Pr)^{14}$

$= 1 - (1 - 0.0059)^{14}$

= 0.08 (to 2 d.p.) > 0.05, i.e. not significant at the 5% level

It is likely that the true probability lies somewhere between these two values. As a concession to both extremes, if the number of loci showing different inheritance patterns according to the data in **Table 14** (i.e. 46-13=33) is used to calculate the probability, then:-

Probability of obtaining observed result or better, after 33 independent tests

$Pr''' = 1 - (1-Pr)^{33}$

$= 1 - (1 - 0.0059)^{33}$

= 0.17 (to 2 d.p.) > 0.05, i.e. not significant at the 5% level

Although these statistics revealed that the association of the *PCNA* locus with chloroquine susceptibility could have occurred by chance within a confidence interval of 95%, no other marker showed such a marked association. It was decided to analyse further clones from the AJ/AS(3CQ) cross to determine whether a chloroquine resistance locus might indeed exist on chromosome 11.

5.5 Analysis of additional progeny clones

Ten further clones were isolated from the uncloned progeny of the cross, using parasites from the second mouse on which infected mosquitoes had been allowed to feed (deep freeze stabilate no. 1523). It was hoped that cloning from these parasites would produce novel clones with genotypes distinct from those previously isolated. These were subjected to the same chloroquine susceptibility tests and RFLP analysis as the previous 11 clones.

5.5.1 Inheritance of parental markers

The clones were analysed for the inheritance of fourteen markers taken from chromosomes 1/2, 3, 5, 7, 8, 10, 11, 12, 13, and 14. **Table 17** shows the inheritance data of all ten clones.

Only two novel genotypes appear to have been isolated. Clone 131 / 1 was clearly of a different genotype from any clone isolated before. The remaining nine clones shared the same RFLP inheritance pattern as each other, but were also different from any clone isolated previously. It seems likely that these nine clones are identical, having originated from the same parasite; this is discussed further in **Chapter 6**. Clone 115 / 9 was selected as representative of all nine clones, and tested for susceptibility to chloroquine with clone 131 / 1.

5.5.2 Chloroquine susceptibility

Clones 131 / 1 and 115 / 9 were tested for chloroquine susceptibility twice as described previously, and the results are shown in **Table 18**. Both were found to be chloroquine-sensitive.

Table 17. Inheritance of RFLP markers in ten further progeny from the AJ x AS(3CQ) *P. chabaudi* cross.

			Progeny Clones									
Probe No.	Probe Name[a]	Chrom. No.	131 / 1	115 / 9	119 / 5	159 / 2	133 / 3	122 / 2	124 / 8	131 / 3	132 / 5	133 / 7
49	Ag3020	1	AJ[d]	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ
47	Ag3003 A	3	AS[d]	AS	AS	AS	AS	AS	AS	AS	AS	AS
50	Ag3024	5	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ
6	*DHFR*	7	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ
4	*MSP-1*	8	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
14	*5s rRNA*	10	AS	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ
29	*PCNA*	11	AJ	AJ	AJ	ND	ND	AJ	ND	AJ	AJ	ND
76	OPL-04	11	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ
11	*H2A*	11	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ
27	*Topo I*	11	AJ	AS	AS	ND	ND	AS	AS	AS	ND	ND
8	*TBP*	11	AJ	AS	AS	AS	AS	AS	AS	AS	AS	AS

Table 17. continued.

Probe No.	Probe Name	Chrom. No.	131 / 1	115 / 9	119 / 5	159 / 2	133 / 3	122 / 2	124 / 8	131 / 3	132 / 5	133 / 7
83	*pcmdr1*	12	AS	AS	AS	AS	AS	AS	AS	AS	AS	AS
84	pc-ps590.7	13	AJ	AS	AS	AS	AS	AS	AS	AS	AS	AS
59	**Ag-3040**[f]	14	AS	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ	AJ

Legend as for **Table 13**.

Table 18. Chloroquine susceptibility results of recombinant clones 131 / 1 and 115 / 9.

	Day 5 undrugged control	Day 11 drugged	Day 13 drugged	Day 15 drugged	Day 17 drugged	Conclusion
AJ test 1	1 +++++ 2 +++++	1 - 2 - 3 -	1 - 2 - 3 -	1 - 2 - 3 -	1 +- 2 +- 3 +-	sensitive parent
AJ test 2	1 +++++ 2 +++++	1 - 2 - 3 -	1 - 2 - 3 -	1 - 2 +-- 3 +--	1 - 2 +++ 3 ++	
AS(3CQ) test 1	1 +++++ 2 ++++	1 +- 2 +- 3 +++	1 +++ 2 +++ 3 ++++	1 ++++ 2 ++++ 3 +++++retics	1 ++++retics 2 ++++retics 3 +++++retics	resistant parent
AS(3CQ) test 2	1 +++++ 2 +++++	1 +- 2 +- 3 ++	1 ++ 2 ++ 3 ++	1 +++pp 2 +++pp 3 +++pp	1 ++pp 2 ++pp 3 +++pp	
131 / 1 test 1	1 +++++ 2 +++++	1 - 2 - 3 -	1 - 2 - 3 -	1 +- 2 - 3 -	1 +++ 2 + 3 +-	sensitive
131 / 1 test 2	1 +++++ 2 +++++	1 - 2 - 3 -	1 - 2 - 3 -	1 - 2 +- 3 -	1 +- 2 +++ 3 -	
115 / 9 test 1	1 ++++++ 2 ++++	1 - 2 - 3 -	1 - 2 - 3 -	1 +- 2 - 3 +	1 ++ 2 + 3 ++++	
115 / 9 test 2	1 ++++ 2 ++++	1 - 2 - 3 -	1 - 2 - 3 -	1 +- 2 + 3 +-	1 + 2 ++++ 3 ++++	sensitive

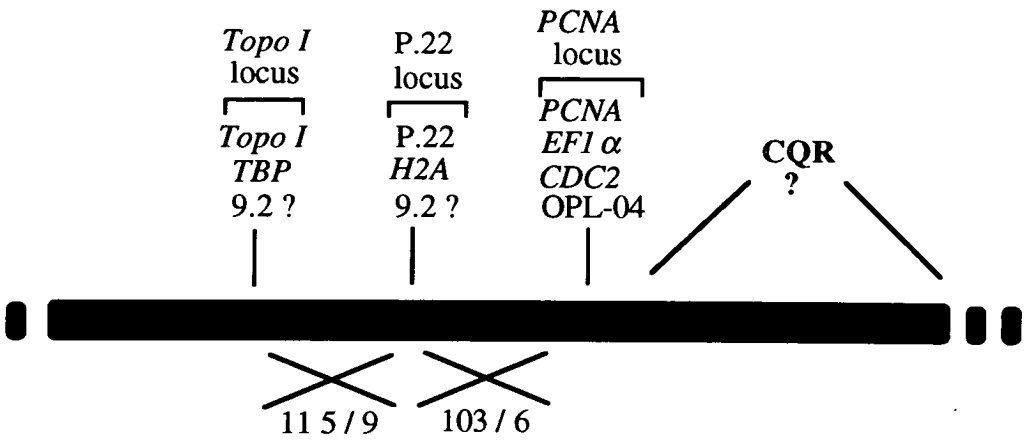
Legend as for **Table 16**.

5.5.3 Linkage analysis of all 13 clones

The chloroquine phenotype results and chromosome 11 inheritance data of all 13 clones are shown in **Table 19**.

It was clear from these results that a recombination event had occurred in clone 115 / 9, and because of this it was possible to order markers within the P.22 locus. **Figure 27** is a revised linkage map of chromosome 11 incorporating these changes.

Figure 27. Revised genetic linkage map of *P. chabaudi* chromosome 11



Revised linkage map of a section of chromosome 11 showing the positions of nine markers relative to each other and to a possible chloroquine resistance locus (**CQR?**), as determined from the inheritance data in **Table 19**. The two crossing-over events which gave rise to recombinant clones 103 / 6 and 115 / 9 are marked. It is not clear whether marker 9.2 segregates with the *Topo I* or P.22 locus, and this is represented as '9.2 ?'. The map is not to scale.

It is interesting to note that the demarcation of *TopoI* and *TBP* into a locus separate from the *PCNA* and P.22 loci correlates with the synteny data shown in **Table 12**. Two conserved syntenies are shown to exist on *P. chabaudi* chromosome 11, consisting of *PCNA*, *CDC2* and *EFIα* in one and found on *P. falciparum* chromosome 13, and *TBP* and *Topo I* in another, found on *P. falciparum* chromosome 5. *H2A* is not found in either synteny group, and this is reflected in its position in the separate P.22 locus and on *P. falciparum* chromosome 6.

Table 19. Inheritance of chromosome 11 RFLP markers in 13 progeny clones.

		Chloroquine-resistant clones								Chloroquine-sensitive clones					
Probe No.	Probe Name[a]	38 / 9	39 / 1	39 / 2	72 / 2	74 / 5	103 / 6	43 / 8	63 / 3	39 / 3	62 / 3	62 / 8	131 / 1	115 / 9	Linkage Ratio[b]
Chromosome 11															
16	*CDC2*	AS[d]	AS	AS	AS	AS	AS	AS	AS	AJ[d]	AS	AJ	ND	ND	10/11
15	*EF1α*	AS	AS	AS	AS	AS	AS	AS	AS	AJ	AS	AJ	ND	ND	10/11
29	*PCNA*	AS	AS	AS	AS	AS	AS	AS	AS	AJ	AS	AJ	AJ	AJ	12/13
76	OPL-04	AS	AS	AS	AS	AS	AS	AS	AS	AJ	AS	AJ	AJ	AJ	12/13
70	P. 22	AS	AS	AS	AS	AS	AJ	AS	AS	AJ	AS	AJ	ND	ND	9/11
11	*H2A*	AS	AS	AS	AS	AS	AJ	AS	AS	AJ	AS	AJ	AJ	AJ	11/13
12	**9.2**[f]	AS	AS	AS	AS	AS	AJ	AS	AS	AJ	AS	AJ	ND	ND	9/11
27	*Topo I*	AS	AS	AS	AS	AS	AJ	AS	AS	AJ	AS	AJ	AJ	AS	10/13
8	*TBP*	AS	AS	AS	AS	AS	AJ	AS	AS	AJ	AS	AJ	AJ	AS	10/13

Legend as for **Table 14.**

5.5.4 Statistical analysis of the linkage data

Linkage analysis of all 13 clones revealed that 12 of them showed association of markers OPL-04 and *PCNA* with chloroquine susceptibility. The probability that this association had occurred simply by chance was calculated using the binomial probability, as described previously:

For n= 13 and r = 12, then

$$\Pr = \left[\frac{13!}{12!(13-12)!}\right](0.5)^{12}(0.5)^{1} + \left[\frac{13!}{13!(13-13)!}\right](0.5)^{13}(0.5)^{0}$$

$$= 13\,(0.5)^{12}\,(0.5) + 1\,(0.5)^{13}$$

$$= 0.0017 \text{ (to 4 d.p.)} < 0.05, \text{ i.e. significant at the 5\% level}$$

This represents a 1 in 588 chance of the association being spurious, which is highly significant using a confidence interval of 95%.

Correcting for the total number of markers analysed, which will produce the most conservative estimate of an association not having occurred by chance:

Probability of obtaining observed result or better, after testing 46 markers (i.e 46 different tests), is:-

$$\Pr' = 1 - (\text{probability of obtaining a worse result})^{46}$$

$$= 1 - (1-\Pr)^{46}$$

$$= 1 - (1 - 0.0017)^{46}$$

$$= 0.075 \text{ (to 3 d.p.)} > 0.05, \text{ i.e. not significant at the 5\% level}$$

This represents a significant decrease in the probability that the association has occurred by chance, from 0.24 (as determined from 11 progeny, see **Section 5.4.2**) to 0.075.

According to the revised inheritance data, 46-12=34 loci appear to have different inheritance patterns. If this value is used to calculate the probability, then:

Probability of obtaining observed result or better, after testing 34 independent markers

$$\Pr'' = 1 - (1-\Pr)^{34}$$

$$= 1 - (1 - 0.0017)^{34}$$

$$= 0.056 \text{ (to 3 d.p.)} > 0.05, \text{ i.e. close to significance at the 5\% level}$$

This probability is still quite conservative, given that some of the 34 markers are known to be on the same chromosome. Indeed, a probability of 0.024 (to 3 d.p.), i.e. a 1 in 42 chance, of obtaining false linkage is produced when taking the number of independent tests is taken as 14. This is a highly significant figure at the 5% level.

Thus a range of probability values can be calculated for the data, depending upon the method of calculation. Analysis of more progeny clones and chromosome markers

is needed in order to determine with greater significance the likelihood that a chloroquine resistance locus exists on chromosome 11 in *P. chabaudi.*

5.6 Construction of a genetic linkage map of *P. chabaudi*

A genetic linkage map of the *P. chabaudi* genome was constructed using all the data obtained from development of over 100 markers and linkage analysis of 13 progeny clones. Results are shown in **Figure 28**.

The 44 polymorphic markers are ordered along each chromosome according to the inheritance data, although the distance between them bears no relationship to the actual distance. At least one marker was identified for each chromosome, and some chromosomes had as many as 20 (e.g. chromosome 5). Certain markers could not be allocated to a particular chromosome because of insufficient separation during PFGE, and others hybridised to several chromosomes and were classsified as multi-copy. The map also includes markers hybridised to *P. chabaudi* chromosome separations during collaborative work with C. Janse and A. Waters (see Janse *et al.*, 1994).

Figure 28. Chromosome map of the genome of *P. chabaudi* clone AS; chromosomes 1-7.

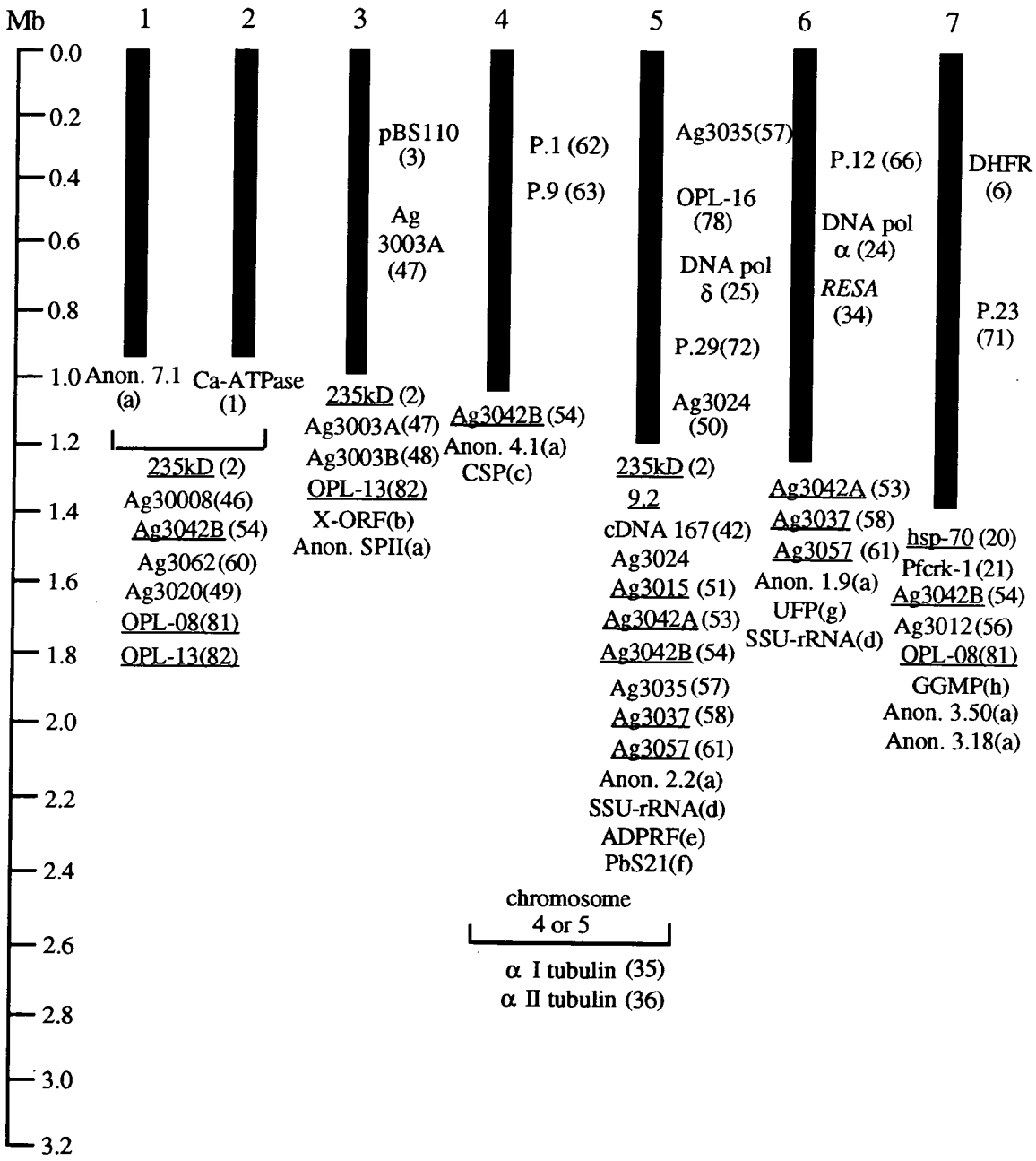
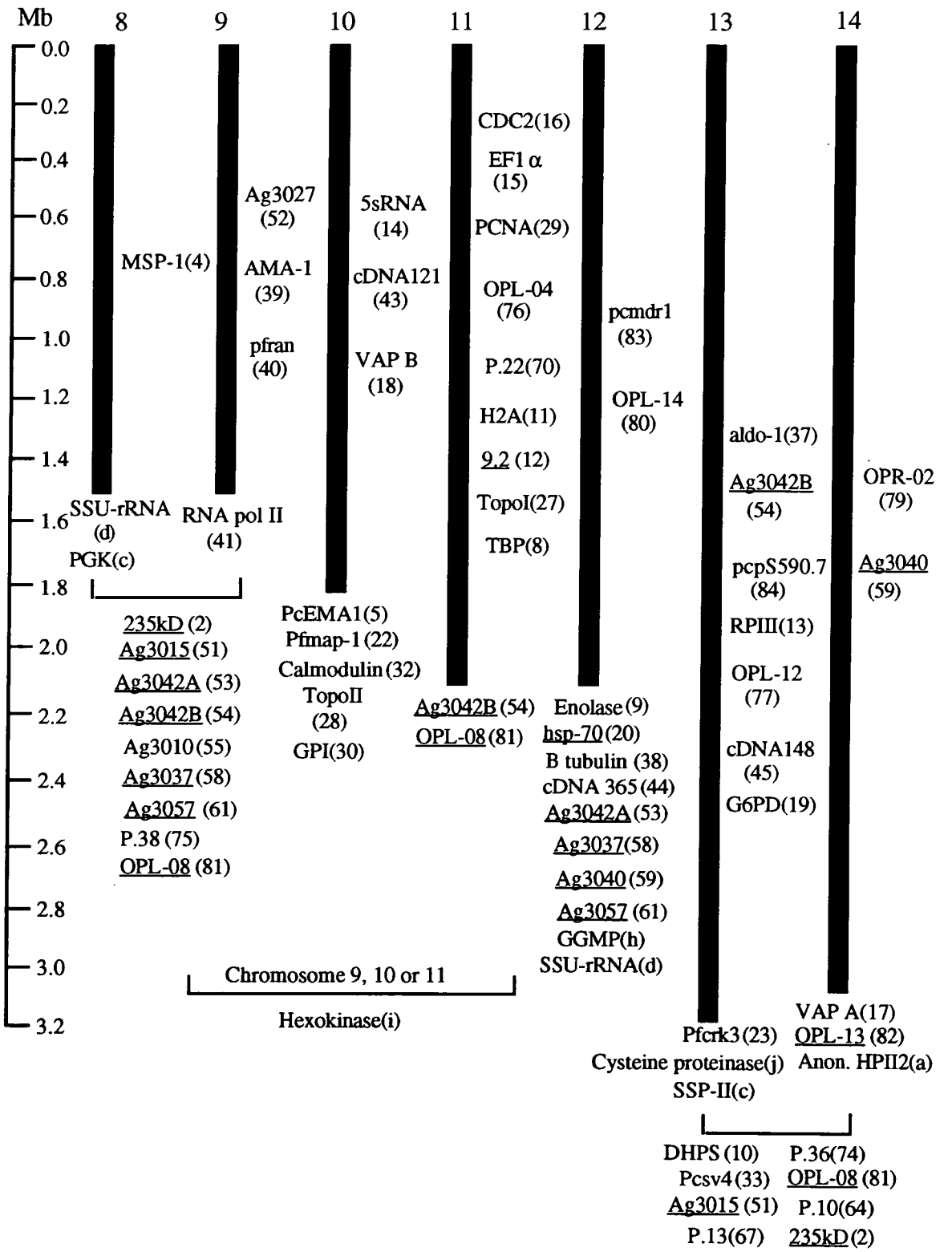


Figure 28. Chromosome map of the genome of *P. chabaudi* clone AS; chromosomes 8-14.



Each chromosome is drawn to scale. Chromosome-specific markers are arranged under their respective chromosomes, and polymorphic markers used to analyse the products of the cross are ordered alongside chromosomes. Markers represented more than once in the genome are underlined. Numbers in parenthesis refer to the Probe Number as shown in **Tables 5, 9** and **10**. Letters in parentheses refer to the reference for probes not previously mentioned, as follows:-

(a) anonymous DNA probe (Ponzi *et al.*, 1990)

(b) probe containing an unidentified open reading frame; A. Thomas, Rijswijk, The Netherlands (Janse *et al.*, 1994)

(c) see **Table 12** for reference

(d) rRNA small sub-unit DNA probe (Dame and McCutchan, 1983)

(e) ADP ribosylation factor gene, A. Waters, Leiden University, The Netherlands (Janse *et al.*, 1994)

(f) 21-kDa ookinete surface antigen gene (Paton *et al.*, 1992)

(g) ubiquitin fusion protein gene (Ohmachi *et al.*, 1989)

(h) gene encoding a protein with multiple GGMP repeats (Langsley *et al.*, 1993)

(i) hexokinase gene (Olafsson *et al.*, 1993)

(j) cysteine proteinase gene (Rosenthal, 1993)

6. Discussion

6.1 Summary of the results

The principal findings of this project can be summarised as follows:

(1) Analysis of the *P. chabaudi* genome.

The first genetic linkage map of *P. chabaudi* has been made, using DNA markers developed by a number of different methods, including the novel RAPD-PCR technique. Improvements in chromosome separation have provided the means to characterise the *P. chabaudi* karyotype, and show that the number of chromosomes present is fourteen. Studies of the inheritance of markers has enabled the identification of over 40 cross-over events. A comparison of genes conserved between *P. chabaudi* and *P. falciparum* has resulted in the identification of at least 10 conserved syntenies between the two genomes.

(2) Analysis of the genetic basis of chloroquine resistance.

A cross between chloroquine-resistant and chloroquine-sensitive parasites has been made, and the recombinant progeny analysed for their chloroquine susceptibility and inheritance of 46 markers. The *P. chabaudi* homologue of the *P. falciparum* MDR gene was isolated, and found not to be linked to chloroquine susceptibility in the progeny studied. A possible homologue of the marker claimed to be linked to a locus determining chloroquine resistance in *P. falciparum*, pS590.7, was isolated from *P. chabaudi*, but showed no association with the chloroquine resistance trait. A possible chloroquine resistance locus on chromosome 11 was identified through linkage of markers in 12 out of 13 progeny clones studied.

The results are discussed here with regard to work by others on these subjects.

6.2 Is chloroquine resistance a multigenic trait?

Resistance of *P. falciparum* to the antimalarial drug pyrimethamine arose independently from various geographical foci within 2 years of the introduction of the drug. The ease of development of resistance suggested that mutation of a single locus was responsible. Subsequently the genetic basis of pyrimethamine resistance was found to be due to a single point mutation in the DHFR gene. This is in marked contrast to the appearance of chloroquine resistance, which first became evident 20 years after its introduction to the field. This has led some researchers to conclude that the genetic basis of chloroquine resistance probably involves an additive effect of mutations at more than one locus, i.e that it is a multigenic trait (e.g. Cowman and

Foote, 1990).

As described in the **Introduction**, initial work by Foote *et al.* (1989) produced evidence that the *pfmdr1* gene of *P. falciparum* was involved in chloroquine resistance. This was disputed by Wellems *et al.*, who took the view that resistance was caused by a single mutant gene, and subsequently produced evidence that this was not *pfmdr1* (Wellems *et al.*, 1990). Linkage analysis of a cross between chloroquine-resistant and chloroquine-sensitive parasites detected a single locus on chromosome 7 which segregated with the chloroquine resistance phenotype (Wellems *et al.*, 1991). However, Foote *et al.* (1990b) provided evidence of a strong link between *pfmdr1* genotype and chloroquine resistance, when the chloroquine susceptibility of 34 of 36 isolates was correctly predicted from sequencing the *pfmdr1* gene. A 'competent *mdr* theory' was proposed to explain these results, which suggested that both a *pfmdr1* allele competent for chloroquine resistance and a mutation in a second unknown gene (possibly Wellems' chromosome 7 gene) were required for chloroquine resistance in *P. falciparum* (for comment, see Newbold, 1990).

The single-gene/multi-gene nature of chloroquine resistance remains unresolved. This prompts the question of whether it is possible to shed light on this subject from studies of laboratory-induced chloroquine-resistant rodent malaria mutants, and in particular from the results produced during this project.

The manner in which chloroquine-resistant rodent malaria mutants have been selected may give an indication as to how many mutant genes are involved in the mechanism of resistance. Only a continuous low drug selection pressure method has resulted in the appearance of stable, resistant parasites (Powers *et al.*, 1969; Rosario, 1976b). High levels of resistance have been produced by gradual increments in the initial drug pressure (Padua, 1980). These results suggest that low levels of chloroquine resistance may be caused by mutations at several loci, and that the accumulation of these in a single parasite results in high levels of resistance. The cross between a highly chloroquine resistant *P. chabaudi* mutant, AS(30CQ), and the sensitive clone AJ, produced progeny with intermediate levels of resistance (Padua, 1981), and thus would appear to provide evidence for this theory.

The manner in which *P. chabaudi* clone AS(3CQ) was selected and its low level of chloroquine resistance, suggested that mutations in a few genes, or even a single gene, might be responsible for the resistance (Rosario, 1976a). Unfortunately, analysis of the progeny from the first cross between AS(3CQ) and a sensitive clone AJ, which might have resolved the question of how many genes were involved, was inconclusive; intermediate levels of chloroquine resistance were recorded for some of the recombinants, but it was not clear whether this was due to a multigenic phenotype

or to host effects (Rosario, 1976a).

The results of the work presented here could be explained by a single gene on chromosome 11 being responsible for the chloroquine resistance of AS(3CQ). However, certain findings suggest that more than one gene could be involved. Thirteen recombinant progeny clones were examined, five of which were phenotyped as chloroquine-sensitive and eight as chloroquine-resistant. The resistant progeny could be further sub-divided; six clones showed similar resistance to the AS(3CQ) parent clone, whereas two clones showed an intermediate level of resistance. These results could be explained if resistance is caused by mutations at more than one locus. During the original selection for resistance in AS(3CQ), parasites were treated with the lowest level of drug which produced stable resistance, and drug pressure was stopped two passages after resistance had been established (Rosario, 1976a). This does not exclude the possibility that mutants selected for resistance to less than 3 mg/kg chloroquine might contain mutations in only one gene. Tanabe *et al.* (1990) have shown that *P. chabaudi* AS parasites are unaffected by chloroquine at a concentration of 1 mg/kg, but that they are marginally suppressed at 2 mg/kg, suggesting that parasites exhibiting levels of resistance intermediate between 1 mg/kg and 3 mg/kg could be produced.

A simple genetic model can be formulated based on the discovery of two resistant phenotypes among the progeny of the cross:-

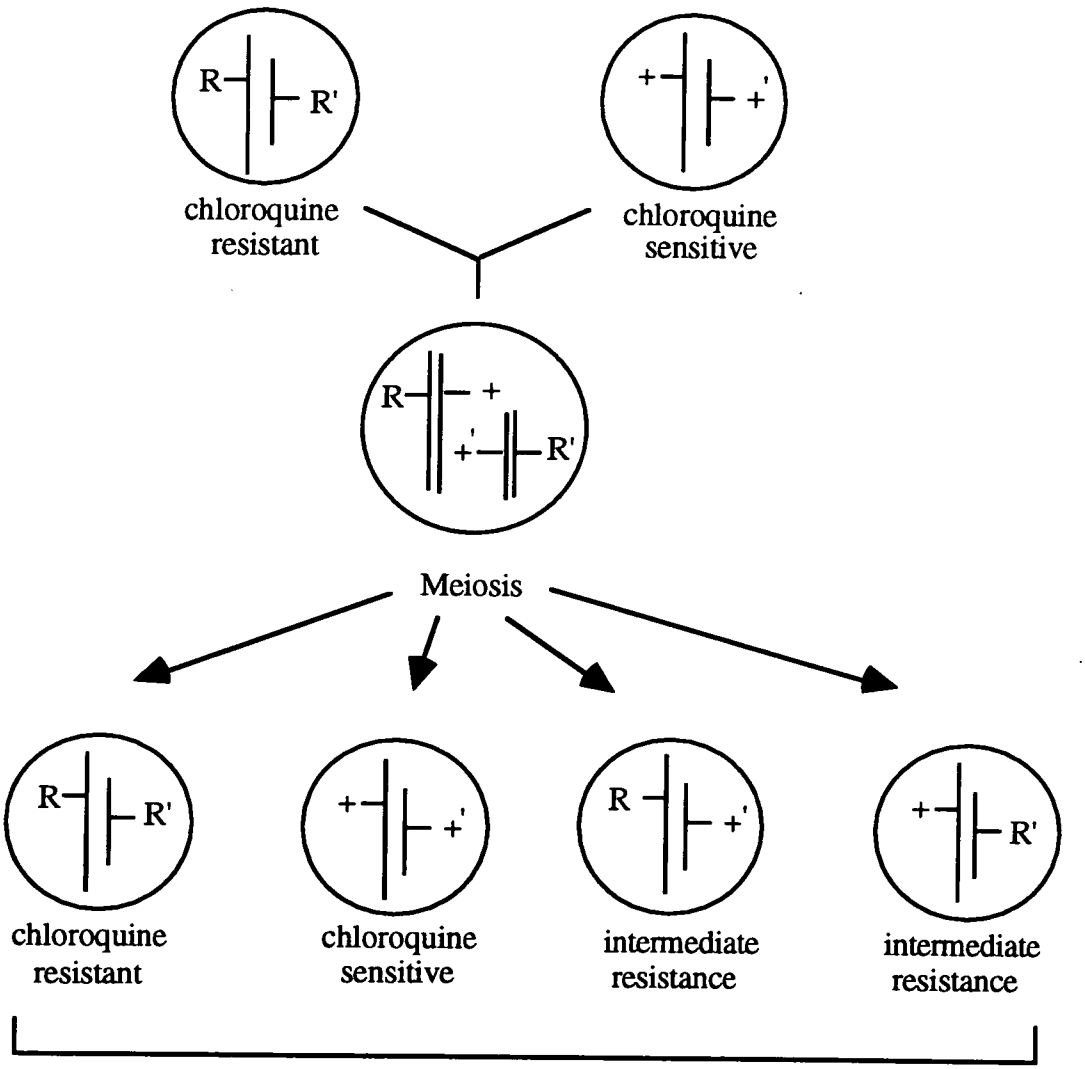
1) Two mutant genes at separate loci confer chloroquine resistance in AS(3CQ)

Let the two mutant genes be referred to as R and R', the wild-type sensitive forms being + and +'. Each mutant gene confers a low level of resistance, but in combination they produce a higher level of resistance. The progeny of a cross between RR' (AS(3CQ)) and ++' (AJ) would show 3 phenotypes: (i) sensitive (++'), (ii) a high level of resistance (RR'), and (iii) a lower level of resistance due to the segregation of either R or R', producing R+' or +R'. Twice as many progeny exhibiting the intermediate level of resistance would be expected compared with the number exhibiting the high level of resistance, among the recombinant progeny. A diagram of this model is shown in **Figure 29**.

If the two-gene model predicted from the phenotypic data is accurate, it should fit with the available genetic data. Linkage analysis of the 13 recombinant clones identified only a single locus on chromosome 11 which may be involved in chloroquine resistance. No other markers showed such an association. A second locus required by the two-gene model was not detected; this may have been because an insufficient number of markers were examined for their inheritance. For example, the

progeny clones were examined for the inheritance of only one marker, *MSP1*, specific for chromosome 8. Analysis of *MSP1* produced a linkage ratio of 8/13, suggesting that there was no association of the marker with chloroquine susceptibility. However, this result does not exclude the possibility that other markers are present on the chromosome which are linked to resistance genes.

Figure 29. Diagram of the segregation and phenotypes expected if two genes, R and R', confer chloroquine resistance.



It is not possible to draw conclusions from the observed numbers of clones of each phenotype compared with the expected number, because of the small number of progeny analysed.

Clone 62 / 3, which is chloroquine-sensitive but has the AS(3CQ) alleles of all chromosome 11 markers, does not fit well with the two gene model unless a cross-over between the *PCNA* locus and the putative chloroquine resistance locus is assumed, with the corollary that the *PCNA* locus is not tightly linked to the resistance locus. Alternatively, a second model may fit the results better:

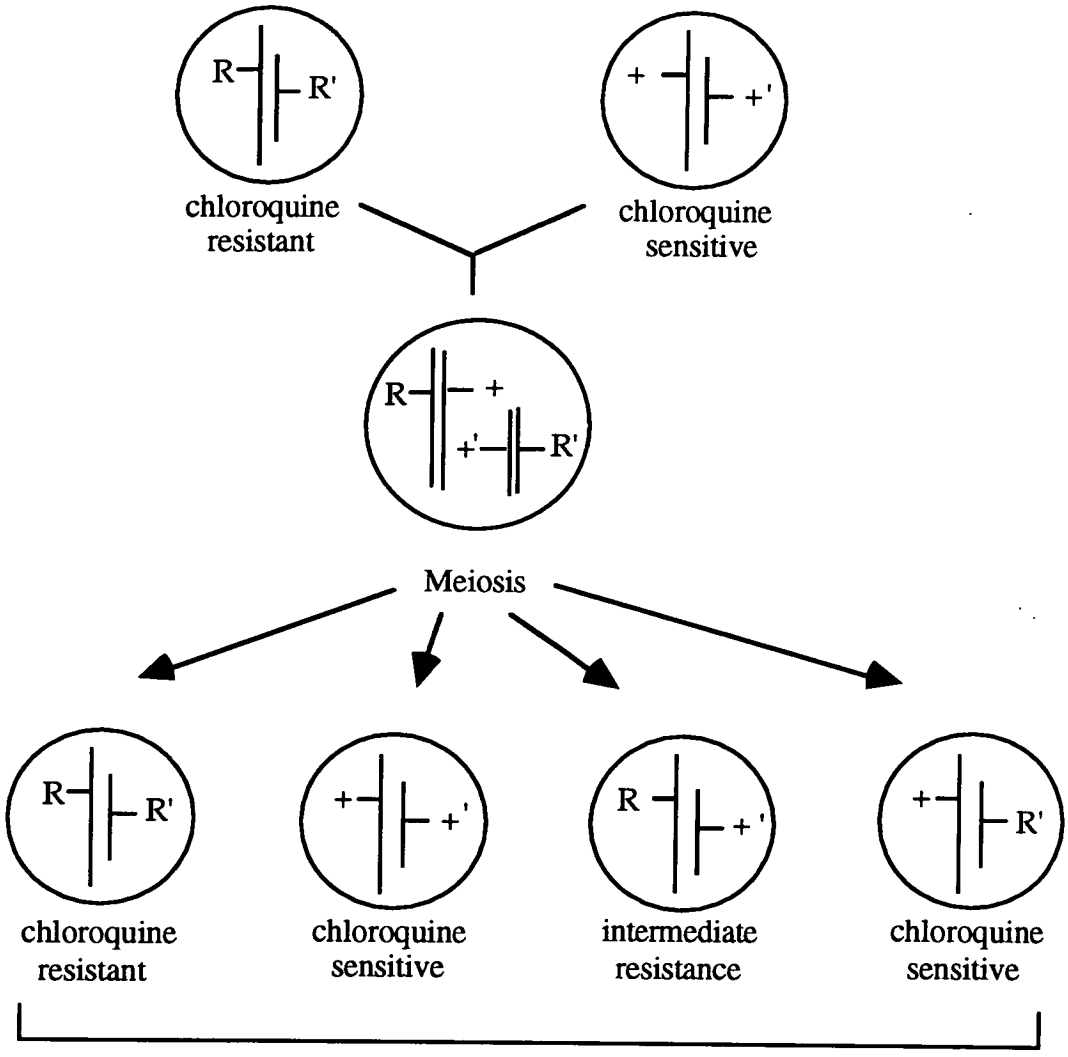
2) A single mutant gene confers a basal level of chloroquine resistance, which is enhanced by other competent gene(s) that alone cannot produce resistance

Let the mutant gene that confers a basal level of resistance be referred to as R, and a second gene that enhances resistance as R'; the wild-type of these genes are denoted as + and +' respectively. R is epistatic to R'. The progeny of a cross between the clone RR' (AS(3CQ)) and ++' (AJ) would exhibit 3 phenotypes: (i) sensitive (genotypes ++' and +R'), (ii) resistant (RR'), and (iii) a lower level of resistance, due to the segregation of R only. Twice as many sensitive progeny would be expected compared with each of the resistant phenotypes, among the recombinant progeny. A diagram of this model is shown in **Figure 30**. The model is reminiscent of the competent MDR theory suggested by Foote *et al.* (1990b) as mentioned above.

In this model, a second resistance locus would be less discernible by linkage analysis because the primary resistance locus is epistatic to it. Thus the model also predicts that some recombinant progeny could be typed as chloroquine sensitive even though they contain competent allele(s). This could explain the result of clone 62 / 3; it may be competent at the chromosome 11 locus but lack the epistatic gene which is an absolute requirement for chloroquine resistance. The competent gene model therefore appears to fit both the phenotypic and genotypic data of the cross. However, it is not possible to draw conclusions from the observed numbers of clones of each phenotype because of the small number of progeny analysed.

As discussed previously, chloroquine resistance in *P. chabaudi* appears to be a stable character and inherited in a Mendelian fashion (Rosario, 1976b), i.e it is not due to a temporary physiological change. It has been shown that clone AJ grows faster than AS(3CQ) in the absence of chloroquine (**Figure 25**). In the presence of chloroquine, AJ does not grow to the same extent as AS(3CQ), indicating that chloroquine resistance gene(s) exist in the latter which are not present in the former. Thus the presence of growth genes and the presence of drug resistance genes are two different components. This does not exclude the possibility that the resistance phenotype could be enhanced by independently segregating growth genes, but it does rule out the prospect that progeny typed as having intermediate levels of resistance are actually fast growing chloroquine sensitive clones.

Figure 30. Diagram of the segregation and phenotypes expected if gene R confers chloroquine resistance and is epistatic to a second mutant gene R' which alone cannot produce resistance.



To conclude, an explanation of the cross results reported here is that at least two genes are involved in the mechanism of chloroquine resistance in AS(3CQ). If the locus identified on chromosome 11 plays a rôle in resistance, then it may be as a secondary locus, competent forms of which increase the level of resistance in conjunction with a major gene.

The results and model presented above do not preclude the possibility that the chloroquine resistance exhibited by AS(3CQ) may be a phenotypically continuous trait, caused by mutations at many loci which have an additive effect. This is because the *in vivo* drug tests may not be sufficiently sensitive to distinguish between additional classes of drug susceptibility (discussed in more detail in **Section 6.7**).

6.3 Candidate chloroquine resistance genes

The nature of the gene, or genes, at the chromosome 11 locus identified during this work, or of genes which might be present at other chloroquine resistance loci, is not known. In the absence of chloroquine, AS(3CQ) is morphologically identical to the sensitive clone from which it was derived, consequently no gene product differences are visible. However, several genes have been proposed as candidate chloroquine resistance genes in *P. falciparum*, and some of these have been analysed in this project. These are discussed in relation to the chromosome 11 locus as follows:

1. *pfmdr1*.

Cross-hybridisation studies during this work suggested that the *P. chabaudi* homologue, *pcmdr1*, is on chromosome 12. Thus the chromosome location of this gene excludes it as being the putative chloroquine resistance locus found during this study. The possibility that *pcmdr1* might play a rôle in chloroquine resistance in AS(3CQ) is discussed in more detail in the following section.

2. pS590.7.

Cross-hybridisation of this marker to *P. chabaudi* chromosomes 1/2 and 12 ruled out the possibility that the *P. chabaudi* homologue might have been closely linked to the chromosome 11 locus. The putative *P. chabaudi* homologue of pS590.7 is discussed in more detail in **Section 6.10**.

3. Genes coding for the vacuolar ATPase subunits A and B (*VAP A* and *VAP B*).

Both *P. chabaudi* homologues of *VAP A* and *VAP B* were found on chromosomes other than chromosome 11, excluding the possibility that they might be the chromosome 11 locus genes. No RFLPs were identified for the *VAP A* homologue, but a *Hind* III RFLP found within *VAP B* homologue enabled the cross progeny to be examined for inheritance of the gene. Limited results from only nine of the clones showed no evidence of the AS(3CQ) allele segregating with either the chloroquine-resistant or the intermediate chloroquine-resistant progeny. In *P. falciparum*, attempts at showing a link between mutations in these genes and a proposed chloroquine resistance-linked, vacuolar acidification defect have been unsuccessful (Karcz *et al.*, 1993b; Karcz *et al.*, 1994). The genetic results presented here support these findings. Further work to illuminate the part played by the proton pump in the accumulation of chloroquine within the lysosome could include a comparison of the enzymatic properties of the vacuolar H^+ ATPase holoenzyme from chloroquine-resistant and sensitive parasites (Karcz *et al.*, 1994). *P. chabaudi* clone AS(3CQ) would be an ideal

model for this because it is isogenic with the chloroquine-sensitive line AS(sens).

4. Calmodulin.

Calmodulin was first proposed as having a rôle in chloroquine resistance by Scheibel (1987), but subsequent studies involving the gene cloned from *P. falciparum* found these claims to be unfounded, at least in the few clones studied (Cowman and Galatis, 1991). Calcium transport and regulation in *P. chabaudi* have been studied (Tanabe *et al.*, 1982) and it is likely that similar mechanisms operate in this species and *P. falciparum*. Therefore, it was of interest to see what rôle the gene might play in chloroquine resistance in AS(3CQ). The *P. falciparum* gene was found to cross-hybridise to *P. chabaudi* chromosome 10. However, cross-hybridisation to Southern blots was weak and RFLPs were not detected, preventing examination of the AJ/AS(3CQ) cross progeny for segregation of the marker with chloroquine resistance. The observation that the *P. chabaudi* homologue is on chromosome 10 rules out this gene as being the chromosome 11 resistance locus.

5. Genes involved in drug resistance in other organisms.

Studying mechanisms of drug resistance in other organisms may provide clues as to possible chloroquine resistance genes. For example, clinical isolates of *Pseudomonas aeruginosa* are resistant to aminoglycosides such as gentamycin, because of altered cell wall porins which cause reduced uptake of the drug (see review by Neu (1992)). This prompts the question that perhaps a mechanism of chloroquine uptake could be involved in resistance. However, it is not known how chloroquine is taken up. One theory proposes that a permease pumps the drug into the food vacuole (Warhurst, 1986). The only known permease on the surface of the vacuole, with the exception of the proton pump, is the product of *pfmdr1*, Pgh1 (Cowman *et al.*, 1991), but it is not thought to transport chloroquine directly (van Es *et al.*, 1994b). An understanding of the mechanism of chloroquine uptake may help to identify other genes which could be involved in chloroquine resistance.

The pleiotropic drug resistance gene *PDR1* of *S. cerevisiae* also provides an interesting example of a resistance mechanism which may be relevant to malaria parasites. The amino acid sequence of *PDR1* has homology to regulatory proteins involved in the control of gene expression (Balzi *et al.*, 1987). Mutations in *PDR1* are known to produce changes in the protein which interfere with the transcriptional control of several genes coding for membrane proteins. These membrane proteins have been proposed as regulators of drug import and/or efflux (Balzi *et al.*, 1987), and therefore alterations in their expression could alter drug accumulation. The gene

coding for one such protein, *PDR5*, has been cloned and found to be a putative membrane pump which confers multidrug resistance when caused to be over-expressed by *PDR1* (Balzi *et al.*, 1994). There is also evidence that alternative *PDR1* mutations could affect the expression of other target genes with distinct efficiencies.

The identification of a single resistance phenotype (identical chloroquine efflux and IC_{50} values) among the progeny of the Dd2/HB3 cross, and cloning of a single locus on chromosome 7 which segregates with chloroquine susceptibility (Wellems *et al.*, 1991), could be due to a single gene having multiple effects through regulation of a number of other genes. Different mutations at the locus could produce different levels of resistance. However, this molecular mechanism does not explain why resistance to chloroquine took so long to appear after the introduction of the drug in the field.

6.4 The rôle of P-glycoprotein genes in drug resistance

Genes coding for parasite P-glycoproteins have been cloned from *Trypanosoma cruzi* (Dallagiovanna *et al.*, 1994), *Schistosoma mansoni* (Bosch *et al.*, 1994), *Trichomonas vaginalis* (Johnson *et al.*, 1994), *Entamoeba histolytica* (Descoteaux *et al.*, 1992), and *Leishmania* (reviewed by Ouellette *et al.*, 1994). A distinction exists between P-glycoproteins thought to be involved in the mechanism of resistance to a variety of structurally unrelated drugs, and those involved in resistance to specific drugs. For example, the *L. major* P-glycoprotein gene *pgpA* is involved in resistance to arsenite and antimonite (Callahan and Beverley, 1991; Papadopoulou *et al.*, 1994), but not to a spectrum of drugs to which MDR mammalian cells are resistant (Légaré *et al.*, 1994), and to which amplification of the *ldmdr1* gene of *L. donovani* produces resistance (Henderson *et al.*, 1992). Moreover, not all parasite P-glycoproteins have been found to be involved in drug resistance. The lack of a consistent relationship between, for example, metronidazole resistance and levels of expression of a *T. vaginalis* P-glycoprotein gene, *Tvpgp1* (Johnson *et al.*, 1994), has led to the suggestion that the P-glycoprotein in protozoan parasites may be involved in a general stress-related state that allows the organism to survive until a specific response, such as a resistance mechanism, is mounted (Ouellette and Borst, 1991).

These points should be kept in mind when considering the rôle that MDR genes might play in drug resistance in *Plasmodium*. Latterly, the two MDR genes cloned from *P. falciparum*, *pfmdr1* and *pfmdr2*, have been considered to be involved in chloroquine resistance rather than multiple drug resistance. Although the concept of parasites exhibiting cross-resistance between structurally unrelated drugs has been discussed (see Peters (1987) for example), such isolates are difficult to appraise *in vivo* because of the problems in assessing whether the parasite population is clonal or

a mixture of parasites, each with a different drug susceptibility (Cowman and Foote, 1990). The widespread use of many antimalarial drugs makes it possible that parasites have successively acquired resistance to a number of drugs, rather than acquiring resistance to one drug which renders the parasite resistant to many others. Thus the term 'cross-resistance' should ideally be used to refer to parasites which are resistant to structurally related compounds, and 'multidrug resistance' to parasites which are resistant to many drugs, however the resistance mechanism was acquired.

Historically, two lines of phenotypic evidence indicated that the failure of chloroquine therapy in *P. falciparum* infections might be attributed to a multidrug resistance mechanism analogous to that observed in MDR mammalian cells; (1) the demonstration that chloroquine resistance is associated with impaired drug accumulation (Fitch, 1970; Verdier *et al.*, 1985; Yayon *et al.*, 1985) and enhanced drug efflux (Krogstad *et al.*, 1987; Krogstad *et al.*, 1992); and (2) the reversal of chloroquine resistance by calcium channel blockers (Martin *et al.*, 1987; Bitonti *et al.*, 1988; Kyle *et al.*, 1990).

Recent studies have called these lines of evidence into question. Firstly, no difference has been found in chloroquine efflux rates between some sensitive and resistant parasites (Bray *et al.*, 1992), suggesting that chloroquine resistance may not be caused by a mechanism which pumps out chloroquine. Secondly, Bray *et al.* (1994) showed that the reversing effect of the calcium modulator verapamil could not be accounted for by its effect on chloroquine accumulation. Thirdly, models based upon existing data indicate that the difference in drug accumulation between resistant and sensitive parasites could be due to a decrease in vacuolar proton pump activity (Geary *et al.*, 1990; Ginsburg and Stein, 1991; Ginsburg and Krugliak, 1992), or the presence of a drug-importer in chloroquine sensitive parasites (Ferrari and Cutler, 1991), without having to invoke the presence of an MDR export pump .

Thus the relationship between the MDR phenotype of mammalian tumour cells and the chloroquine resistance phenotype of *Plasmodium* is not as clear-cut as was once suggested. This is also clear from genetic studies on *pfmdr1* and *pfmdr2* as described in the **Introduction** and reviewed in Ginsburg (1991) and Foote and Cowman (1994).

The *P. chabaudi* homologue of *pfmdr1*, *pcmdr1*, was an obvious candidate marker to be used in analysis of the AS(3CQ)/AJ cross in order to assess the rôle it might play, if any, in chloroquine resistance in *P. chabaudi*. Moreover, reports that calcium antagonists reversed resistance in AS(3CQ) (Tanabe *et al.*, 1990), and that resistant parasites accumulated chloroquine at a reduced rate compared with sensitive parasites (attributed to enhanced efflux of the drug in the resistant line, although no efflux

studies were carried out) (Miki *et al.*, 1992), strengthened the phenotypic similarities between chloroquine resistance in *P. falciparum* and *P. chabaudi* and made the assessment even more appropriate.

The sequence data and cross-hybridisation studies with *pfmdr1* suggested that part of the *pcmdr1* gene had been isolated in the current work. As an RFLP of the homologue was to be used as a marker for linkage analysis of the progeny, it was not thought necessary to isolate and sequence the complete gene. The presence of a single copy of the gene on *P. chabaudi* chromosome 12 and data from probing Southern blots of genomic DNA appeared to exclude the possibility that the fragment was part of a pseudogene. This is a significant finding because of an earlier report of two P-glycoprotein pseudogenes, each with a frame shift and stop codon in identical places within the amino ATP-binding site, in *E. histolytica* (Descoteaux *et al.*, 1992). Further evidence that the fragment does not originate from a pseudogene comes from the apparent duplicative transposition of the gene to a second chromosome in several *P. chabaudi* lines selected for mefloquine resistance (Bisoni, 1994). Evidence that amplification of *pfmdr1* causes mefloquine resistance in *P. falciparum* was discussed in the **Introduction** (Barnes *et al.*, 1992; Cowman *et al.*, 1994; Peel *et al.*, 1994).

It was also important to determine the copy number of the gene because of results showing amplification of *pfmdr1* in some chloroquine-resistant isolates of *P. falciparum* (Foote *et al.*, 1989; Wilson *et al.*, 1989). Southern blot data suggests that the gene exists as a single copy in the genome (unless it is amplified as a tandem array with no disruption of restriction sites). Further proof comes from PFG separations of chromosome 12 from AS(3CQ) and AS(sens) parasites, which showed no difference in size between the chromosomes, unlike chloroquine-resistant *P. falciparum* isolates with *pfmdr1* amplicons, which show considerable variations in the size of chromosome 5 compared with sensitive isolates (Foote *et al.*, 1989).

Linkage analysis of the progeny clones with *pcmdr1* failed to show segregation of the gene with chloroquine resistance (**Table 14**). Thus chloroquine resistance in AS(3CQ) and in the progeny does not appear to be caused exclusively by a mutant allele of *pcmdr1*. Moreover, a competent form of *pcmdr1*, <u>in addition</u> to a major resistance gene, was not recognised as enhancing chloroquine resistance among the progeny; two out of the six resistant clones inherited AJ alleles of the gene, and one of the two intermediate resistant clones inherited the AS(3CQ) allele of the gene. Foote *et al.* have proposed that <u>both</u> a competent *pfmdr1* allele and a mutation in a second gene are necessary for chloroquine resistance (Foote *et al.*, 1990b). If the AJ allele of *pcmdr1* is 'naturally' competent for chloroquine resistance, this might explain the lack of segregation of the AS(3CQ) allele with the resistance phenotype.

However, this does not exclude the possibility that *pcmdr1* may play some rôle in chloroquine resistance. If resistance in *P. chabaudi* is a continuous trait, it may be caused by the accumulation of many small changes in a number of genes. The expression of *pcmdr1* may be influenced by other genes that have yet to be identified, and which complicate the segregation pattern of genes seen in **Table 14**. This was also suggested by Newbold (1990), who noticed that in the *P. falciparum* HB3/Dd2 cross, both parental clones had the competent form of *pfmdr1* hypothesised by Foote *et al.* (1990b), but HB3 had one copy of the gene whereas Dd2 had 4 copies. The phenotypes of the recombinant clones, whether they possessed a single *pfmdr1* copy from HB3 or between two and four copies from Dd2, had identical IC_{50} values and chloroquine efflux rates. As Newbold states, this implies that the *pfmdr1* genotype and copy number cannot alone be responsible for these characters, and other genes are probably more important in their determination.

If *pfmdr1* is not directly associated with chloroquine resistance, the question remains as to its function. Suggested functions include transportation of chloroquine (Barnes *et al.*, 1992; van Es *et al.*, 1994a) or mefloquine (Cowman *et al.*, 1994), or regulation of vacuolar pH (van Es *et al.*, 1994b). Alternatively, Pgh1 may be involved in a stress-related response, as mentioned previously (Ouellette and Borst, 1991; Barnes *et al.*, 1992). There is insufficient experimental evidence so far to determine if any of these functions could be applied to *pcmdr1* too.

Although the evidence for *pcmdr1* or *pfmdr1* playing a rôle in drug resistance is contradictory, the possibility still exists that other P-glycoprotein genes could be involved in chloroquine resistance in *Plasmodium*. Low stringency washing of PFG blots hybridised with a fragment of *pcmdr1* identified at least two other loci on chromosomes 3 and 6, which may be homologous genes encoding P-glycoproteins (**Chapter 3**). In *Leishmania*, a P-glycoprotein gene family was identified by similar cross-hybridisation techniques (Légaré *et al.*, 1994). Cloning these *P. chabaudi* genes and examining them for increased levels of expression in drug resistant lines may identify possible mechanisms of resistance.

6.5 The inheritance data

During this work, a comparison was made between linkage analyses using data obtained from uncloned drug-treated progeny and data obtained from cloned progeny of the cross. The results showed the importance of analysing individual recombinant clones. For example, analysis of the uncloned, chloroquine-treated progeny for inheritance of the marker *MSP-1* appeared to show segregation of the AS(3CQ) form of the allele with the chloroquine resistance phenotype (**Table 12**). Analysis of the

13 progeny clones, however, detected two clones which were chloroquine-resistant and had inherited the AJ-type allele of *MSP-1*. This is evidence that the drug-selection procedure causes certain genotypes to be favoured over others, as proposed in **Results 5.2.2**, and that these two clones were selected against during drug treatment. Alternatively, clones with these genotypes may have been present in such low numbers that detection of their DNA by radioactive probe hybridisation was not possible.

Uncloned, chloroquine-treated progeny of the HB3/Dd2 *P. falciparum* cross were analysed for inheritance of *pfmdr1* in this way, and no linkage of the gene with chloroquine resistance was found (Wellems *et al.*, 1990). It is a matter of speculation whether the treated progeny were truly representative of all the genotypes produced during the cross. Selection of particular genotypes during multiplication in the chimpanzee host or during *in vitro* culturing, has been noted in genetic crossing work with other *P. falciparum* clones (Ranford-Cartwright *et al.*, 1993).

The pattern of RFLP markers inherited by the cloned progeny of the cross showed that extensive recombination and reassortment had occurred among the chromosomes during meiosis. RFLPs of most loci exhibited approximately even distribution in the progeny. However, two chromosomes in particular showed skewed inheritance of RFLPs among the progeny; chromosome 5 markers inherited by the progeny were predominantly AJ-type, and chromosome 11 markers inherited by the progeny were predominantly AS(3CQ)-type. The skewed inheritance of chromosome 5 markers is discussed later with regard to the selective advantage that an AJ background appears to have. The skewed inheritance of chromosome 11 markers is of more interest however, because of the possible association with a chloroquine resistance locus.

109 polymorphic sites were tested on chromosome 11 among the 13 progeny clones, and 78 of these were AS(3CQ)-type. Of further interest is that only two chromosome 11 cross-overs were found to have occurred in the progeny, whereas many more were found in the progeny for chromosomes which were examined with fewer markers: chromosome 13 was examined with seven markers, for example, and four cross-overs were detected. These observations may not be significant if it is accepted that all nine chromosome 11 markers, which were chosen at random, happened to be closely linked by chance; if this were the case, the detection of so few cross-overs would not be surprising, because only a small area of the chromosome was examined.

However, six of the chromosome 11 markers are known to be genes which are conserved between *P. falciparum* and the rodent malaria species (Janse *et al.*, 1994,

and this work). Synteny data shown in **Table 11** suggests that they were not sufficiently closely linked to each other as to have prevented chromosomal rearrangements in a primitive ancestor of malaria parasites. Taken together, the lack of cross-overs and skewed inheritance of so many AS(3CQ) alleles is strong evidence for a locus or loci of some importance on this chromosome.

6.6 Selective advantage of the AJ genotype

Certain genotypes appeared to be over-represented in the progeny of the AJ/AS(3CQ) cross. For example, six of the 34 progeny clones isolated were found to be AJ parental-type whereas none were AS(3CQ) parental-type, and many of the independent recombinant clones inherited a preponderance of AJ alleles for markers on chromosome 5. This suggests that selection favouring certain progeny genotypes has occurred during the making of this cross. Moreover, growth tests in which equal numbers of parasites were inoculated into mice revealed that clone AJ outgrows clone AS(3CQ).

The advantage AJ has over AS has been noted in other work. Rosario (1976a; 1976b) observed a disproportionately high number of AJ-type clones among the progeny of a cross between AJ and AS(3CQ). From 70 clones obtained from his cross, 32 were found to have the parental AJ forms of four markers, compared with only four having the parental AS(3CQ) forms of the same markers. He suggested that this could have been due to: (a) AJ-type parasites outgrowing AS-type in splenectomised rats, (b) a disadvantage of pyrimethamine-resistant forms in splenectomised rats, and (c) an error of sampling. An excess of AJ parental type clones in the progeny of two separate crosses between AJ and a highly chloroquine-resistant line, AS(30CQ), was also noted by Padua (1980). The results obtained from the work presented here are difficult to interpret because of the small numbers of clones isolated, but they are consistent with previous results.

The advantage of an AJ genetic background in recombinant progeny has also been noted. Padua tested 97 clones from one of her AJ/AS(30CQ) crosses for forms of two alloenzymes, LDH and 6PGD (Padua, 1980). 62 were found to have the parental AJ forms of the two markers, compared with only 27 having the parental AS(3CQ) forms of the same markers; the AJ parental form of both markers was expected to occur in only a quarter of the total number of clones, assuming random mating. Also, competition experiments between the drug sensitive AS(sens) and AJ lines, and AS(30CQ) and a recombinant progeny clone AJ(30CQ), resulted in the AJ line and AJ(30CQ) recombinant clone outgrowing the other two clones. Padua concluded that parasites having an AJ genetic background were at an advantage, and that the apparent

selective advantage of the recombinant clone could be explained in terms of 'recombinant vigour', equivalent to 'hybrid vigour' but pertaining to a haploid organism. Once again, it is difficult to draw comparisons because of the small number of recombinant clones isolated from the progeny of the present cross, but it is interesting that many of them had inherited AJ alleles of the markers specific to chromosome 5.

The difference between AJ and AS(3CQ) in rates of growth may be due to the action of a gene or genes determining, for example, the number of merozoites produced during schizogony, or the ability of the intracellular parasite to extract nutrients from the host cytoplasm. It is probably not due to the action of a gene or genes which reduce the length of the erythrocytic cycle, as periodicity remains unchanged in the two clones. It may be that the gene(s) are to be found on chromosome 5, because 43 of the 55 polymorphic sites examined on this chromosome were of AJ-type. Markers Ag3035 and OPL-16 may be particularly relevant because ten of the eleven recombinants inherited the AJ allele of both markers. Rosario reported that AS(3CQ) appeared to enter schizogony 2-3 hours earlier than AS(sens), and that this could account for its advantage in competition experiments, but it is unlikely that this is an explanation for the differences in growth seen between AJ and AS(3CQ) because of its occurrence so early on in the infections, when host erythrocytes are in plentiful supply.

It is also interesting to note that skewed RFLP distributions were found in the progeny of the HB3/Dd2 *P. falciparum* cross on four chromosomes (Walker-Jonah *et al.*, 1992). It was thought that some of these could be explained by biological phenomena rather than statistical variation. On *P. falciparum* chromosome 13, for example, the gene coding for a histidine-rich protein, HRP-III was inherited from the Dd2 parent in 14 out of 16 progeny. Genetic studies of the HB3/3D7 *P. falciparum* cross (Walliker *et al.*, 1987) showed similar selection against the HB3 allele of this gene. It was suggested that progeny possessing the HB3 allele proliferated at a slower rate relative to those without it (Wellems *et al.*, 1987).

The selection of advantageous genotypes is probably responsible for other cross progeny results. Nine of the ten recombinant clones isolated from the second mouse on which infected mosquitoes had fed, had identical inheritance patterns for 14 markers. These nine clones were probably derived from the same original recombinant clone and single meiotic event. Their genotype may have been at a selective advantage over other genotypes in the uncloned progeny, resulting in selection either within the mosquito salivary glands, or more likely during *in vivo* passage, where differences in growth between clones would have enabled some clones

to out-grow others. Recently, Viriyakosol *et al.* (1994) showed that approximately 70% of 51 genotypically distinct mixtures of *P. falciparum* altered in composition during *in vitro* cultivation. It is likely that similar alterations in the composition of parasite populations occur during passage of rodent malaria parasites *in vivo*.

To conclude, 28 of the 34 cross progeny clones were found to be recombinant, but only 13 of these were found to be genotypically distinct from each other, probably because of selection of particular genotypes over others. An alternative explanation may be that an inadequate number of independent meioses had occurred in each infected mosquito, resulting in the production of few independent recombinants. Evidence that this may have been the case comes from the number of oocysts found to be present on dissected midguts of mosquitoes infected with a mixture of clones AJ and AS(3CQ); of six mosquitoes dissected, only two were found to be infected with one and eight oocysts respectively (**Results 5.1**). The small number of mosquitoes dissected and few progeny clones isolated make it difficult to draw further conclusions from these data.

6.7 The chloroquine susceptibility tests

The drug test used during this project was based upon parasite recrudescence following drug treatment of 3 mg chloroquine/kg for 8 days; resistant parasites emerged on or before D_{13}, whereas sensitive parasites appeared after this. Rosario (1976b) used a drug dose of 3 mg chloroquine/kg for six days, and defined resistant parasites as those appearing on or before D_8. The two extra doses given to parasites in this work enabled an increase in the time lapsed between the emergence of resistant and sensitive parasites, producing more accurate phenotyping.

Variation in parasitaemia levels was apparent between repeated drug tests of the same clone. This has been noted in other work (Rosario, 1976b; Padua, 1981), and it prevented any estimation of the number of mutations responsible for the expression of chloroquine resistance in AS(3CQ) (Rosario, 1976b). The age, sex and strain of mouse used, and host diet, are variables known to affect parasite development. For example, genetic studies by Stevenson *et al.* (1982; 1988) have shown that differences exist between inbred strains of mice in the level of resistance to infection by *P. chabaudi* clone AS. The level of resistance is genetically determined by a major dominant gene, *Pchr*, which was found to be autosomal but to be influenced by the sex of the mouse, female mice exhibiting a superior resistance to parasite infection. However, Ott (1969) has suggested that the course of infection of *P. chabaudi* in mice can be highly reproducible under various conditions, one of which is the use of young mice, ideally approximately 20 g in weight. The variation in parasite growth due to

host diet has also been established (Gilks *et al.*, 1989). To minimise the effect of these host factors on the results, all drug tests carried out during this work used male CBA/Ca mice, 7-8 weeks old (approximately 17-23 g), maintained on a constant diet with drinking water supplemented with 0.05% PABA.

Excluding variation due to sex, age, diet and mouse strain type, the variation noted during this work is probably due to differences in the response of parasites and mice to chloroquine, and the immune status of individual mice. For example, the rate at which drug is concentrated in red blood cells is dependent upon the rate of drug absorption from the alimentary canal (Vessel *et al.*, 1971). Also, the trophozoite stage of the parasite is known to be most susceptible to chloroquine, while the mature schizont, merozoite and ring stages are only partially susceptible (Cambie *et al.* (1991) as cited by Cambie *et al.* (1994)). *P. chabaudi* infections are approximately 80% synchronous, and so chloroquine efficacy depends upon the timing of treatment.

In vitro drug tests have been used in previous studies in an attempt to standardise the test by eliminating host factors (Rosario, 1976a; Padua, 1980). The tests were based upon culture of parasites from trophozoite to schizont stage in the presence of different concentrations of chloroquine. The tests were not successful for several reasons, such as the requirement for mouse dissection to be carried out asceptically, and the impracticable necessity of calculating individual parasitaemias and blood cell counts (Rosario, 1976a). It was concluded that the most reliable criterion for determining resistance was either the presence or absence of healthy parasites after drug treatment, rather than actual levels of parasitaemias (Padua, 1980). This was the theory behind the method of determining drug susceptibility during this project.

The *in vivo* drug tests used in this work may not have been sensitive enough to distinguish between additional classes of drug susceptibility among the progeny clones. This was also noted by Padua (1980). Recently, a test for inhibition of [^{3}H]hypoxanthine uptake in *P. falciparum* (Desjardins *et al.*, 1979) has been modified for use with *P. chabaudi* (Sohal and Arnot, 1993), which obviates the need for large numbers of mice and time-consuming parasitaemia counts. This automated method may be sensitive enough to define further levels of resistance. However, the relationship between *in vitro* and *in vivo* drug tests is not clear, and future work would be served best by the use of both tests to determine parasite drug responses.

6.8 Construction of a genetic linkage map

During this project, the chromosome number and karyotype of the *P. chabaudi* genome was finally resolved, mainly due to advances in the PFGE technique. The genome was found to contain 14 chromosomes, a number which is consistent with all

other *Plasmodium* species studied (Weber, 1988). Three size polymorphisms between chromosomes of clones AJ and AS were found. Those involving chromosomes 5 and 6 have been described previously (Sharkey *et al.*, 1988), but this is the first report of size differences between clones of *P. chabaudi* involving chromosome 11.

Advances in the technology used to map genomes over the past decade (for reviews see Wicking and Williamson, 1991; Collins, 1992) have resulted in the development of genetic linkage maps for a variety of organisms. Over 4,000 markers have been mapped in the laboratory mouse *M. musculus* for example (Dietrich, 1994), and 5,000 markers now exist for the free-living nematode *Caenorhabditis elegans* (Waterston *et al.*, 1992).

The work presented here describes the development of the first genetic linkage map of *P. chabaudi* (**Figure 28**). 44 of approximately 100 probes identified as chromosome markers were found to be polymorphic between the *P. chabaudi* clones AJ and AS(3CQ). Most were RFLPs identified through probing Southern blots of genomic DNA with known genes from other species of *Plasmodium*, or anonymous probes collected from a *P. chabaudi* library. A few were polymorphic markers developed using the novel PCR technique, RAPD-PCR; the advantages of this procedure are discussed later.

6.9 Chromosome maps of other *Plasmodium* species

Chromosome maps can be distinguished from linkage maps in that the former show the chromosome location of markers, whereas the latter include the order of and often the distance between the markers. Besides the linkage maps of *P. falciparum* described above, chromosome maps of other species of *Plasmodium* have also been made. For example, Sharkey *et al.* (1988) mapped 11 markers to chromosome separations of *P. chabaudi* clones AS and CB, and Sheppard *et al.* (1989b) mapped 9 markers to separated chromosomes of *P. chabaudi*, *P. berghei* and *P. vinckei*..

During the construction of the chromosome map of *P. chabaudi* described here, large-scale chromosome maps of three other rodent malaria parasite species were made in collaboration with Drs. C. Janse and A. Waters of Leiden University, The Netherlands. The chromosomal location of fifty markers, mostly genes, was established in three clones of *P. berghei*, one clone of *P. vinckei vinckei*, one clone of *P. chabaudi adami* and one clone of *P. yoelii yoelii*, as well as clone CB of *P. chabaudi chabaudi* (Janse *et al.*, 1994). All species were found to contain 14 chromosomes, ranging in size from 0.5 to 3 Mb. Polymorphisms in the sizes of homologous chromosomes were found to occur both between the species, and between different clones of a single species, as has been described in previous work

(Janse *et al.*, 1989) (see **Introduction 1.4.2**).

The principal finding of the work was that the location of genes on polymorphic chromosomes of all of the four species appeared to be well conserved. Of the 50 genes studied, only the location of 3 were not conserved among the species. This was not expected, since homologous chromosomes of the species show differences in size (Sheppard, 1989), and because large scale chromosomal rearrangements have been reported to occur quite frequently (Cowman and Lew, 1989; Janse *et al.*, 1992; van Dijk *et al.*, 1994).

As described in the **Introduction**, such chromosome polymorphisms may be caused by changes in the number of repeat sequences in the subtelomeric regions of chromosomes (Ponzi *et al.*, 1990), or through unequal crossing-over between (Janse *et al.*, 1992), and amplification and translocation of genes (Cowman and Lew, 1989; van Dijk *et al.*, 1994). However, several of the described large scale rearrangements have been found in parasites maintained in an artificial environment, for example cultured *in vitro* (Wellems *et al.*, 1988) or passaged through laboratory hosts (Janse *et al.*, 1989), and it is uncertain whether these parasites would be able to survive in nature. Therefore, it was important to find out whether the genome plasticity regularly affected the chromosome location of genes in parasites from field isolates, resulting in shuffling of linkage groups. The principle conclusion which can be drawn from the chromosome maps of the four rodent malaria species described in Janse *et al.* (1994) is that there is no evidence that large-scale rearrangements frequently affect the gene composition and linkage groups of their chromosomes.

An explanation for the conserved location of genes on chromosomes among the four species of rodent malaria might be that internal chromosomal rearrangements occur less frequently in parasites which multiply under natural conditions than in parasites which are maintained under laboratory conditions. A more likely explanation might be that parasites with internal chromosomal rearrangements have a selective disadvantage in nature. It has been proposed that carriers of most types of chromosomal rearrangements suffer from sterility, and because of this, many types of chromosomal change cannot be fixed as a new species characteristic (Ohno, 1970). If this is the case, the shuffling of genes into novel linkage groups will be severely limited in natural *Plasmodium* populations.

6.10 Synteny between the genomes of *P. falciparum* and *P. chabaudi*

The conclusions drawn from a comparison of the locations of genes of the four rodent malaria species, as outlined above, show the value of comparative mapping between closely related species. Comparative mapping between distantly related

species may also be useful, for example in answering questions concerning genome evolution, and in predicting the location of genes in one species given their locations in another.

During this project, a comparison was made between the location and synteny of genes in *P. falciparum* and *P. chabaudi*. A further study gauged the level of sequence conservation between chromosome 7 of *P. falciparum* and chromosome 13 of *P. chabaudi*.

6.10.1 Synteny between genomes

25 out of 40 homologous genes localised to single chromosomes in *P. chabaudi* and *P. falciparum* appear to be conserved in their location within 10 synteny groups. Some of these associations may have occurred by chance. For example, genes coding for β tubulin and enolase are found on chromsome 10 in *P. falciparum* and their homologues are found on chromosome 12 in *P. chabaudi*. If the genes are far apart on each chromosome, it is possible that they are present on the same chromosome in the two species purely by chance. If, however, the genes are closely linked to each other, then it is likely that both chromosome regions are conserved and represent a relic of the primitive ancestor from which both species evolved.

Unfortunately, none of the genes in this study have been mapped to particular regions of chromosomes, which makes the assessment of conserved linkage difficult. The one exception is the *P. falciparum* genes EF1α and pfPK5 which are known to be on the same 4.5 kb Hind III fragment on chromosome 13, and to lie within 1 kb of each other (D. Williamson, personal communication). These two genes are also found on the same *P. chabaudi* chromosome number 11. It may be that the *P. chabaudi* homologues are as tightly linked to each other as they are in *P. falciparum*, in order for the linkage to have been conserved since the two species diverged from each other.

Some of the *P. falciparum* chromosomes have groups of genes on the same chromosome which show synteny conservation with more than one *P. chabaudi* chromosome. For example, *P. falciparum* chromosome 14 has two conserved syntenies: Group 1 contains *5s rRNA*, *Topoisomerase II*, *Calmodulin*, *PGI*, *Pfmap-1*; and Group 2 contains *Aldolase* and *G6PD*. These two groups are not found on the same *P. chabaudi* chromosome; Group 1 is found on chromosome 10 and Group 2 on chromosome 13. *P. chabaudi* also has split regions of synteny.

If the conserved syntenies shown here for a limited set of genes are representative of all the homologous genes shared between *P. falciparum* and *P. chabaudi*, then more than 60% of the total number of genes are likely to be within a conserved synteny. Moreover, this figure is only an estimate, as chromosomes 2, 7 and 12 of *P.*

falciparum and chromosomes 2 and 3 of *P. chabaudi* were not included in the analysis because of the lack of cloned genes from these chromosomes. It has been estimated that the *Plasmodium* genome contains 7,500 genes (Reddy, 1995), and only a small fraction of these have been examined in this work. Analysis of additional genes will be necessary to obtain a more precise indication of synteny between the two species.

Although this work has shown that there is significantly less conservation of syntenies between *P. chabaudi* and *P. falciparum* compared to the conservation observed between the rodent malaria species (Janse *et al.*, 1994), it presents exciting possibilities for genome analysis. Comparative mapping data are currently available for more than 25 species of mammals (Nadeau, 1989), for five species of *Leishmania* (Ravel *et al.*, 1995) and for some invertebrate species (e.g. Weller and Foster, 1993). The map of homology segments for mouse and man is the closest to saturation for any pair of species, and it provides an example of the applications that comparative mapping has. Of 2616 loci mapped to the mouse genome, 917 have homologues which have been mapped in the human genome, marking 101 segments of conserved linkage homology (Copeland *et al.*, 1993). From these data, it has been estimated that approximately 150 chromosomal rearrangements have occurred since the divergence of the lineage leading to humans and mice, roughly 1 per million years (Nadeau and Taylor, 1984). Using the data from comparative mapping of the rodent malaria species and *P. falciparum*, it should be possible to determine the location and number of rearrangements that have occurred during evolution of the species. If data from other *Plasmodium* species are included, an evolutionary map of the genus could be drawn.

Other applications of comparative mapping centre around the question of why syntenic groups are stable. As mentioned previously, it may be that chromosome rearrangements often lead to meiotic disturbances and are selected against (Ohno, 1970). Alternatively, perhaps not enough evolutionary time has passed to allow the break-up of a sufficient number of conserved linkages to cause a random pattern in respect of distantly related species. A third view is based upon the preservation of linkage groups due to function (Lundin, 1993). If a group is conserved over a long period of time, it may be as a consequence of some vital structure of the chromatin necessary for the satisfactory expression of the genes within it. In prokaryotes, genes specifying enzymes of the same metabolic pathway are often clustered and coordinated as a group (or operon) because they transcribe a single polycistronic mRNA. The best known example is the *lac*-operon of *E.coli* which contains genes used for the metabolism of lactose. Scrutiny of conserved syntenies may therefore provide information on genome organisation and gene regulation.

Perhaps the most significant application of comparative mapping, in terms of this project, is in predicting the location of loci in one species from their locations in another. The scope for identifying genes in *P. falciparum* after their initial identification in the rodent malaria species is of considerable importance, especially considering the functional information of candidate genes which can be obtained through genetic crossing work and transfection studies (C.Janse and A.Waters, personal communication), which are more easily carried out in the rodent malaria models.

Examples of genes suited to isolation across species by comparative mapping include those coding for antigens, which are difficult to clone because of the problems involved in cross-species DNA hybridisation. Genes coding for enzymes could also be located, for example the gene for LDH in *P. falciparum* has been cloned (Simmons *et al.*, 1985) and its chromosomal location could be used to locate the *P. chabaudi* homologue which has not been cloned. Of more relevance to this project are the isolation of genes implicated in mechanisms of drug resistance. A recent example of this was the discovery of an insecticide resistance gene on chromosome 5 in the housefly *Musca domestica* and chromosome 2R of *D. melanogaster*. Comparative mapping of other loci on these chromosomes suggests that when resistance to this drug arises in the sheep blowfly *Lucilia cupina*, it will be due to a gene on chromosome 6 (Weller and Foster, 1993). An attempt to locate the locus in *P. chabaudi* implicated in chloroquine resistance in *P. falciparum* by comparative mapping is discussed in the following section.

6.10.2 Search for synteny with *P. falciparum* chromosome 7

Wellems *et al.* located a marker, pS590.7, on *P. falciparum* chromosome 7, which segregated with the chloroquine resistance phenotype in 16 progeny from a cross between clones HB3 and Dd2 (Wellems *et al.*, 1991). During this work, an attempt was made to isolate the *P. chabaudi* homologue of pS590.7, and to identify regions of synteny between *P. falciparum* chromosome 7 and other *P. chabaudi* chromosomes.

The marker pcpS590.7 was isolated from *P. chabaudi* DNA by PCR. The evidence for it being the homologue of pfpS590.7 is ambiguous:-

(i) single amplified bands were produced from both *P. falciparum* and *P. chabaudi* DNA using the same primers

(ii) a high annealing temperature of 52.5^{o}C was used

(iii) the amplified bands had similar sizes, 456bp for pfpS590.7 and 400bp for pcpS590.7

(iv) both sequences were present as single copies in their respective genomes

(v) the *P. chabaudi* product cross-hybridised to *P. falciparum* chromosome 7. However, neither fragment contained an open reading frame, and sequence comparisons at the nucleotide level are difficult to interpret because of the high A + T content of pcpS590.7. Subsequently, the *P. falciparum* marker pS590.7 was found to hybridise to chromosome 12 of *P. chabaudi*, whereas the fragment amplified from *P. chabaudi* hybridised to chromosome 13.

These ambiguous results led to the search for regions of synteny between *P. falciparum* chromosome 7 and *P. chabaudi* chromosome 13. The results of these studies involving hybridisation of eight chromosome 7 *P. falciparum* probes were also too ambiguous to deduce whether conserved syntenies exist between these chromosomes. Weak hybridisation between the *P. falciparum* probes and *P. chabaudi* chromosomes may have been the result of lack of homology, or a result of the non-coding nature of the probes.

Recently, a gene belonging to the heat shock protein-90 family has been cloned from the chloroquine resistance locus on *P. falciparum* chromosome 7 (Su and Wellems, 1994). It is likely to be highly conserved between the *P. chabaudi* and *P. falciparum* genomes. Cross-hybridisation of the gene to *P. chabaudi* chromosome separations should enable a *P. chabaudi* homologue to be located; its location may resolve the relationship between chromosome 13 of *P. chabaudi* and 7 of *P. falciparum.*

6.11 RAPD-PCR technique: conclusions

The RAPD-PCR technique was used in an attempt to produce genome markers more quickly than had been possible using cloned genes. It represents an advance over the more traditional method of identifying RFLPs by probing Southern blots with markers because (i) it is faster and more effective at detecting large numbers of anonymous polymorphic markers; (ii) a great many randomly chosen primers can be used for the screening; (iii) it requires little parasite material and is technically easy to perform; and (iv) the anonymous markers obtained represent independent loci which are not biased towards particular sequences, and so are representative of the genome.

However, in its simplest form, the technique proved not to be reproducible. It was found necessary to purify the variable bands obtained by initial RAPD-PCR amplification, and to hybridise them to genomic DNA blots to obtain stable single locus RFLP markers. This increased the time and complexity of the procedure. Nevertheless, the initial RAPD-PCR screening was advantageous because it identified polymorphic loci for further analysis, unlike anonymous markers which are uncharacterised before screening. This means that RAPD-PCR markers are more

likely to produce an RFLP when screened than unknown markers. Evidence for this comes from a comparison between the number of RFLPs obtained using the RAPD-PCR technique and the number obtained from the anonymous library screened during this project. 43% (6 out of 14) recombinant plasmids containing unique sequence DNA detected RFLPs, whereas with the RAPD-PCR technique, 100% (5 out of 5) of the polymorphic bands recognising a single locus detected RFLPs. Some caution is necessary when interpreting these results because of the small sample size analysed.

To conclude, the RAPD-PCR technique promises to be of significant value in developing markers for use in mapping the *Plasmodium* genome.

6.12 Relevance of *P. chabaudi* work to *P. falciparum*

Using *P. chabaudi* as a model for *P. falciparum* is relevant for several reasons:

(1) Of the four rodent malaria models available, *P. chabaudi* shares the most biological traits with *P. falciparum*. For example, both have a preference for mature erythrocytes, both show synchronous schizogony, and both have an innate sensitivity to antimalarial drugs.

(2) Pyrimethamine-resistant parasites from both species have been shown to contain equivalent point mutations within the *DHFR* gene (Cowman *et al.*, 1988; Peterson *et al.*, 1988; Cowman and Lew, 1990). Genetic studies have shown the *P. falciparum* allele to be inherited by all the resistant progeny of a *P. falciparum* cross (Peterson *et al.*, 1988). Analysis of the uncloned, pyrimethamine-treated progeny of the AS(3CQ)/AJ cross for inheritance of the *P. chabaudi DHFR* gene presented during this work (**Table 13**), has produced the first genetic evidence for the rôle of *DHFR* in resistance to this drug in *P. chabaudi*. Thus it appears that the molecular genetic mechanism of at least one type of drug resistance is the same between the two malaria parasite species. Further studies on cloned progeny are required to confirm this result, for the reasons mentioned in **Section 6.5**.

(3) A genetic linkage map has been made for *P. falciparum* from the data of 85 RFLP markers inherited among 16 progeny of the Dd2/HB3 cross (Triglia *et al.*, 1992; Walker-Jonah *et al.*, 1992) and from other chromosome map studies (Kemp *et al.*, 1987; Walliker *et al.*, 1987). The limiting factor in its future development is likely to be the number of independent recombinant progeny clones available. Cloning and examination of more clones is likely to be expensive and time consuming. In an attempt to circumvent the difficulty of performing classical genetic analyses with *P. falciparum*, and in the absence of a stable *P. falciparum* transfection system, physical mapping and sequencing of the genome is being undertaken by groups within the Wellcome Trust Malaria Genome Collaboration (for the objectives and approaches

being undertaken, see Craig and Langsley, 1993).

However, genetic crosses between clones of *P. chabaudi* are easier to perform than between clones of *P. falciparum*, because the process involves using laboratory rodents which are cheap, easy to maintain and ethically more acceptable than primates. Moreover, studies presented here show that a high proportion of genes homologous between *P. chabaudi* and *P. falciparum* are likely to be within conserved synteny groups. Taken together, exciting possibilities exist for the isolation of genes determining important phenotypic traits by linkage analysis and positional cloning using *P. chabaudi* crosses. Once located to a particular chromosome and cloned, examination of conserved synteny groups between the *P. falciparum* and *P. chabaudi* genomes could identify the chromosome location of the *P. falciparum* homologue.

6.13 Finding the chloroquine resistance gene(s); money well spent?

Determining the molecular genetic mechanism of chloroquine resistance has almost become the 'Holy Grail' of malaria research. Novel molecular techniques and genetic mapping methods have greatly aided the task. One question often left unasked, however, is how relevant an understanding of the genes(s) and molecular mechanism involved in resistance would be in combating the disease.

Firstly, determining the number of mutations involved in chloroquine resistance may provide practical information for its continued use in the field. If chloroquine resistance is a multigenic trait, it is possible that resistance would not have emerged if use of the drug had been restricted and higher doses used. This principle could be adhered to in areas where chloroquine resistance is not yet a problem. It is not known whether chloroquine resistant *P. falciparum* persists in the absence of drug pressure; reports that reversion occurs (e.g. Thaithong *et al.*, 1988) are interesting in the light of a possible inverse relationship between chloroquine resistance and mefloquine resistance.

Determining the number of mutations would also be valuable in the construction of models which predict the rate of spread of resistance. Curtis and Otoo (1986) have proposed a model which determines the rate of spread of resistance to two unrelated drugs, caused by mutations in two unlinked genes. They concluded that when only a small fraction of the population is taking drugs, and when both resistance genes are initially very rare, random mating and free recombination between the loci will break up linkage disequilibrium, and so delay the build-up of resistance to both drugs. Thus, administering a mixture of drugs would be preferable to administering the drugs in sequence.

Secondly, if the molecular mechanism of chloroquine resistance is known, the

possibility exists to develop new antimalarials which would not induce the same resistance mechanism. This requires detailed knowledge of the structure of proteins involved in the mechanism, obtainable through experimental techniques such as X-ray crystallography and nuclear magnetic resonance. In the past, new drugs were developed from an initial 'blanket' screening of many compounds, and their mode of action was often not determined. Now there is a move towards more rational chemotherapy which aims to design drugs tailored to known biochemical pathways or parasite molecules which differ between parasite and host (Hyde, 1990b). Determining the molecular basis of chloroquine resistance will enable an overall understanding of parasite mechanisms of resistance.

Finally, if the number of molecular mutations required to produce chloroquine resistance is known, it may be possible to monitor the spread of drug resistance in natural populations more effectively. The current method of monitoring resistance using *in vitro* drug tests is inefficient and may not correlate with *in vivo* results. Mutation-specific PCR is now available for observing pyrimethamine resistance in samples of blood taken directly from infected patients (Zolg *et al.*, 1990; Gyang *et al.*, 1992). Determining DNA mutations which cause chloroquine resistance, and their detection by simple molecular methods, would represent a significant step towards monitoring this resistance directly.

Appendix 1. The composition of buffers, solutions and media

All solutions were sterilised either by autoclaving or by filtration, unless otherwise indicated.

Citrate saline
0.9% (w/v) NaCl
1.5% (w/v) Na citrate

Complete medium
incomplete medium with the following:
0.2% (w/v) $NaHCO_3$ (42 ml/l 5% $NaHCO_3$)
10% (v/v) heat inactivated human serum
Stored at 4°C. Used within 1 week.

Deep-freeze solution
28% (v/v) glycerol
3.0% (v/v) sorbitol
0.65% (v/v) NaCl
Sterilised by filtration.

Electra-HR buffer
18g Tris Barbital-Sodium Barbital, pH 8.6-9.0

Gel-loading buffer (6 x)
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
30% (w/v) glycerol in water
Not sterilised. Stored at 4°C

Incomplete medium
RPMI 1640
25 mM HEPES buffer (5.94 g/l)
50 µg/ml gentamycin sulphate
Sterilised by filtration. Stored at 4°C for up to 4 weeks.

Luria-bertani (LB) medium
per litre: 10 g bacto-tryptone
5 g bacto-yeast extract
10 g NaCl
pH adjusted to 7.5 with NaOH

Lysis solution
0.1 mM EDTA pH 8.0
0.5 % (w/v) sarcosyl

Mammalian Ringer solution
27 mM KCl
27 mM $CaCl_2$
0.15 M NaCl

PBS (phosphate buffered saline)
137 mM NaCl
2.7 mM KCl
4.3 mM $Na_2HPO_4 \cdot 7H_20$
1.4 mM KH_2PO_4

PCR buffer
50 mM KCl
10 mM Tris-HCl pH 8.8
2.5 mM $MgCl_2$

PCR dNTP solution (100 x)
75 µM dGTP
75 µM dCTP
75 µM dTTP
75 µM dATP

Phosphate buffer pH 7.5
for 1 litre:
4.53 g $Na_2HPO_4 \cdot 7H_20$
10.61 g $NaH_2PO_4 \cdot 7H_20$

PFG lysis solution

0.5 M EDTA

0.01 M Tris pH 9.5

1% (w/v) sarcosyl

Pre-hybridisation solution

for 1 litre:

100 g dextran sulphate (sodium salt, MW ~500,000, Sigma)

100 ml 10% SDS

58 g NaCl

846 ml H_2O

Mixed at 65°C for 45 minutes, aliquoted and frozen at -20°C

RAPD-PCR Clontech Buffer

10 mM Tris-Cl pH 8.8.

50 mM KCl

1.5 mM $MgCl_2$

0.1% (v/v) Triton X-100

Serum Ringer

50% (v/v) heat inactivated calf serum

50% (v/v) mammalian Ringer solution

20 units heparin/ml mouse blood

Anachem Sequencing Gel Solution

7 M urea

6% (w/v) acrylamide

0.16% (w/v) bis-acrylamide

in 1 x TBE

TEMED and 25% (w/v) ammonium persulphate added to final concentration of 0.6µl/ml

SSC (saline-sodium citrate)

3 M NaCl

0.3 M sodium citrate

Supra-heme buffer

0.025 M TBE, pH 8.2-8.6

TAE buffer (Tris-acetate/EDTA)

0.04 M Tris-acetate

0.002 M EDTA

TBE buffer (Tris/borate/EDTA)

0.09 M Tris base

0.09 M boric acid

0.002 M EDTA

TE buffer (Tris/EDTA)

10 mM Tris-HCl

1 mM EDTA

Used at the required pH of 7.4 to 8.0

Thawing solutions

i. 12% (w/v) NaCl

ii. 1.6% (w/v) NaCl

iii. 0.2% (w/v) dextrose, 0.9% (w/v) NaCl

Appendix 2. Operon primers used for RAPD-PCR analysis

Kit E 5' 3'		
OPE-01 CCCAAGGTCC	OPE-08 TCACCACGGT	OPE-15 ACGCACAACC
OPE-02 GGTGCGGGAA	OPE-09 CTTCACCCGA	OPE-16 GGTGACTGTG
OPE-03 CCAGATGCAC	OPE-10 CACCAGGTGA	OPE-17 CTACTGCCGT
OPE-04 GTGACATGCC	OPE-11 GAGTCTCAGG	OPE-18 GGACTGCAGA
OPE-05 TCAGGGAGGT	OPE-12 TTATCGCCCC	OPE-19 ACGGCGTATG
OPE-06 AAGACCCCTC	OPE-13 CCCGATTCGG	OPE-20 AACGGTGACC
OPE-07 AGATGCAGCC	OPE-14 TGCGGCTGAG	
Kit L 5' 3'		
OPL-1 GGCATGACCT	OPL-8 AGCAGGTGGA	OPL-15 AAGAGAGGGG
OPL-2 TGGGCGTCAA	OPL-9 TGCGAGAGTC	OPL-16 AGGTTGCAGG
OPL-3 CCAGCAGCTT	OPL-10 TGGGAGATGG	OPL-17 AGCCTGAGCC
OPL-4 GACTGCACAC	OPL-11 ACGATGAGCC	OPL-18 ACCACCCACC
OPL-5 ACGCAGGCAC	OPL-12 GGGCGGTACT	OPL-19 GAGTGGTGAC
OPL-6 GAGGGAAGAG	OPL-13 ACCGCCTGCT	OPL-20 TGGTGGACCA
OPL-7 AGGCGGGAAC	OPL-14 GTGACAGGCT	
Kit O 5' 3'		
OP0-1 GGCACGTAAG	OP0-8 CCTCCAGTGT	OP0-15 TGGCGTCCTT
OP0-2 ACGTAGCGTC	OP0-9 TCCCACGCAA	OP0-16 TCGGCGGTTC
OP0-3 CTGTTGCTAC	OP0-10 TCAGAGCGCC	OP0-17 GGCTTATGCC
OP0-4 AAGTCCGCTC	OP0-11 GACAGGAGGT	OP0-18 CTCGCTATCC
OP0-5 CCCAGTCACT	OP0-12 CAGTGCTGTG	OP0-19 GGTGCACGTT
OP0-6 CCACGGGAAG	OP0-13 GTCAGAGTCC	OP0-20 ACACACGCTG
OP0-7 CAGCACTGAC	OP0-14 AGCATGGCTC	
Kit R 5' 3'		
OPR-1 TGCGGGTCCT	OPR-8 CCCGTTGCCT	OPR-15 GGACAACGAG
OPR-2 CACAGCTGCC	OPR-9 TGAGCACGAG	OPR-16 CTCTGCGCGT
OPR-3 ACACAGAGGG	OPR-10 CCATTCCCCA	OPR-17 CCGTACGTAG
OPR-4 CCCGTAGCAC	OPR-11 GTAGCCGTCT	OPR-18 GGCTTTGCCA
OPR-5 GACCTAGTGG	OPR-12 ACAGGTGCGT	OPR-19 CCTCCTCACTC
OPR-6 GTCTACGGCA	OPR-13 GGACGACAAG	OPR-20 ACGGCAAGGA
OPR-7 ACTGGCCTGA	OPR-14 CAGGATTCCC	

Appendix 3. Conference abstracts.

British Society for Parasitology Spring Meeting
University of Edinburgh, Scotland 10th-12th April 1995.
Oral presentation: The genetics of mefloquine resistance in the rodent malaria *Plasmodium chabaudi.*
Jane Carlton, Laura Bisoni and David Walliker.

'Variation and Immune Responses to Infections'
Two day symposium on current research on HIV, Malaria and Nematode infections, University of Edinburgh, Scotland 5th-6th January 1995.
Oral presentation: Drug resistance in malaria.

British Society for Parasitology Malaria Meeting
University of Liverpool, England 18th-21st September 1994.
Oral presentation: The genetics of chloroquine resistance in the rodent malaria *Plasmodium chabaudi.*
Jane Carlton and David Walliker.

Scottish Universities Molecular Parasitology Group Summer Meeting
Kirkmichael, Perthshire 6th-8th May 1994.
Winner of the best oral presentation: A locus for chloroquine resistance in *Plasmodium.*
Jane Carlton and David Walliker.

Australian Society for Parasitology Meeting
Heron Island, Queensland 28th-30th September 1993.
Oral presentation: Unravelling the genetics of chloroquine resistance in malaria parasites.
Jane Carlton and David Walliker.

Royal Society of Tropical Medicine and Hygiene Annual Meeting
Royal College of Physicians, Edinburgh, Scotland 5th-7th July 1993.
Poster presentation: A rapid technique for the detection of DNA polymorphisms in *Plasmodium.*
Jane Carlton, Janet Howard, Jim Jensen and David Walliker.

Appendix 4. Publications arising from this study.

Copies of these manuscripts are at the end of the **References**.

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RESEARCH BRIEF

A Rapid Technique for the Detection of DNA Polymorphisms in *Plasmodium*

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We describe here the use of the RAPD–PCR technique (random amplified polymorphic DNA–polymerase chain reaction) to detect DNA polymorphisms in malaria parasites. This has proved to be a powerful and rapid method for detecting polymorphic genetic markers in other organisms. It requires little parasite material and is technically easy to perform. A large number of anonymous markers can be readily obtained, representing independent loci which are not biased towards particular sequences, and so are representative of the genome. Such markers can be used in population and phylogenetic studies, in the identification of parasite strains and species and in the comparison of field isolates. The markers may also be used to detect genomic polymorphisms for use in linkage analysis of simple and complex phenotypic traits, for example, drug resistance and virulence.

RAPD–PCR involves amplifying fragments of genomic DNA with short, single primers of arbitrary sequence (Welsh and McClelland 1990; Williams *et al.* 1990). No prior sequence information is required, unlike conventional PCR of loci of known sequences (Saiki *et al.* 1988). This feature allows the analysis of genomes for which little sequence information exists, for example, isolates taken from the field. The reaction takes place under conditions of low stringency that encourage the simultaneous amplification of DNA at a number of loci. The amplified products are separated by agarose gel electrophoresis and visualised by ethidium bromide staining. The majority of the products are expected to be identical in different individuals of a single species. A small proportion are likely to be polymorphic, variant forms shown by the presence or absence of amplified bands in different parasite strains, or by variations in their size or intensity due to differences in primer binding sites caused by mutation, deletion, or insertion of DNA sequences.

We have used RAPD–PCR to examine polymorphisms in the genomes of two genetically distinct cloned lines of the rodent malaria species *Plasmodium chabaudi*. The clones, denoted AJ and AS, were derived originally from thicket rats of the Central African Republic and maintained by passage in laboratory mice (Carter and Walliker 1975). Parasites were extracted from host cells and DNA was prepared following the method of Snounou *et al.* (1988). RAPD–PCR reactions were performed on this DNA using each of 80 decamer primers (Operon Technologies, Kits E, L, O, and R, each kit containing 20 decamers with a G + C content of at least 60%). Each reaction was carried out in 15 μl containing 0.4 μ*M* of one primer in 1× reaction buffer (10 m*M* Tris–Cl, pH 8.8, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.1% Triton X-100, Clontech), 200 μ*M* each dATP, dCTP, dTTP, dGTP, 2.5 m*M* $MgCl_2$, 0.6 units *Taq* DNA polymerase (Clontech), and 200 ng of *P. chabaudi* DNA. Reactions were overlaid with 100 μl light mineral oil (Sigma). Negative controls for each primer contained all of the above components except for 2 μl of TE in place of *P. chabaudi* DNA. Cycling conditions were as follows: 92°C for 3 min initially, then for 1 min at the start of each cycle; 36°C for 1 min 45 sec; 72°C for 2 min and for 7 min at the end of all 35 cycles. Amplification products were electrophoresed on 1.5% agarose gels and visualised by ethidium bromide staining.

In an initial screening, 51 of the 80 primers tested produced one or more polymorphic bands which were different in clones AS and AJ. As an example, Fig. 1A shows the amplification products of primer OPL-16. Several fragments were amplified, ranging in size from

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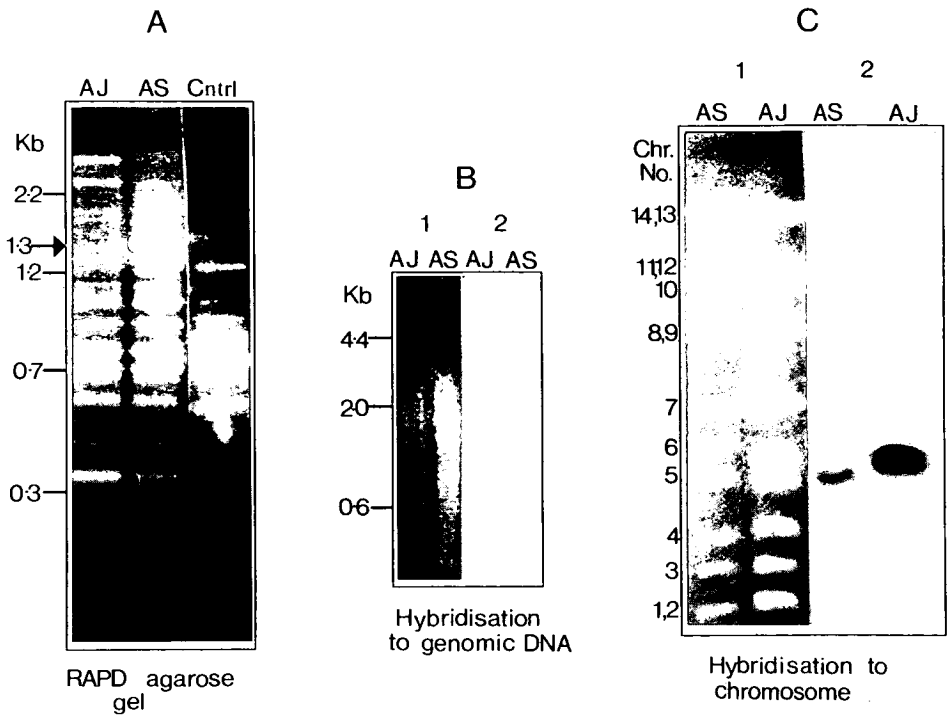

FIG. 1. DNA polymorphisms in *P. chabaudi* revealed by RAPD primer OPL-16. (A) Products of initial amplification from clones AS and AJ; arrow indicates 1.3-kb band in AS used in subsequent work. (B) Lane 1, *Alu*I digests of genomic DNA of clones AS and AJ. Lane 2, Autoradiograph of Southern blot probed with purified 1.3-kb band. (C) Lane 1, PFG separation of *P. chabaudi* AS and AJ chromosomes; electrophoresis conditions were 140 V, 120-sec pulse time for 24 hr; 130 V, 300-sec pulse time for 24 hr; 140 V, 180-sec pulse time for 24 hr, using a contour-clamped homogeneous electric field apparatus (CHEF DR II, Bio-Rad) and a 1% chromosomal grade agarose gel (Bio-Rad). Lane 2, Southern blot probed with purified 1.3-kb band showing hybridisation to chromosome 5; note that chromosome 5 of clone AJ is larger than in AS, while chromosome 6 is smaller.

0.3 to 2.4 kb. Most of the bands were present in both AJ and AS. However, some were present in one clone but absent from the other. In the example shown, an amplified band of approximately 1.3 kb was present in AS but absent from AJ. Also, a difference in the intensity of a band of approximately 0.35 kb existed between the two clones, the AJ 0.35-kb band being more intense than the AS band. Using other RAPD primers, differences in the sizes of certain bands in the two clones could be seen (data not shown), which represents a third type of polymorphism due to insertion or deletion of sequences between the primer binding sites at a single locus; alternatively, such bands could be presence and absence types of polymorphism at two loci.

The OPL-16 negative control (Fig. 1A) contains amplified products in the range 0.6 to 1.2 kb. We found that the majority of RAPD primers produced bands in their negative controls and that the pattern and sizes of bands in these control lanes varied from one reaction to another. The possibility of contaminating parasite DNA was eliminated by radiolabelling all of the negative control products and attempting to hybridise them to a Southern blot of restricted parasite DNA. No hybridisation to the blots was seen (data not shown). Recently it has been shown that contamination of *Taq* polymerase with *T. aquaticus* DNA is a common occurrence (Bottger 1990). We believe that the most likely explanation for the presence of these bands is amplification of small amounts of such contaminating DNA. In the presence of an excess of *P. chabaudi* DNA, primers are able to anneal to this DNA at more sites and so compete out the contaminating material.

Some polymorphic bands could not be amplified reproducibly in repeated experiments. This has been noted in studies of other organisms (Ellsworth *et al.* 1993; Kernodle *et al.* 1993; Meunier and Grimont 1993; Riedy *et al.* 1992; Schierwater and Ender 1993). Some laboratories have optimised the PCR reagents and conditions for the parasite under study (Dias Neto *et al.*

1993; Tighe *et al.* 1993; Waitumbi and Murphy 1993). We have circumvented this problem by screening polymorphic bands for RFLPs in restriction enzyme digests of genomic DNA. Polymorphic bands obtained with 7 of the 51 primers successfully used in this work (oligonucleotides OPL-04, OPL-12, OPL-16, OPR-02, OPR-14, OPL-08, and OPL-13; Table I) were excised from agarose gels and the DNA was purified (Vaux 1992). The DNA was radiolabelled by random priming (random primed DNA labelling kit, Boehringer Mannheim) and used to probe Southern blots (Hybond N^+, Amersham International) of *Alu*I, *Dra*I, *Hind*II, *Hin*fI, *Sau*3A, *Rsa*I, and *Eco*RI digested DNA of each parasite clone. The blots were washed at 65°C in 0.5× SSC, 0.1% SDS for 2 × 30 min and exposed to Kodak XAR-5 film. Figure 1 shows the OPL-16 1.3-kb polymorphic band hybridised to blots of *Alu*I-digested DNA and to *P. chabaudi* chromosomes in a pulsed-field gel. An RFLP of this marker is clearly seen in digests of AS and AJ genomic DNA (Fig. 1B), and it appears to exist as a single copy on chromosome 5 (Fig. 1C).

Polymorphic bands obtained from five of the seven primers produced clear and reproducible RFLPs which appear to exist as single copies in the genome of each clone (Table I). The remaining two, OPL-08 and OPL-13, produced amplified bands which were present on more than one chromosome and may contain repetitive sequences or be members of a multigene family. Such repetitive markers have potential as fingerprinting probes, which can be used to distinguish different lines or clones. Products of all seven RAPD–PCR primers were reamplified using the same primers and reagents as described above, but with 2 μl of the purified DNA of the excised polymorphic band in place of *P. chabaudi* DNA. Amplification conditions were 92°C for 30 sec; 50°C for 30 sec; 72°C for 1 min 30 sec for 25 cycles. The reamplified products were cloned into the vector pCR II using the TA cloning kit (Invitrogen) and stored as glycerol stocks for future use as probes.

Although we used DNA prepared by standard methods, the technique could utilise material prepared by other means. For example, several methods have been described for preparing DNA from field isolates collected on filter paper (Kain *et al.* 1992; reviewed in McCabe 1991) and from finger-prick samples (Foley *et al.* 1992). Preparing DNA samples using these alternative methods would be faster and eliminate the need for noxious chemical reagents such as phenol.

In summary, the RAPD-PCR technique has proved a fast and effective method for detecting large numbers of anonymous polymorphic markers in this parasite. A great many randomly chosen primers can be used for the screening of different parasite lines, and results are obtained in a few hours. This has considerable advantages over the traditional method of probing Southern blots with anonymous markers because the initial screening quickly identifies polymorphic loci for further characterisation. The method outlined here of purifying variable bands and hybridising them to genomic DNA blots allows stable single locus RFLP markers to be obtained readily and thus overcomes the problem of the reproducibility of the basic RAPD–PCR technique. Although we exploited only 7 of the 51 primers in this way here, the method could be extended to any of the RAPD–PCR products obtained with these types of primer. We are currently using the RAPD–PCR markers obtained through this work to map drug resistance genes to chromosomes of *P. chabaudi*. Such linkage analysis has been hindered in the past by the absence of suitable DNA markers. The RAPD–PCR technique has the potential to provide many more markers for the *Plasmodium* genome.

TABLE I

RAPD Primers Which Produced Polymorphic Bands, Subsequently Used to Detect RFLPs in *P. chabaudi*

Primer No.	Sequence	RFLP	Chromosome No.
OPL-04	5′-GACTGCACAC-3′	*Eco*RI	11
OPL-12	5′-GGGCGGTACT-3′	*Hinc*II	13
OPL-16	5′-AGGTTGCAGG-3′	*Alu*I, *Rsa*I	5
OPR-02	5′-CACAGCTGCC-3′	*Alu*I, *Hinc*II, *Hin*fI, *Rsa*I	14
OPR-14	5′-CAGGATTCCC-3′	*Hinc*II	12
OPL-08	5′-AGCAGGTGGA-3′	*Alu*I, *Dra*I, *Hinc*II, *Hin*fI, *Sau*3A, *Rsa*I, *Eco*RI	1/2, 7, 8/9, 11, 13/14
OPL-13	5′-ACCGCCTGCT-3′	*Alu*I, *Dra*I, *Hinc*II, *Hin*fI, *Sau*3A, *Rsa*I, *Eco*RI	1/2, 3, 4, 5 (AJ only), 14

Note. The chromosomal location of each RAPD–PCR product is shown. OPL-08 and OPL-13 recognised multiple sites; their precise chromosomal location was unclear in some instances, due to incomplete separation of the chromosomes during PFG electrophoresis.

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Conserved location of genes on polymorphic chromosomes of four species of malaria parasites

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Conserved location of genes on polymorphic chromosomes of four species of malaria parasites

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Abstract

The number of chromosomes and the chromosomal location and linkage of more than 50 probes, mainly of genes, have been established in four species of *Plasmodium* which infect African murine rodents. We expected that the location and linkage of genes would not be conserved between these species of malaria parasites since extensive inter- and intraspecific size differences of the chromosomes existed and large scale internal rearrangements and chromosome translocations in parasites from laboratory lines had been reported. Our study showed that all four species contained 14 chromosomes, ranging in size between 0.5 and 3.5 Mb, which showed extensive size polymorphisms. The location and linkage of the genes on the polymorphic chromosomes, however, was conserved and nearly identical between these species. These results indicate that size polymorphisms of the chromosomes are more likely due to variation in non-coding (subtelomeric, repeat) sequences and show that a high plasticity of internal regions of chromosomes that may exist does not frequently affect chromosomal location and linkage of genes.

Keywords: Malaria parasite; Chromosome; Polymorphism; Gene location

1. Introduction

The genome of the unicellular eukaryotic parasites which are members of the genus *Plasmodium*, comprises 14 chromosomes, ranging in size between 0.5 and 3.5 megabases (Mb) (for review see [1]). These organisms exhibit a remarkable plasticity of their genomes as shown by the frequent occurrence of intra-specific size differences between homologous chromosomes. For example, size differences of up to 0.5 Mb have been found between homologous chromosomes of different cloned lines of *Plasmodium falciparum* [2–4]. Extensive size polymorphism of chromosomes has also been observed in other *Plasmodium* species [5,6]. Size polymorphisms in these organisms arise as a result of various processes, such as unequal crossing-over between homologous chromosomes during meiosis [4,7], deletion and insertion of repeat-sequences [8,9], gene amplification [10] and chromosome breakage, followed by 'healing' by the process of telomere addition [11]. Most of these rearrangements affect only

Abbreviations: FIGE, field inversion gel electrophoresis; CHEF, contour clamped homogenous electric field electrophoresis

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the subtelomeric regions of chromosomes, while the internal regions appear to be less prone to large scale rearrangements [9,12]. However, chromosome translocations resulting in exchange of genes between non-homologous chromosomes have also been reported [13]. Moreover, evidence has been presented for the occurrence of mitotic recombination between non-homologous chromosomes resulting in the exchange of DNA-sequences [14]. Analysis of the patterns of sequence diversity of ribosomal genes also suggest that genetic exchange may occur between genes which are located on non-homologous chromosomes (A.P. Waters and C.J. Janse, unpublished). Large scale rearrangements can even result in duplication of chromosomes, by which parasites arise with 15 instead of 14 chromosomes ([15]; unpublished observations).

Large-scale genome rearrangements in *Plasmodium* have mainly been found in parasites from laboratory cultures or in parasites under strong selective pressure [1]. It is not known whether these kinds of rearrangements play a significant role in changes in location and linkage of genes on chromosomes of parasites in natural populations. In this study we have compared the location and linkage of a large number of genes on the polymorphic chromosomes of four distinct species belonging to the genus *Plasmodium*. These species infect African murine rodents and are closely related [16]. We show that the location and linkage of genes on homologous chromosomes are conserved between these species despite extensive intra- and interspecific size polymorphisms of their chromosomes. This result indicates that large scale rearrangements involving inter-chromosomal translocations of genes do not significantly contribute to size polymorphisms of chromosomes in *Plasmodium* species which infect rodents. Apparently, parasites with large internal rearrangements have a selective disadvantage in nature.

2. Materials and methods

2.1. Parasites

Four distinct species of malaria parasites of African murine rodents have been recognized: *Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium vinckei* and *Plasmodium yoelii*. The clones and lines of several of the (sub)species which were used in this study are listed in Table 1.

2.2. Separation of chromosomes

Field inversion gel electrophoresis (FIGE) and contour clamped homogenous electric field (CHEF) conditions were used to separate the chromosomes. Preparation of parasites and of agarose blocks containing the chromosomes was performed as described [9]. FIGE conditions to separate the chromosomes of the rodent parasites were as described previously [9] and specified in the legends to the figures. CHEF separations were performed using a BioRad CHEF-DR II apparatus. Conditions are specified in the legends to the figures.

Table 1
Different clones and lines of four species of *Plasmodium* which infect African murine rodents

Species	Line	Origin	Clone	Reference/source
P. berghei	ANKA	Katanga	8417	[5]
P. berghei	ANKA	Katanga	PE	[30]
P. berghei	K173	Katanga	1	[13]
P. berghei	NYU2	Kasapa	1 [a]	[31], T.F. McCutchan, Bethesda, MD
P. vinckei vinckei	V-67	Katanga	1 [a]	[32], M. Wery, Antwerp
P. yoelii yoelii	17X	Central African Rep.	1 [a]	[16], M. Wery, Antwerp
P. chabaudi chabaudi	CB	Central African Rep.	1 [a]	[33], G. Snounou, London
P. chabaudi chabaudi	AS	Central African Rep.	1 [a]	[33], G. Snounou, London
P. chabaudi chabaudi	AJ	Central African Rep.		[33], D. Walliker
P. chabaudi adami		Brazaville	1 [a]	[20], G. Snounou, London

[a] Clones derived from the lines in our laboratory.

Table 2
Molecular probes which have been mapped to the chromosomes of the rodent malaria parasites

Probe	Marker [a]	Reference/source
Actin-I	14,1	[34]
Anonymous: HPI12	14,2	[9]
VAP A (Vaculoar ATPase)	14,3	[35]
Aldolase-I,II	13,1,2	[36]
SSP-II (Sporozoite Surface Protein 2)	13,3	[37]
Cysteine Proteinase	13,4	[38]
G6PD	13,5	D. Kaslow, Bethesda; O'Brien and Luzzatto (submitted)
RNA pol III	13.6	[39]
Small subunit (SSU) rRNA	12,1	[17]
chab 451	12,2	[40]
(HSP70)	(12,3)	[41]
(GGMP)	(12,4)	[18]
Pcmdr1 (multidrug resistance)	12,5	J.M.R.C., Edinburgh
β-tubulin	12,6	[42]
PCNA (proliferating cell nuclear antigen	11,1	[43]
CDC2	11,2	R. Vinkenoog, Leiden
Ef1α (Elongation factor 1α)	11,3	D. Williamson, London
Histone 2a	11.4	[44]
VAP B (Vacuolar ATPase subunit B)	10,1	Karcz, Herrman, Trottein and Cowman (submitted)
Topoisomerase II	10,2	B. Kilbey, Edinburgh
Calmodulin	10,3	[45]
5S-RNA	10,4	[46]
AMA-I (Apical Membrane Antigen-1)	9,1	[47]
Hexokinase	9–11,2	[48]
RAN-I (Ras related nuclear antigen)	9,2	A. Sultan, Edinburgh
PGK (3-phosphoglycerate kinase)	8,1	[49]
MSA-I (merozoite surface antigen)	8,2	[50]
RNA-polymerase-II	8,3	[51]
DHFR (dihydrofolate reductase)	7,1	[15]
SSU-rRNA	7,2	[17]
Anonymous: 3.18	7,3	[9]
Anonymous: 3.50	7,4	[9]
HSP70	7,5	[41]
GGMP	7,6	[18]
CRK-1 (CDC2-related kinase)	7.7	C. Doerig, Edinburgh
SSU-rRNA	6.1	[17]
Ubiquitin fusion protein	6,2	[52]
Anonymous: 1.9	6,3	[9]
DNA polymerase-α	6,4	[54]
PbS21 (21-kDa ookinete surface antigen)	5,1	[29]
ADPRF (ADP ribosylation factor)	5,2	A.P. Waters, Leiden
SSU-rRNA	5,3	[17]
α-Tubulin	5,4	[53]
Anonymous: 2.2	5,5	[9]
DNA polymerase-δ	5,6	[54]
CSP (Circumsporozoite Protein)	4,1	[55]
α-Tubulin	4,2	[53]
Anonymous: 4.1	4,3	[9]
Anonymous: SPII	3,1	[9]
X-open reading frame	3,2	A. Thomas, Rijswijk
Ca^{2+} ATPase	2,1	[56]
Anonymous: 7.1	1,1	[9]

[a] Probes are numbered according to their location on the chromosomes of clone 8417 of *P. berghei*. The location of the genes on the separate chromosomes of the group of 9, 10, and 11, which cannot be separated in *P. berghei*, has been established in *P. chabaudi* where size polymorphisms between different lines allow the separation of these chromosomes.

2.3. Restriction digests of separated chromosomes

Individual chromosomes or groups of chromosomes were excised as small agarose blocks from FIGE gels after ethidium bromide staining of the gel. The blocks were rinsed once for 15 min with 10 mM Tris-HCl/1 mM EDTA, pH 8, once with double distilled water and 3 times for 15 min in fresh restriction buffer. Thereafter blocks were incubated for 24 h in 200 μl restriction buffer containing 100 μg ml^{-1} of bovine serum albumin (BSA; Promega) and 10 U of the restriction enzyme. Restriction fragments were separated using FIGE [13]. Electrophoretic conditions are specified in the legends to the figures.

2.4. Blotting, labelling and hybridization

Agarose gels were blotted to Hybond-N plus membranes (Amersham). DNA probes were radiolabelled by random priming. Hybridization was performed under standard conditions at 60°C. Blots were washed at 60°C as follows: 3 × 15 min in 3 × SSC/0.5% SDS, 3 × 15 min in 1 × SSC/0.5% SDS (1 × SSC: 0.15 M NaCl, 0.015 M Na citrate). A further 30 min wash in 0.1 × SSC/0.5% SDS at 60°C was performed when appropriate.

2.5. Description of probes

Table 2 shows the probes which were used in this study. Details of most probes and sequence of the genes have been published elsewhere (for references see Table 2). All probes selected have been obtained from *Plasmodium* species, except the probe for Ubiquitin Fusion Protein. All housekeeping genes which have been cloned from *P. falciparum* showed a strong cross-hybridization with the rodent malaria parasites. In contrast many of the cloned genes encoding antigens of the human parasites did not show cross-hybridization (results not shown). To establish the location of antigen-genes we therefore selected the genes cloned from the rodent parasites which showed cross-hybridization with all four rodent species. Nearly all probes hybridized to only one of the fourteen chromosomes. The few exceptions were: The SSU-rRNA gene which is present in four copies in the genome [17]. The HSP70 and the GGMP gene (a protein homologous to HSP70 encoding glycine-glycine-methionine-proline repeats), which are located on chromosome 7 [18]. Both probes show a consistent cross-hybridization with chromosome 12 of all species. The α-tubulin gene, which is present in two different forms in the genome of *P. falciparum* [19].

3. Results

3.1. Location and linkage of genes on chromosomes

Each of the four species, *P. berghei, P. yoelii, P. chabaudi* and *P. vinckei*, has 14 chromosomes (see below and Fig. 2). We have mapped more than 50 probes to the chromosomes of isolates of each species by hybridization to blots of pulsed field gels (Table 2). Fig. 1 shows the chromosomal location of each probe in each species. Chromosomal location and linkage of the genes appear to be well conserved between the species, with certain exceptions as detailed below.

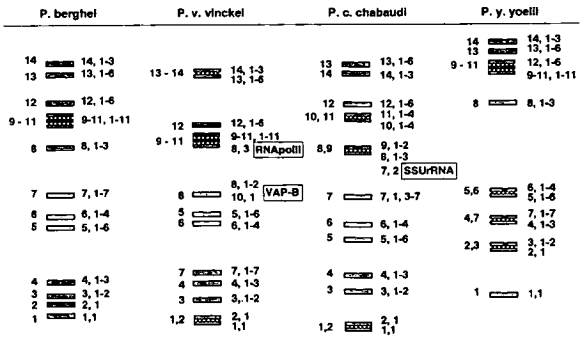

Fig. 1. Schematic representation of the karyotypes and the location and linkage of probes on different chromosomes of four species of *Plasmodium*. Chromosomes were separated by pulsed field gel electrophoresis (FIGE or CHEF conditions). The number of chromosomes in groups of co-migrating chromosomes in pulsed field gels was determined as described in Fig. 2. The karyotype of clone 8417 of the ANKA strain of *P. berghei* (see Fig. 2) is used as the reference karyotype to number the chromosomes (see left hand side of the karyotypes). The chromosomal location of the probes was established by hybridization of radio-labelled probes to the chromosomes (see right-hand side of the karyotype for the numbers of the probes and Table 1 for the description of the probes). The three genes which show a deviant location compared to the conserved location and linkage of most genes are boxed.

As noted in previous work, polymorphisms in the sizes of homologous chromosomes occur both between the species, and between different isolates and clones of a single species. Results obtained for each species are as follows.

P. berghei

The number of chromosomes and size polymorphisms in a number of cloned lines of several distinct strains of *P. berghei* have been previously studied in detail [9]. The genome comprises 14 chromosomes ranging in size between 0.5 and 3 Mb. The molecular karyotype of clone 8417 of the ANKA strain has been chosen as the reference karyotype to number the chromosomes from 1 (the smallest chromosome) to chromosome 14 (the largest) (Fig. 2). Chromosomes 13 and 14 co-migrate in FIGE gels and we were not able to separate them by this method. However, by applying CHEF conditions these chromosomes can readily be separated (Fig. 3). Chromosomes 9, 10 and 11 always co-migrate as a condensed group, using both FIGE and CHEF conditions. The number of chromosomes in this group has been determined previously by counting the number of telomeric restriction fragments after digestion of this group with rare cutting enzymes and hybridization with a telomere specific probe [9] (see also Fig. 4).

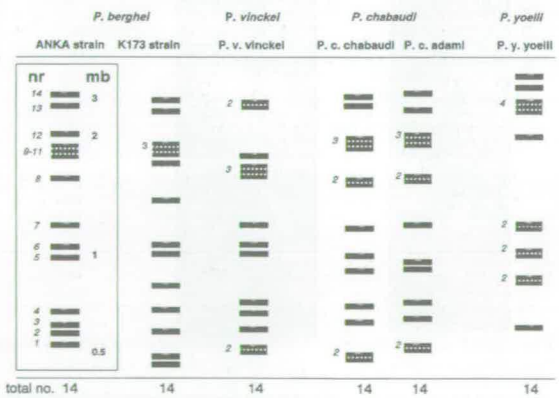

Fig. 2. Schematic representation of the karyotypes and the total number of chromosomes of four species of *Plasmodium*. The total number of chromosomes have been established by separation of chromosomes by pulsed field gel electrophoresis (both FIGE and CHEF conditions; see also Fig. 3). In each species several of the 14 chromosomes have the same size and co-migrate as a group in pulsed field gels. The exact number of the chromosomes in all those groups (see the number at the left hand side of the karyotypes) was established by counting the number of telomeric fragments after digestion of the chromosomes by *Apa*I (see Fig. 4).

P. chabaudi

Two subspecies are recognised, *P. c. chabaudi*, which occurs in the Central African Republic, and *P. c. adami* in the Congo, Brazaville [20]. As in *P. berghei*, some chromosomes co-migrate in pulse field gels, and the exact number of 14 has been established after digestion of chromosomal bands with *Apa*I and hybridization with a telomeric probe (Fig. 4).

Size polymorphisms of homologous chromosomes are seen between *P. c. chabaudi* and *P. c. adami* (Fig. 2). In addition size polymorphisms occur between different *P. c. chabaudi* lines [21]. For example, in line AS, chromosome 11 migrates with chromosome 12 but in AJ-line, chromosome 11 migrates with chromosome 10. Also, chromosome 5 of line AJ is larger than chromosome 6 of the same line, and chromosome 5 of line AS has an intermediate size between the two.

P. vinckei

Four subspecies have been recognised [16]. Here we have studied only *P. v. vinckei* in detail, which has 14 chromosomes. Two other subspecies, *P. v. lentum* and *P. v. brucechwatti*, had nearly identical molecular karyotypes as *P. v. vinckei* (unpublished results).

P. yoelii

Three subspecies have been recognized [16]. Here the 17 × strain of *P. y. yoelii* is studied in detail. The molecular karyotype appears at first sight quite different from the other three rodent species (Figs. 1 and 3). The same is true for the subspecies *P. y. killicki* (unpublished results). In general the chromosomes appear to be larger than the chromosomes of the other three species and the low number of separated bands in ethidium bromide-stained pulsed field gels suggest that the total number of chromosomes is lower than 14. However, many chromosomes co-migrate and are difficult to separate both with FIGE and CHEF conditions. Counting of restriction fragments of *Apa*I digested chromosomes which hy-

bridize to the telomeric probe indicates that the genome of this species also comprises 14 chromosomes (Fig. 4).

3.2. Chromosomal rearrangements

While the linkage groups appeared to be conserved in the parasite lines studied, there were certain examples where they were not. Two examples of this are shown in Figs. 5 and 6 and are the dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene and the small subunit ribosomal (SSUrRNA) genes. In most parasites the DHFR-TS gene is located on chromosome 7. However, in one laboratory line of *P. berghei* this gene is on chromosome 13 (Fig. 5, lane c) as a result of a chromosome translocation [13]. In another laboratory line of the same species, the gene is present in two copies (Fig. 5, lane e) as a result of a chromosome duplication (unpublished results).

In all species four rRNA gene units are present which are unlinked in the genome and are located on four different chromosomes (Fig. 6). In most lines of the different species they are located on chromosome 5, 6, 7 and 12. In *P. chabaudi*, however, chromosome 7 lacks a copy, while a gene unit is present on chromosome 8/9 (Fig. 6, lane d). None of the other markers for either chromosome 7 or 8/9 are affected. In the laboratory line of *P. berghei*, EP, the rRNA gene unit of chromosome 7 is on chromosome 13 as a result of a chromosome translocation [13] (Fig. 6, lane c).

We observed only two further cases in which the location of genes deviated from an otherwise con-

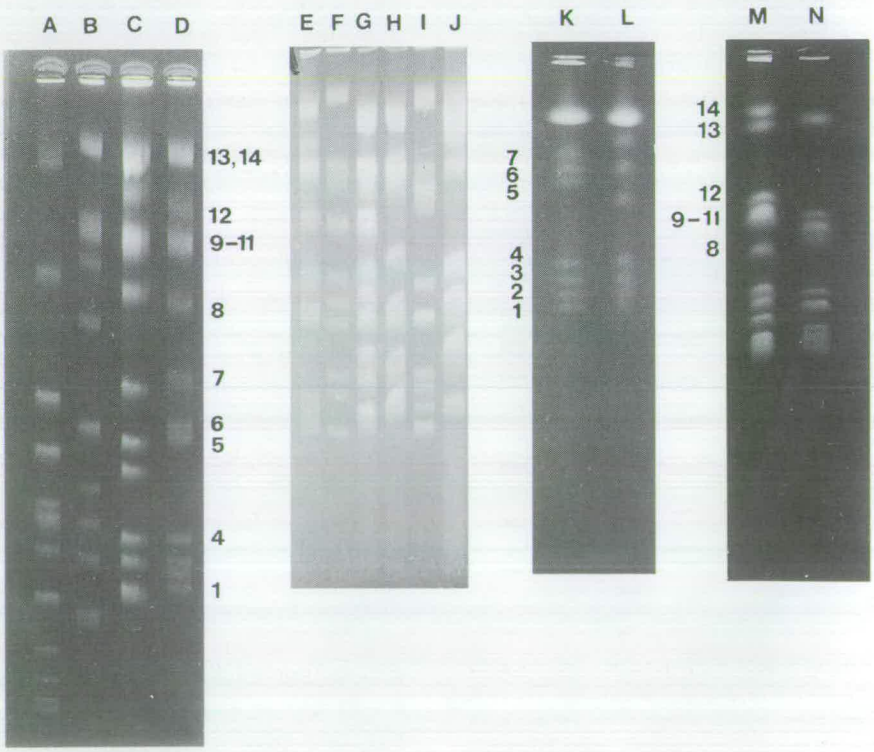

Fig. 3. Separation of chromosomes of four species of *Plasmodium* by pulsed field gel electrophoresis under different FIGE and CHEF conditions. FIGE conditions were used to separate the chromosomes in the complete size range from 0.5–3.5 Mb (lanes a–j). Different CHEF conditions (lanes k–n) were used to separate chromosomes which co-migrate in groups under FIGE conditions. Lanes A-D: *S. cerevisiae* (A), *P. berghei* K173 (B), *P. berghei* ANKA (C,D). (FIGE: 100 h, 3.5 V cm^{-1}, pulse time from 30-550 s). Lanes E-J: *P. berghei* ANKA (E), *P. c. chabaudi* (F,G), *P.v. vinckei* (H,I), *P. y. yoelii* (J). (FIGE: 90 h, 3.5 V cm^{-1}, pulse time from 60-500 s). Lanes K,L: *P. berghei* ANKA (CHEF: 24 h, 80–120 s pulse time; 24 h, 130–180 pulse time; 24 h, 180-240 pulse time; 4.5 V cm^{-1}) Lanes M,N: *P. berghei* ANKA (M), *P. v. vinckei* (N). (CHEF: 60 h, 500-700 s pulse time; 25 h, 300–500 s pulse time; 3.5 V cm^{-1}).

served gene linkage (Fig. 1). In *P. vinckei* the RNA polymerase-II gene is on one of chromosomes 9, 10 or 11 while in the other species the gene is linked to genes on chromosome 8. In the same species the VAP-B gene is on chromosome 8 but found on chromosome 10 in all other species.

4. Discussion

The principal novel finding here is that location and linkage of genes on polymorphic chromosomes of four malaria parasites which infect rodents appear to be well conserved. This conservation of linkage groups was not expected since the chromosomes of the four species showed considerable inter- and intra-specific size differences and since large scale chromosomal rearrangements have been reported frequently.

Large scale rearrangements in chromosomes, such as chromosome translocation, gene amplification and chromosome duplication we now know to occur in most eukaryotes. Most of these large scale rearrangements occur infrequently and do not belong to developmentally regulated DNA rearrangements. These rearrangements seem to have no specific function and may simply be aberrant processes during DNA replication and recombination [22]. Cells bearing

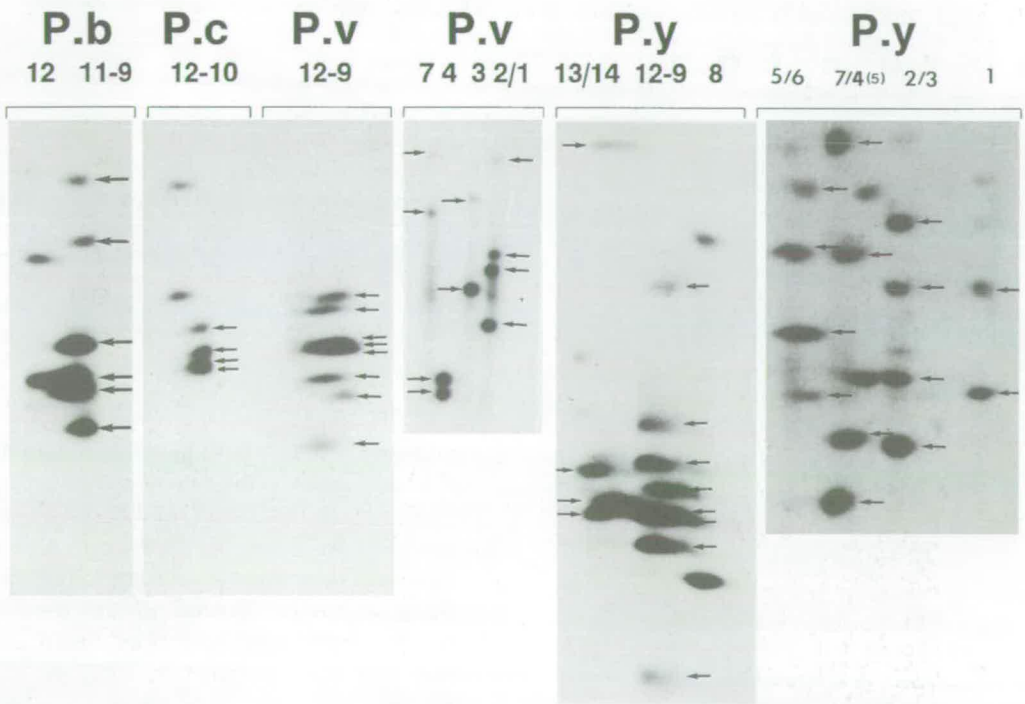

Fig. 4. Telomeric fragments of (co-migrating) chromosomes of four species of *Plasmodium*. Chromosomes were separated by pulsed field gel electrophoresis (see Fig. 3) and groups of chromosomes were excised as small agarose blocks from the gels (see Fig. 1 for the numbering of the chromosomes). These chromosomes were digested by *Apa*I and the restriction fragments separated using FIGE conditions [13]. The fragments were hybridized to a probe which is specific for the telomeres of *Plasmodium*. Arrows show the telomeric fragments in groups of chromosomes which co-migrate in pulsed field gels. P.b. = *P. berghei* ANKA; P.c. = *P. c. chabaudi*; P.v. = *P. v. vinckei*; P.y. = *P. y. yoelii*. The exact number of the telomeric fragments for all species have been determined after interpretation of results from different experiments. This is neccessary since the large difference in size of the fragments requires different FIGE conditions for optimal separation of the fragments. In addition, individual chromosomes separated by FIGE are often contaminated with (fragments) of other chromosomes, resulting in additional weak hybridization bands. Larger *Apa*I fragments always show a weaker signal than the smaller telomeric fragments, which is possibly due to a less efficient transfer of the large fragments during the blotting procedure. In *P. yoelli* the chromosomes are shown from a heterogenous population with regard to chromosome 5, which is present in two different size forms.

chromosomes with large scale rearrangements often show abnormal functions in the progression of the normal cell cycle. There is, however, no doubt that chromosomal rearrangements have played a large role in the evolution of eukaryotic genomes [23].

Species belonging to the genus *Plasmodium* exhibit an extensive genome plasticity. Chromosome size polymorphism and large scale rearrangements have been frequently reported. Many of the observed size polymorphisms are due to variations in the number of subtelomerically located, repeat sequences [9]. Besides structural changes in the subtelomeric areas, large scale rearrangements can also affect the internal regions of chromosomes. Several lines of evidence indicate that large scale rearrangements can also cause exchange of genetic material between non-homologous chromosomes of *Plasmodium* [13,14]. It was unknown whether such large scale rearrangements involving translocation of genes between non-homologous chromosomes occur frequently in nature. Several of the described large scale rearrangements have been found in parasites which had been cultured in vitro or in artificial laboratory hosts and it is uncertain whether these parasites would be able to survive in nature. An interesting question was therefore whether the genome plasticity frequently affected the chromosomal location of genes in parasites from field isolates, resulting in reshuffling of linkage groups and whether this plasticity could be a specific feature of chromosomes of *Plasmodium*.

The comparison of the location and linkage of genes on chromosomes of four murine *Plasmodium*

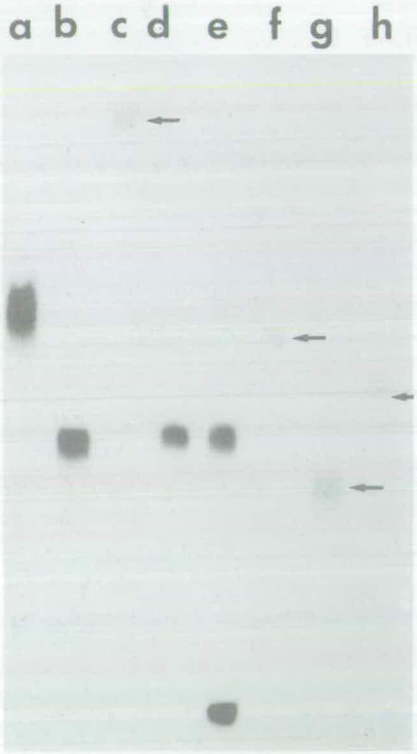

Fig. 5. Chromosomal location of the DHFR-TS gene on polymorphic chromosomes of four species of *Plasmodium*. This gene is usually located on chromosome 7, ranging in size between 1 and 1.5 Mb (see Fig. 1). In line EP of *P. berghei* ANKA this gene is translocated to chromosome 13 of about 3 Mb (lane c) and in line NYU2 of *P. berghei* two copies are found as a result of a chromosome duplication (lane e). Lanes a–e, *P. berghei*; lane f, *P. c. chabaudi*; lane g, *P.v. vinckei*; lane h, *P.y. yoelii*.

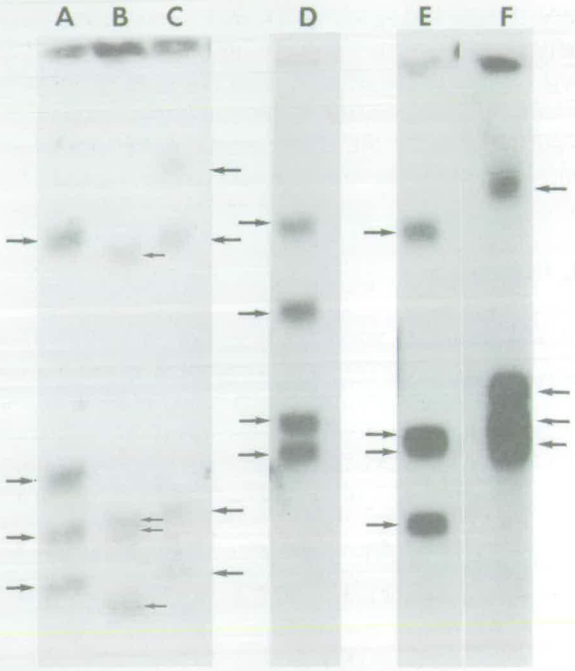

Fig. 6. Chromosomal location of the rRNA units on polymorphic chromosomes of four species of *Plasmodium*. In all species only four copies are present which are unlinked and reside on chromosome 5, 6, 7 and 12 in *P. berghei* (lane A,B), *P.v. vinckei* (lane E) and *P. y. yoelii* (lane F). In *P.c. chabaudi* (lane D) the genes are located on chromosome 5,6,8/9 and 12. In line EP of *P. berghei* ANKA the unit of chromosome 7 is translocated to chromosome 13 of about 3 Mb (lane C).

species reported here, has produced no evidence that large-scale rearrangements frequently affect the gene composition of their chromosomes. An explanation for the conserved location and linkage of genes on chromosomes of the four rodent species might be that internal chromosomal rearrangements occur less frequently in parasites which multiply under natural conditions than in parasites which are maintained under laboratory conditions. A more likely explanation might be that parasites with rearranged chromosomes have a selective disadvantage in nature. It is known that chromosomal rearrangements often lead to meiotic disturbances and are selected against [24]. This will substantially limit the reshuffling of genes. Internal large scale rearrangements in *Plasmodium* have mainly been found in laboratory lines which only proliferate by asexual multiplication without the occurrence of meiosis which takes place during the sexual cycle in the mosquito [1].

Studies on chromosome structure of the human parasite *P. falciparum* points to the existence of conserved internal regions of chromosomes [12] and the less frequent appearance of rearranged chromosomes in field isolates compared to parasites cultured in the laboratory [25]. Studies on the molecular basis of pyrimethamine resistance of *P. falciparum* for example showed that in culture induced resistance could coincide with duplication of genes and chromosome rearrangements. In resistant parasites from field isolates this kind of rearrangements have so far not been detected, and resistance has been found to be exclusively due to point mutations [26].

Despite the conserved linkage of the genes under study, the chromosomes of the four rodent species showed considerable inter- and intraspecific size differences. Although we localized only a small proportion of the total number of genes present in the genome, it seems unlikely that all these size differences result from differences in the chromosomal location of as yet unlocalized genes. If we assume therefore that the probes selected are representative of a reasonable proportion of the genome and no large variation exists in the total number of genes between the different species, the observed size polymorphisms are most likely caused by differences in number and structure of non-coding intervening sequences. Studies on different sized chromosomes from *P. berghei* have shown that size differences of up to 0.5 Mb were almost exclusively due to variations in the number of 2.3-kb repeats, which are subtelomerically located [9]. Large variations in those repeats did not influence the viability of the parasites and apparently did not exclude cross-fertilization between parasites containing different sized chromosomes (unpublished results).

We found a few exceptions to the conserved linkage of the genes in these species. In *P. chabaudi* chromosome 7 lacks a copy of the rRNA gene unit, while a copy is present on chromosome 8/9. In the other three rodents a copy is present on 7 and the copy on 8/9 is missing. The organization of rRNA genes in *Plasmodium* is unusual in that only a low number of unlinked gene units are present and unique in that they can be divided in two different types, A- and C-type units [27,28]. We recently showed that in *P. berghei* the A-type genes are on chromosome 7 and 12 and the C-type genes on chromosome 5 and 6 (unpublished results). Since all rodent species appear to have four copies, it is likely that the ancestor of these species had the same number of units. Most probably these genes were located in the ancestor on chromosome 5, 6, 7 and 12 since in three out of four species the units reside on those chromosomes. In the case of *P. chabaudi* the 'deviant' location on chromosome 8/9 could be the result of a chromosome translocation event, comparable to the translocation of a rRNA gene from chromosome 7 to chromosome 13 in a laboratory line of *P. berghei*. Since rodent parasites are usually maintained by asexual multiplication in artificial hosts in the laboratory, it is still possible that the few exceptions of deviant locations of genes are caused by rearrangements under laboratory conditions, and do not occur in field isolates. In the case of *P. chabaudi* this is unlikely since isolates of both, geographically isolated, subspecies have the rRNA gene copy on chromosome 8/9 (see also [21]). The other two deviant locations were found in *P. vinckei* and involved genes located on chromosome 8. Interestingly, in three subspecies of *P. vinckei* (*P. v. vinckei, P. v. lentum, P. v. brucechwatti*) chromosome 8 has the same size and is small compared to the same chromosome in the other three rodent species. This may suggest that in the ancestor of these subspecies a translocation of a region of chromosome 8 to a larger chromosome has occurred. These few exceptions of

deviant gene locations suggest that occasionally viable parasites with inter-chromosomal translocations do arise in nature, although the frequency of these events appears to be lower than expected. More importantly, the results indicate that a high plasticity of internal regions of chromosomes is not a clear and specific feature of the chromosomes of *Plasmodium*.

Comparison of linkage of genes between closely and distantly related species might be useful to establish information about the evolution of genomes and species. Recently a comparison was made between the chromosomal linkage of genes in the rodent parasites and the linkage groups in the human parasite *P. falciparum*. This work has shown that there is some conservation of linkage groups between the rodent species and *P. falciparum*, although this is significantly less than is observed between the four rodent species (J.M.R. Carlton, unpublished observations). The presence of conserved linkage groups of genes in distantly related species might be an indication of the existence of functional relationships between linked genes [23]. For example, coordination of timing of expression of a set of genes might require linkage. Interestingly, in the rodent malaria parasites several genes which are expressed during early sexual development (C-type rRNA [28], unpublished observations), PbS21 [29], α-tubulin [19]) are located on chromosome 5 in the rodent malaria parasites. Deletions in this chromosome coincided with the loss of the capacity to produce sexual cells [30]. Characterization and localization of more genes involved in sexual development is necessary to establish whether this chromosome might play a crucial role in the sexual cycle. If indeed genes involved in sexual development appear to be clustered in the genome of *Plasmodium*, the study of the region might provide information about genes which determine the initial steps in the sexual differentiation of *Plasmodium*. Knowledge of stable linkage groups within the genus *Plasmodium* may also be applied in a predictive sense to aid the search for homologous genes in other human parasites.

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Maternal inheritance of extrachromosomal DNA in malaria parasites

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The journal provides a medium for the rapid publication of investigations of the molecular biology, molecular immunology and biochemistry of parasitic protozoa and helminths and their interactions with both the definitive and intermediate host. The main subject areas covered are: chemical structure, biosynthesis, degradation, properties and function of small molecular weight substances, DNA, RNA, proteins, lipids and carbohydrates – intermediary metabolism and bioenergetics – molecular and biochemical studies on the mode of action of antiparasitic drugs – molecular and biochemical aspects of membrane structure and function – molecular and biochemical aspects of host–parasite relationships including analysis of parasitic escape mechanisms – characterisation of parasite antigen and parasite and host cell surface receptors – characterisation of genes by biophysical and biochemical methods, including recombinant DNA technology – analysis of gene structure, function and expression – mechanisms of genetic recombination.

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Abstract

Plasmodium falciparum has two extrachromosomal genomes, the mitochondrial 6-kb DNA element and the 35-kb circular DNA. The mitochondrial gene cytochrome *b* on the 6-kb element has been shown to be inherited uniparentally. In order to ascertain whether the route is maternal or paternal we have examined preparations of male and female gametes of the closely related *Plasmodium gallinaceum* for the presence of extrachromosomal DNA. DNA from purified preparations of gametes was hybridised to probes for both the 6-kb and 35-kb extrachromosomal genomes. Both probes hybridised to the preparation of *Plasmodium gallinaceum* female gametes but not to that of the males. We conclude that the extrachromosomal DNAs of malaria parasites are transmitted maternally.

Key words: Maternal inheritance; *Plasmodium falciparum*; *Plasmodium gallinaceum*; Extrachromosomal genomes

1. Introduction

Malaria parasites have two forms of extrachromosomal DNA; a multi-copy 6-kb linearly reiterated molecule and a low copy number 35-kb circle [1]. Subcellular fractionation [2] suggested that the 6-kb element is mitochondrial in origin unlike the 35-kb molecule which has yet to be assigned to a specific organelle. In a previous study of a cross between two clones of the human malaria parasite *Plasmodium falciparum*, we showed that in individual hybrid oocysts the cytochrome *b* gene located on the 6-kb element was inherited from one or other but not both parents [3]. We interpreted this to mean that the 6-kb element may be present in only one of the parental gametes and we now describe experiments designed to distinguish whether inheritance is through the male or the female route.

Our approach has been to hybridise preparations of DNA from purified male and female gametes with probes representing the 6-kb element (the mitochondrial cytochrome *b* gene) or the 35-kb circle (the rpo*B* gene). Since it is not yet possible to obtain purified male gametes from *P. falciparum*, we prepared male and female gametes from the related parasite *P. gallinaceum* [4] whose male gametes are sufficiently robust to withstand the purification procedure [5].

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2. Materials and methods

Gamete preparations. Two types of gamete preparation were derived from the blood of chickens infected with *P. gallinaceum*. One consisted of purified male gametes prepared by fractionation followed by differential centrifugation as previously described [5]. The other was a mixture of female gametes and fertilised zygotes, hereafter referred to as the female gamete preparation, and was prepared also by fractionation and centrifugation as previously described [6]. Both preparations were approximately 98% pure, with respect to other contaminating cell material, as assessed by microscopic examination.

Preparation of DNA blots. DNA was obtained by phenol/chloroform extraction [7], from both male and female gamete preparations, and the DNA concentrations were estimated following electrophoresis. The male gamete preparation was not diluted since the concentration of the DNA was very low. The female preparation was diluted to give a sample in the same range of concentration as that of the male. Both samples were digested at 37°C overnight, with *Hin*dII, and electrophoresed on a 1% agarose gel at 70 V. The gel was blotted overnight onto nylon membrane (Hybond N+, Amersham) by alkaline transfer, using the method of Southern [7].

Preparation of probes. A fragment of a single copy *P. gallinaceum* nuclear gene, *Pgs25* [8], which encodes the ookinete surface protein, was amplified by the polymerase chain reaction technique using a mixed male and female *P. gallinaceum* gamete DNA template. The amplified product was used as a probe to determine the relative amounts of nuclear DNA in the male and female samples. A fragment of the mitochondrial cytochrome *b* gene [9] was similarly amplified for use as a probe for the 6-kb element. A fragment of the 35-kb molecule overlapping the 5′ end of the rpo*B* gene was amplified from *P. falciparum* DNA using *P. falciparum* primers. An attempt to amplify this fragment from the *P. gallinaceum* gamete DNA using the *P. falciparum*-specific oligonucleotides failed to give reliable results, possibly because of the high 85% AT richness of the 35-kb primers. The various primers cited above and the conditions of the polymerase chain reactions were as follows: (a) for the *Pgs25*, primers 5′-GTA CTA ACA TCT GAA AGT ACC TG-3′ and 5′-CTT CCT TAT CGA AAG TGT AAC C-3′ with 35 cycles of 95°C for 30 s, 50°C for 1 min, and 70°C for 2 min, (b) for the cytochrome *b* gene, primers 5′-TCA ACA ATG ACT TTA TTT G-3′ and 5′-TTT GTT CTG CTA ATA G-3′ with 30 cycles of 95°C for 30 s, 45°C for 30 s, and 72°C for 2 min, (c) for the rpo*B* gene primers 5′-AAT AAT TGA ATA CAT GTT TTA TAT AAT C-3′ and 5′-AAT TTT AAA GAA ATT AAT ATA TTT AAA T-3′ with 35 cycles of 95°C for 30 s, 42°C for 30 s and 72°C for 2 min.

Hybridisation of probes to DNA blots. Both the nuclear and the two extrachromosomal DNA fragments were labelled with [^{32}P]dATP by random-priming [10]. The Southern blot of male and female gamete DNA preparations was hybridised with the nuclear *Pgs25* probe overnight, washed and exposed to film for 4 h before examination. The blot was then stripped and the hybridisation procedure repeated as before using the 6-kb element cytochrome *b* gene probe. The blot was exposed to film for 4, 8, and 25 h. For the 35-kb probe the blot was again stripped and hybridised at a low stringency of 50°C overnight and washed with 2 × saline sodium citrate + 0.1% sodium dodecyl sulphate. The blot was exposed to film for 4 h, overnight and for 6 days.

3. Results

Visual examination of the Southern blots of male and female gamete DNA preparations showed that there was approximately twice the amount of DNA in the male track as in the female track, as measured by the intensity of the nuclear gene probe (Figs. 1A and 2A). The cytochrome *b* gene probe hybridised very strongly to the female track but was undetectable at 4-h exposure in the male track (Fig. 1B). However, following 8-h and 25-h exposure, a faint band in the male track was detectable at the same position as

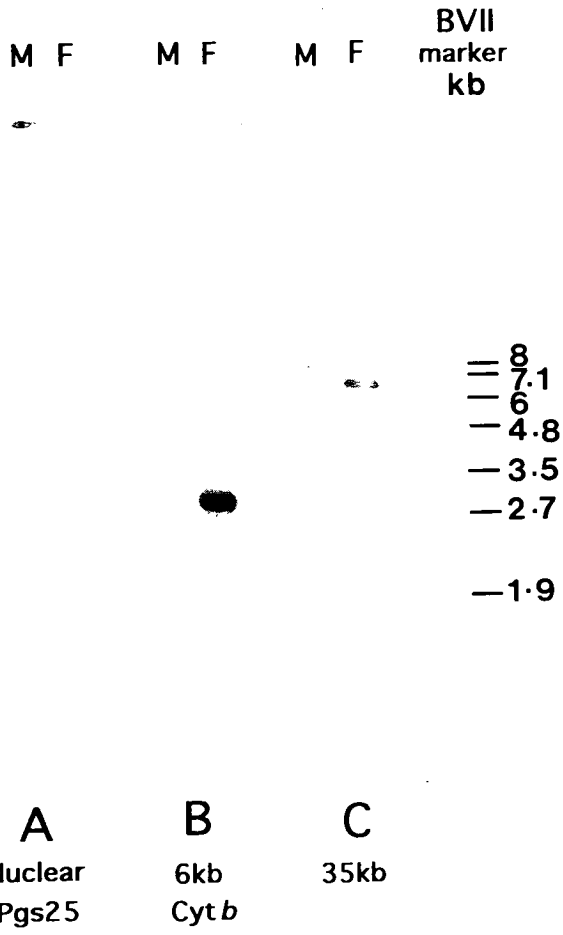

Fig. 1. The same Southern blot of male (M) and female (F) *P. gallinaceum* DNA gamete preparations probed with three different probes: (A) probed for a fragment of the nuclear gene *Pgs25*; (B) probed for a fragment of the 6-kb cytochrome *b* gene; (C) probed for a fragment from the 35-kb circle.

the cytochrome *b* gene probe on the female track. The density of these bands was measured on a densitometer (Fig. 2), and on a Molecular Dynamics Phosphorimager (Fig. 3). The Phosphorimager readings for all three exposures (4, 8 and 25 h) in the female track were in linear progression. In both the 4- and 8-h exposures where a male track signal was detectable, this reading was only 2% of the signal in the female track. Thus in relation to the amount of DNA recognised by the probe for the nuclear genome (female = 0.5 male), there was approximately 1% of the signal for cytochrome *b* in the preparation of male gametes, compared to that in the female gametes.

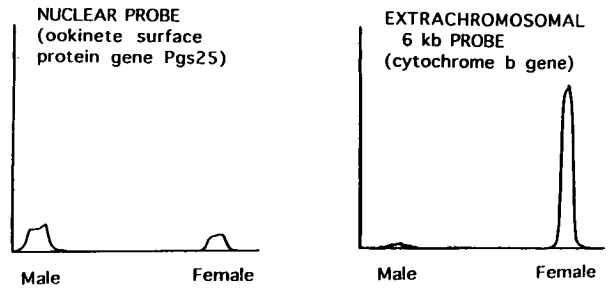

Fig. 2. Densitometric traces of Southern blot of *P. gallinaceum* male and female gamete preparation DNA probed with: (A) a nuclear gene fragment *Pgs25* (integral: male = 3334, female = 1506), and (B) a fragment from the cytochrome *b* gene on the extrachromosomal 6-kb element (integral male = 640, female = 17 710).

The 35-kb probe showed a similar hybridisation pattern to the 6-kb probe with a single clear band in the female track but no hybridisation visible in the male track even after 6 days exposure of the film to the blot (Fig. 1C). The lower intensity of the hybridisation with this probe may be due to

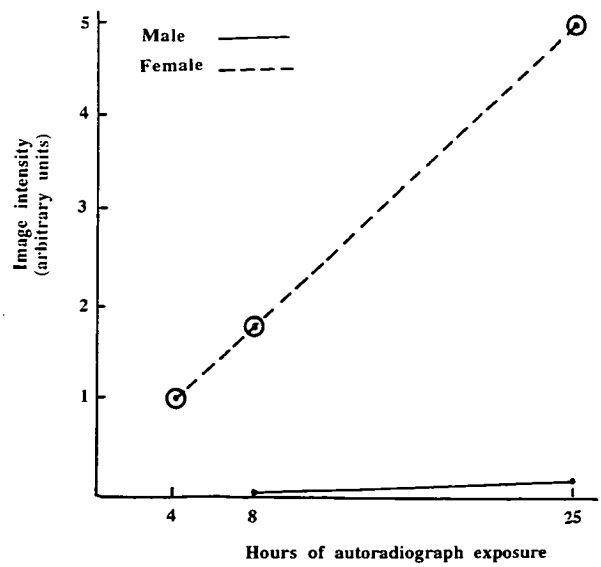

Fig. 3. Readings taken on the Molecular Dynamics Phosphorimager of three different exposures of the male and female *P. gallinaceum* gamete preparations probed with the cytochrome *b* gene probe.

the fact that the probe was from a different species. Furthermore, dot blot analysis shows the 35-kb circle to be present at one, or at the most two, copies per cell compared to around 20 copies of the 6-kb element per cell (P. Preiser and D. Williamson, unpublished data). This may also contribute to the lower intensity of signal.

4. Discussion

We found that with respect to the amounts of nuclear DNA in our two samples, the female gamete DNA contained large amounts of the cytochrome *b* gene fragment compared to the male gamete DNA. The female preparation also contained DNA corresponding to the fragment derived from the 35-kb element, which was undetectable in our male gamete preparation. Similar findings have recently been reported by Vaidya et al. [11]. Whilst our purification technique went a long way towards obtaining pure male and female gametes, we are aware that the preparations were not 100% pure. The female preparation undoubtedly contained some fertilised zygotes and possibly damaged or dead male gametes. The male preparation may have contained female-derived debris and residual body material from the male gametocytes. It is reasonable to suppose therefore, that the small amounts of 6-kb cytoplasmic element detected in the male preparation could be the result of contamination.

Electron microscopic examination of male and female gametes in malaria species indicates that each of the six to eight male gametes, derived from a single male gametocyte, contains little more than a nucleus, surface membrane and an axoneme whereas the female gamete contains a full complement of cytoplasmic organelles [12]. Crosses between two different clones of *P. falciparum* indicated that inheritance of the cytochrome *b* gene was uniparental [3,11]. We conclude from the results presented here that both the 6-kb and the 35-kb cytoplasmic elements are most probably inherited only through the female gamete.

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

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

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Drug response and genetic characterization of *Plasmodium falciparum* clones recently isolated from a Sudanese village

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Abstract

We have isolated 20 clones of *Plasmodium falciparum* from isolates from patients attending a village clinic in Sudan during 10 d in October–November 1989. The clones were genetically diverse, having highly variable molecular karyotypes and a wide range of drug responses. Chloroquine-sensitive (50% inhibitory concentration [IC_{50}] in the 4–15 nM range) and chloroquine-resistant clones (IC_{50} in the 40–95 nM range) co-existed in the population, but no obvious amplification of the P-glycoprotein homologue gene, *Pgh*1 (previously known as the multi-drug resistance gene, *mdr*1) marked the chloroquine-resistant clones. Chloroquine resistance was reversible by verapamil in these clones, although they varied in their susceptibility to verapamil alone. These observations indicate that the biochemical characteristics of the Sudanese chloroquine-resistant *P. falciparum* are similar to those reported from south-east Asian and Latin American isolates, which is consistent with there being a similar molecular basis for this phenomenon.

Introduction

Chloroquine is the only antimalarial drug available at a cost accessible to even a minority of the Sudanese people and as such constitutes the major element of malaria control in Sudan and similar African countries (PETERS, 1987). The emergence of chloroquine-resistant *Plasmodium falciparum* in the Eastern Province since around 1986 (BAYOUMI *et al.*, 1989) is causing acute problems as the episodic, post-rainy season malaria epidemics characteristic of sub-Sahelian Sudan cease to be controlled by this drug.

The mode of action of chloroquine and the mechanism of resistance to the drug are unclear, and several opposing viewpoints exist (HOMEWOOD *et al.*, 1972; KROGSTAD *et al.*, 1987; GINSBURG, 1988; WARHURST, 1988; FOOTE *et al.*, 1990; WELLEMS *et al.*, 1990). Studies *in vitro* on chloroquine resistance in different laboratories have made use of different long-term cultured lines of *P. falciparum* originating mainly from south-east Asia or the Amazon basin. Conclusions drawn from the analysis of such isolates may not accurately reflect the present situation in an area such as Sudan. Although currently spreading rapidly, chloroquine-resistant *P. falciparum* was not reported in Sudan until 25 years after its appearance in south-east Asia and South America, and several years after its appearance in East Africa (AL TAWIL & AKOOD, 1983). Chloroquine is essentially the only antimalarial drug available in most of Sudan, and for economic and climatic reasons usage has not been as heavy as in more prosperous areas with more stable malaria transmission.

In order to study the genetic basis for the spread of chloroquine-resistant *P. falciparum*, we have recently characterized 29 isolates from a single village in eastern Sudan with a developing problem of chloroquine-resistant malaria (BABIKER *et al.*, 1991a, 1991b). Since most of these isolates proved to be mixtures of genetically different parasites, we have now obtained cloned lines from some of the isolates, in order to define parasite genotypes and their biochemical profiles of drug resistance. We showed that the clones had highly variable molecular karyotypes and wide ranges of drug responses. Chloroquine resistance is reversible by verapamil, but no obvious amplification of the P-glycoprotein homologue gene, *Pgh*1 (previously known as the multi-drug resistance gene, *mdr*1) marked the chloroquine-resistant clones.

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Materials and Methods

Study area

The study area in Asar village, 20 km from Gedaref in the Eastern Province of Sudan, has been described elsewhere (BABIKER *et al.*, 1991b). Malaria transmission is seasonal and reaches a peak in October or November following the rainy season. The main *Plasmodium* species present is *P. falciparum*. Chloroquine resistance was first reported in this region in 1986 (BAYOUMI *et al.*, 1989).

Isolation and characterization of P. falciparum *clones*

The initial isolates were obtained with informed consent from villagers attending a local clinic during the October–November malaria season of 1989. Clones were obtained using the limiting dilution method (ROSARIO, 1981), from a selection of isolates known to exhibit a range of sensitivity to chloroquine and to pyrimethamine. Certain of these isolates were known to be mixed infections by their possession of more than one allele of genes for antigens and other proteins (BABIKER *et al.*, 1991a). Following the cloning procedure, the resulting cultures were shown to be pure clones by ensuring that each haploid clone was monoallelic for each of 2 highly polymorphic antigens (merozoite surface protein [MSP]-1 and MSP-2) when tested in immunofluorescence assays with a panel of allele-specific monoclonal antibodies (CONWAY & MCBRIDE, 1991).

Pulsed field gels

P. falciparum chromosomes were separated by pulsed field gradient gel electrophoresis (PFG) as described previously (BABIKER *et al.*, 1991b), with a basic regime of 22 h, 120s pulses, 140 V followed by 22 h, 180s pulses, 140 V, and finally 24 h, 300s pulses, 120 V. Southern blotting, dot-blotting and hybridization were performed using standard techniques (SAMBROOK *et al.*, 1989).

Measurement of drug sensitivity

Hypoxanthine incorporation assay. Chloroquine-induced inhibition of uptake of [^{3}H]hypoxanthine by the clones was measured using the methods of DESJARDINS *et al.* (1979) and GEARY *et al.* (1983). Tests were carried out on unsynchronized cultures, diluted in RPMI medium containing 10% human serum to 1% parasitaemia and 1% haematocrit, in a final volume of 0·2 mL in microtitre plate wells. [^{3}H]hypoxanthine was added to a final concentration of 5μCi/mL. Chloroquine sulphate was added to final concentrations ranging from 10 to 160 nM. After 40–44 h incubation, cells were lysed, washed and harvested on to fibre glass filters. Filters were then baked

and counted in a PPO/POPOP*/toluene scintillation fluid supplemented with 30% Triton X-100®. Results were expressed as percentage inhibition compared to the incorporation of label in control wells without drug. The concentration of chloroquine which produced 50% inhibition of parasite growth (IC_{50}) was obtained from the regression line of dose/response curves of parasite inhibition plotted against the logarithm of drug concentration.

Microscopical determinations. Clones were cultured in microtitre plates in serial dilutions of chloroquine, mefloquine and pyrimethamine in complete RPMI medium for 72 h, essentially according to the method of THAITHONG *et al.* (1983). Each well contained 100 μL, at 1% parasitaemia and 5% haematocrit. After 72 h, thin blood films were made from each well, and the viability assessed by microscopical examination. The results were expressed as the minimum inhibitory concentration (MIC) which killed all, or nearly all, of the parasites.

Effect of verapamil

Drug sensitivity of the parasites to chloroquine, mefloquine and pyrimethamine was assessed with and without the addition of 1 μM verapamil to the cultures. The effect of verapamil alone over a wider range of concentrations (0–5 μM) was also tested on some clones.

Chemicals

[^{3}H]hypoxanthine (40 Ci/mmol) was obtained from Amersham International Ltd, UK; chloroquine sulphate (Nivaquine®) from May and Baker Ltd, UK; pyrimethamine from Wellcome, UK; mefloquine from Hoffman La Roche, Switzerland; and verapamil from Sigma Chemical Company. Glass fibre filters were obtained from Titertek.

of results were obtained.

(i) All the clones from a single isolate possessed chromosomes of identical size, as observed in isolate SUD 111. This suggests that, at the time of cloning, the parasites in these samples were of a clonal type, due either to a clonal-type infection in the patient or to selection of a predominant clone during initial culturing of the uncloned isolate.

(ii) The clones of a single isolate varied in the size of only one or 2 chromosomes. This was most probably due to deletions or accretions in the chromosomes concerned during asexual growth in culture of a clonal-type parasite (WELLEMS *et al.*, 1988). This was seen in isolate SUD 105, in which the 4 clones obtained were karyotypically identical, except that clone 7 had a 300–400 kilobase (kb) deletion of chromosome 10, and clones 9 and 11 both had 200 kb accretions in chromosome 4. These related clones had identical drug sensitivities and possessed the same MSP-1 and MSP-2 alleles.

(iii) Several quite different karyotypes were obtained from a single isolate. This result can be presumed to have been due to the patient's harbouring a mixed infection at the time the parasites were obtained. The clones derived from patients SUD 106 and 124 illustrate this situation. While SUD 106/7 and 106/10 differed from other clones of the same isolate, they were identical to each other, except in the size of 2 chromosomes.

Identical clones have never been isolated from different individuals, a result consistent with the extremely diverse genetic profiles of malaria isolates in general (CREASEY, 1990) and in particular the uncloned Asar isolates from which these clones were derived (BABIKER *et al.*, 1991a, 1991b).

An autoradiograph of a Southern blot of gel 1A probed

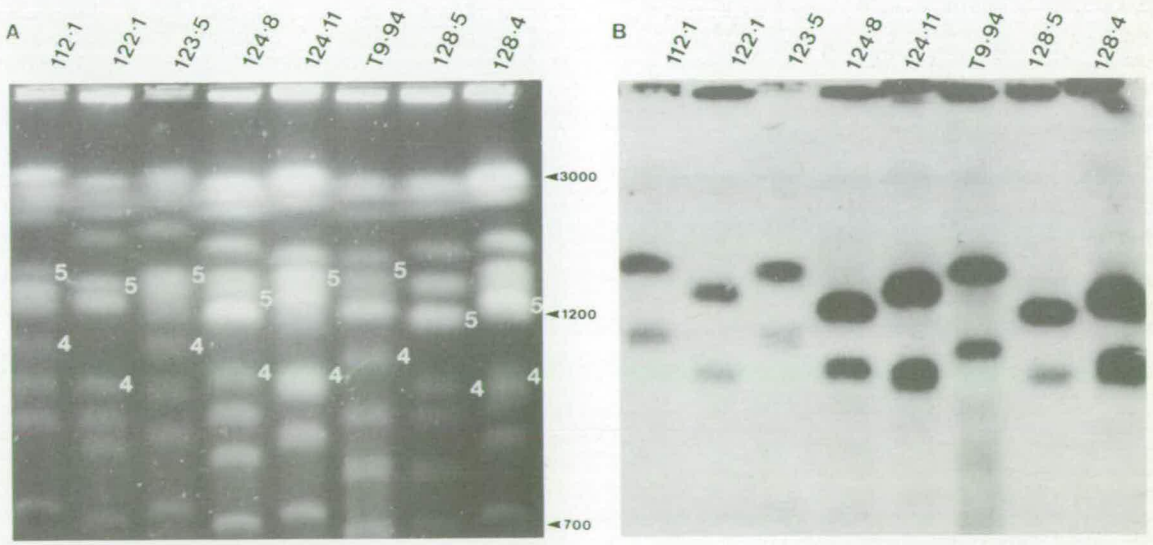

Fig. 1. Karyotypic diversity in some examples of *P. falciparum* clones from Asar village, eastern Sudan. A. Ethidium bromide stained chromosomes separated on a 0·8% agarose gel using a CHEF® electrophoresis apparatus. Seven of the parasites tested are Asar clones, 2 of which, 112/1 and 124/11, were not included in the study (see the Table for the drug sensitivity phenotypes). T9/94 is a Thai clone. Chromosomes 4 and 5 are marked, and approximate chromosome sizes in kilobases are indicated on the right. B. After Southern blotting, gel A was successively probed with gene markers for chromosomes 4 and 5, the *Pgh*1 gene and the *DHFR* gene respectively. The 2 autoradiographs from the same blot have been superimposed before photography for comparison and signal intensities are not proportional to gene copy number.

Results

Clone characterization

All the clones were confirmed as pure by their possession of single alleles of MSP-1 and MSP-2, using monoclonal antibody typing (results not shown).

The clones were examined for chromosomes by pulsed field electrophoresis. Results for a representative sample of clones are shown in Fig. 1A. When clones from individual isolates were compared, the following 3 categories

with the genes encoding the *P. falciparum* dihydrofolate reductase (*DHFR* gene) located on chromosome 4, and *Pgh*1 located on chromosome 5 is shown in Fig. 1B. In each clone the *DHFR* probe always hybridized to the fourth smallest chromosome, which, however, differed in size among the clones. The *Pgh*1 probe hybridized to the fifth smallest chromosome in only 2 clones (SUD 128/5 and SUD 128/4). These results illustrate clearly the considerable size polymorphism of these 2 chromosomes, especially of chromosome 5, among this group of isolates.

*2,5-Diphenyloxazole/1,4-bis[5-phenyl-2-oxazolyl]benzene.

Although there were differences in the relative intensity of hybridization of the probes shown in Fig. 1, deoxyribonucleic acid (DNA) transfer from pulsed field gels is not reliably quantitative and the amount of DNA per lane in this gel was not constant. Using more quantitative dot-blots and Southern blots of DNA digested with restriction enzymes, we have not been able to detect *DHFR* or *Pgh*1 gene amplification in DNA samples of any of the clones, whether drug-resistant or drug-sensitive.

Chloroquine sensitivity

Drug resistance measurements, even with identical clones, may show considerable inter-laboratory variation. To increase confidence in assessing the drug response of a given clone, we have, therefore, employed and compared 2 different assays in this work. IC_{50} values above 35 nM, as assessed by [^{3}H]hypoxanthine incorporation, and MIC values of 16×10^{-7}M, as assessed microscopically, were taken to indicate clear chloroquine resistance. The drug responses of the 20 clones are presented in the Table. They are listed in ascending order of IC_{50} values,

Table. Drug sensitivity phenotypes of twenty *P. falciparum* clones derived from patient blood samples collected in the village of Asar, Sudan, during October and November 1989

	Chloroquine[a] IC_{50}[b] ($\times10^{-9}$M)		Chloroquine[a] MIC[c] ($\times10^{-7}$M)		Mefloquine[a] MIC[c] ($\times10^{-7}$M)		Pyrimethamine[a] MIC[c] (M)	
Verapamil	No	Yes	No	Yes	No	Yes	No	Yes
Experimental clones[d]								
SUD 105/1	4	3	8	4	4	4	10^{-7}	10^{-7}[f]
SUD 105/9	6	5	8	4	4	4	10^{-7}	10^{-7}[f]
SUD 105/11	7	4	8	4	4	4	10^{-7}	10^{-7}[f]
SUD 106/10	7	4	2	2	4	4	10^{-6}[e]	10^{-6}
SUD 105/7	8	4	8	4	4	4	10^{-7}	10^{-7}[f]
SUD 106/9	9	8	4	4	4	4	10^{-6}	10^{-6}
SUD 106/7	13	4	4	2	4	4	10^{-6}[e]	10^{-6}
SUD 106/11	11	5	4	4	4	4	10^{-6}	10^{-6}
SUD 106/1	15	6	2	2	4	4	10^{-6}[e]	10^{-6}
SUD 128/5	43	10	8	4	4	2	10^{-8}	10^{-8}[f]
SUD 128/4	45	11	8	4	4	2	10^{-8}	10^{-8}[f]
SUD 124/8	58	19	16[e]	4	2	2	10^{-5}	10^{-5}[f]
SUD 128/1	64	18	8	4	4	2	10^{-8}	10^{-9}
SUD 123/5	64	18	16	8	8	8[f]	10^{-5}[e]	10^{-5}
SUD 124/5	65	28	16	4	2	2	10^{-5}	10^{-5}
SUD 124/1	73	27	16	8	2	2	10^{-5}	10^{-6}[f]
SUD 126/1	83	18	16[f]	1	2	2	10^{-7}	10^{-7}
SUD 102/1	85	12	16	8	2	2	10^{-8}	10^{-9}
SUD 122/1	92	23	16	4	2	2	10^{-6}	10^{-6}[f]
SUD 111/1	95	21	16	8	2	2	10^{-5}[e]	10^{-5}
Control clones								
3D7[g]	8	3	4	2	4	2	10^{-8}	10^{-9}
Dd2[g]	57	15	16	8	16	8	10^{-6}	10^{-6}[f]

[a]All values are means of 3 or more separate experiments, with ('yes') and without ('no') verapamil (1 μM).
[b]Concentration giving 50% inhibition of parasite growth.
[c]Concentration killing all parasites within 72 h.
[d]Clones with the same 'one hundred' number before the solidus were isolated from a single patient sample.
[e]A few parasites were still viable at the indicated concentrations.
[f]Reduced parasitaemia at the indicated concentrations.
[g]'Control' chloroquine-sensitive and chloroquine-resistant clones, respectively.

with the chloroquine-resistant Dd2 and chloroquine-sensitive 3D7 laboratory-adapted clones as reference controls.

On the whole, the 2 tests of sensitivity *in vitro* to chloroquine gave comparable results. No clone with an MIC of 16×10^{-7}M chloroquine had an IC_{50} <50 nM. Similarly, no clone with an IC_{50} value <35 nM has an MIC $>8\times10^{-7}$M. Certain ambiguities remain, in that some clones of isolate SUD 105 which appeared very chloroquine-sensitive in the IC_{50} test, showed intermediate sensitivity in the MIC test. Apart from this, the combination of both tests appeared to allow differentiation of the parasites into a sensitive group comprising all the SUD 105 and SUD 106 clones, and a resistant group consisting of all the other clones. No clone had chloroquine sensitivity in the IC_{50} range 15–43 nM.

Resistance to mefloquine and pyrimethamine

Most of the clones were sensitive to mefloquine (Table), in agreement with the results of tests on the original 29 uncloned isolates from Asar village (BABIKER *et al.*, 1991b). Again in accordance with our earlier results, 5 clones (SUD 124/1, SUD 124/5, SUD 124/8, SUD 123/5 and SUD 111/1) were highly resistant to pyrimethamine.

One clone, SUD 123/5, was resistant to both chloroquine and pyrimethamine and showed a slight decrease in susceptibility to mefloquine. However, none of the other clones was resistant to all 3 drugs. Mefloquine resistance is clearly not linked to chloroquine resistance. While high level pyrimethamine resistance was found only in chloroquine-resistant clones, many of the latter were sensitive to pyrimethamine.

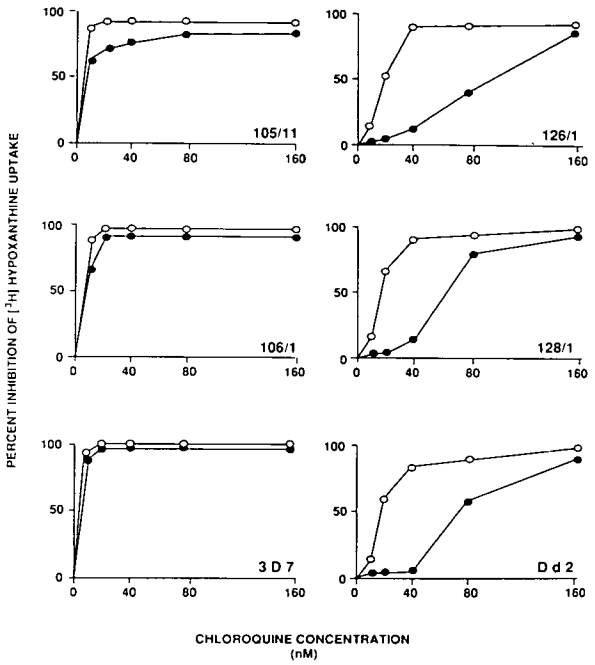

Fig. 2. The effect of 1 μM verapamil on the response of 4 Sudanese and 2 standard laboratory-adapted *P. falciparum* clones to chloroquine. Closed circles (●), without verapamil; open circles (○), with verapamil. SUD 105/11, SUD 106/1 and 3D7 were chloroquine sensitive, SUD 126/1, SUD 128/1 and Dd2 were chloroquine resistant. Points represent the average of simultaneous duplicate experiments. Similar curves have been obtained for all clones tested.

Effect of verapamil

Verapamil, a calcium channel blocker, has been reported to reverse chloroquine resistance in some isolates of *P. falciparum* (MARTIN *et al.*, 1987). We tested the effects of verapamil on our clones (Table) to ascertain whether verapamil reversibility was also characteristic of the chloroquine-resistant parasites studied here. Examples of drug tests where the capacity of increasing concentrations of chloroquine to inhibit [^{3}H]hypoxanthine uptake has been measured, with and without the addition of 1 μM verapamil, are shown in Fig. 2. Clones SUD 105/11, SUD 106/1 and 3D7 were chloroquine-sensitive. Verapamil did not shift the inhibition curve of the 3D7 reference control, although it slightly increased the toxicity of chloroquine to the drug-sensitive Asar (SUD) clones. Clones SUD 126/1, SUD 128/1 and Dd2 were chloroquine-resistant, and verapamil clearly increased their chloroquine sensitivity, although not to the levels of the naturally sensitive clones.

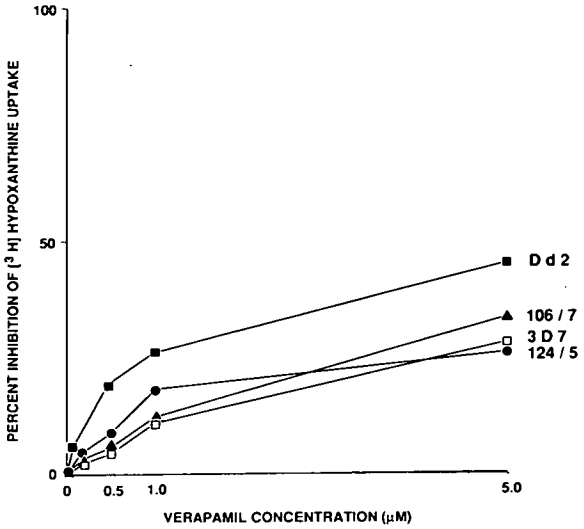

Fig. 3. The effect of verapamil alone on [^{3}H]hypoxanthine uptake over 40–44 h by 2 Sudanese and 2 standard laboratory-adapted *P. falciparum* clones. Dd2 and SUD 124/5 were chloroquine resistant and 3D7 and SUD 106/7 were chloroquine sensitive. Points represent the average of duplicate experiments carried out simultaneously on the same microtitre plate. Three separate experiments gave the same rank order of drug sensitivities.

It has been reported (MARTIN *et al.*, 1987) that verapamil itself has intrinsic antimalarial activity. An example of the effect of verapamil alone on the capacity of parasites to incorporate [^{3}H]hypoxanthine is shown in Fig. 3. Dd2 and SUD 124/5 were chloroquine-resistant, and SUD 106/7 and 3D7 were chloroquine-sensitive. There were large intrinsic differences in their susceptibility to the antimalarial effect of verapamil, Dd2 being particularly sensitive.

The sensitivity of some of the Sudanese clones to mefloquine and pyrimethamine also appeared to increase on addition of verapamil (Table), although to only a limited extent.

Discussion

In our initial survey of uncloned *P. falciparum* isolates from Asar village, we found that no 2 isolates were genetically identical and that it was possible to detect several obviously mixed isolates (BABIKER *et al.*, 1991a, 1991b). The molecular karyotypes of the clones derived from these isolates have further confirmed the highly diverse nature of this small parasite population. Mapping of *DHFR* and *Pgh*1 markers on to chromosomes demonstrates that comparisons of chromosome separations stained with ethidium bromide underestimate the true extent of karyotypic diversity. More detailed genome maps would undoubtedly reveal more radical differences in genome organization between these clones which, it should be emphasized, represent only a small sample of the total population of *P. falciparum* in this small community.

In general, our results indicated that the biochemical characteristics of the Sudanese chloroquine-resistant *P. falciparum* are similar to those reported for south-east Asian and Latin American isolates, and are consistent with there being a similar molecular basis for the phenomenon. The use of genetically pure clones from the 1989 transmission peak permitted a clearer differentiation of some of the characteristics of the drug resistant *P. falciparum* in this village, as follows.

(i) Clones of *P. falciparum* exhibiting low, intermediate and high level chloroquine resistance co-existed with highly sensitive parasites in the population of this village.

(ii) The reversibility of chloroquine resistance of *P. falciparum* by verapamil (MARTIN *et al.*, 1987) also appeared to be characteristic of the chloroquine resistant malaria currently spreading in Asar. However, verapamil has an antimalarial effect of its own, as shown by MARTIN *et al.* (1987) and in our work. The addition of verapamil increased the sensitivity of these clones not only to chloroquine but also, in some instances to mefloquine and pyrimethamine.

(iii) The *Pgh*1 gene has been reported to be amplified in some, but not all, chloroquine-resistant *P. falciparum* isolates (FOOTE *et al.*, 1989). However, we have not been able to detect *Pgh*1 gene amplification in DNA samples from either chloroquine-resistant or chloroquine-sensitive Sudanese *P. falciparum* clones.

(iv) In accordance with our earlier findings with the uncloned isolates, 5 clones were highly resistant to pyrimethamine. This result is of interest, since pyrimethamine was not widely used in Sudan before 1986. Since then, however, Fansidar® (pyrimethamine/sulfadoxine) became available through relief agencies. This may explain the appearance and selection of mutants resistant to this drug. It may also be relevant that clinical pyrimethamine resistance was noted in Sudan as early as 1954 (PHILLIPS, 1954) and, more recently, in the Sennar region (IBRAHIM *et al.*, 1991). Only a few parasites in this and our previous survey (BABIKER *et al.*, 1991b) showed any degree of resistance to mefloquine.

(v) In the survey of the uncloned isolates, some were found to exhibit resistance to all 3 drugs. However, none of the clones examined in this work was multi-drug resistant, with the possible exception of clone SUD 123/5. Since clones resistant to both chloroquine and, at a high level, to pyrimethamine coexisted in this community, and many patients were infected with more than one clone, it is certainly possible that genetic recombination during mosquito transmission could increase the frequency of multi-drug resistant clones.

These results suggest that chloroquine-resistant *P. falciparum* in this small village (and presumably the rest of Sudan) is not a distinct parasite 'strain'. Rather, it appears that genes conferring resistance to this drug are increasing in frequency in the parasite population, probably due to the continuing use of chloroquine in the area. While clinical chloroquine resistance has increased in frequency among patients attending the nearby Gadaref District Hospital during 1986–1990 (unpublished data), most patients in the village appeared to be cured by standard chloroquine therapy. Whether the situation is slowly deteriorating and chloroquine will soon be clinically useless remains to be seen.

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Announcements

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