The inheritance of extranuclear DNA in malaria parasites

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ABBREVIATIONS

bp	-	base pair
ºC	-	degrees centigrade
df	-	degrees of freedom
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
DHFR-TS	-	dihydrofolate reductase-thymidine synthetase
DMSO	-	dimethyl sulphoxide
EDTA	-	ethylenediamine tetraacetic acid disodium salt
f	-	female (gamete)
g	-	gramme
g	-	g force
g	-	gauge
kb	-	kilobase
LSU	-	large subunit
MPC	-	mitochondrial phosphate carrier
mRNA	-	messenger ribonucleic acid
Mb	-	megabase
M	-	molar
µM	-	micromolar
µg	-	microgramme
mg	-	milligram
ml	-	millilitre
mm	-	millimetre
m	-	male (gamete)
mRNA	-	messenger ribonucleic acid
mtDNA	-	mitochondrial deoxyribonucleic acid
ng	-	nanogram
NIMR	-	National Institute for Medical Research, Mill Hill, London NW1 7AA.
nt	-	nucleotide
ORF	-	open reading frame
oligo.	-	oligonucleotide
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
poly(A)	-	polyadenylated
p	-	probability
rbc	-	red blood cell (erythrocyte)
rRNA	-	ribosomal ribonucleic acid
SDS	-	sodium dodecyl sulphate
SSC	-	saline-sodium citrate buffer
SSU	-	small subunit
TBE	-	Tris borate /EDTA buffer
TE	-	TrisHCl /EDTA buffer
TEMED	-	N,N,N,',N'- tetramethylethylene-diamine
tRNA	-	transfer RNA
TGGE	-	temperature gradient gel elecrophoresis
UV	-	ultraviolet
UWGCG	-	University of Wisconsin Genetics Computer Group
x^2	-	chi^2

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I declare that with the assistance mentioned above the work presented and the opinions expressed herein are my own.

[redacted]

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ABSTRACT

The inheritance pattern of extranuclear DNA in malaria parasites has been investigated in a cross between two different clones of the human malaria parasite Plasmodium falciparum. A 1:1 mixture of gametocytes from the two clones, 3D7 and HB3, had been fed to mosquitoes. Single oocysts, each of which is the product of a single fertilisation event, were harvested from the mosquito gut tissue. The self-fertilised parental-type oocysts were distinguished from the cross-fertilised hybrid oocysts by examining polymorphic allelic markers from two nuclear genes MSP-1 and MSP-2, which differ between the two clones and which are known to be inherited in a typical Mendelian manner. Hybrid oocysts possessed both forms of these parental markers [Ranford-Cartwright, 1993].

In this project, a polymorphism between clones 3D7 and HB3 was identified in the highly conserved mitochondrial cytochrome b gene of the extranuclear DNA. When this allelic marker was examined in the oocysts from the cross, each hybrid oocyst showed only one form, and never both forms, of the parental alleles. This indicated that extranuclear DNA in P. falciparum is uniparentally inherited [Creasey, 1993]. Unexpectedly 58 out of the 59 hybrid oocysts examined showed the 3D7 form and only one oocyst showed the HB3 form of the allele.

The sex of the gamete transmitting the extranuclear DNA was investigated using purified preparations of male and of female gametes of the avian malaria parasite P. gallinaceum. Probes for the two extranuclear DNA elements in malaria, the 6kb mitochondrial element and the 35kb plastid-like element, hybridised to the female gamete DNA but not to the male gamete DNA. This led to the conclusion that inheritance of extranuclear DNA is through the female gamete [Creasey, 1994], and implies that the majority of the hybrid oocysts in the 3D7xHB3 cross were of the 3D7female/HB3 male type.

Analysis of cross fertilisation and self fertilisation events in this cross, using nuclear gene alleles, was shown to be in accordance with random mating, falling within the Hardy-Weinberg equilibrium predictions [Ranford-Cartwright, 1993]. The existence of the bias in parental contribution to the hybrids revealed by the extranuclear markers in the present work indicate that simple random mating is not occurring in this cross. The possible implications of these results are discussed.

During the process of studying extranuclear DNA for this project three smaller investigations were stimulated. These were an investigation of the melanisation of P. falciparum parasites in the mosquito midgut, inhibition of an extranuclear gene using antisense oligodeoxynucleotides, and the construction of a phylogenetic tree using extranuclear gene sequences. Preliminary results of these three projects are presented.

Chapter 1 INTRODUCTION

The purpose of this study has been to investigate the pattern of inheritance of extranuclear DNA elements in malaria parasites. The work has involved the identification of polymorphic markers in the sequences of the extranuclear DNA of the human malaria parasite, Plasmodium falciparum, and the following of one of these markers through a parasite cross in mosquitoes. Some of the implications arising from these investigations have been examined.

The first part of this Introduction discusses general features of extranuclear DNA and the particular interest in studying their patterns of inheritance.

This is followed by a description of some of the physical characteristics of the malaria parasite, including its life cycle, its genomes, and particularly its two extranuclear genomes.

The final part of the Introduction describes aspects of the genetics of malaria parasites and outlines a strategy for following extranuclear DNA through a genetic cross between clones of Plasmodium falciparum.

1.1 Features of extranuclear DNA

In most organisms extranuclear DNA accounts for a relatively small proportion of the total DNA. However, it invariably performs crucial and specialised functions within the cell, especially within mitochondria, the centres of energy production (Tzagaloff and Meyers, 1986) and within plant chloroplasts, the centres of photosynthesis (Palmer, 1985). Extranuclear DNA is found also in naturally occurring plasmids which often confer important evolutionary advantages to the host cell (Beale and Knowles, 1978). Sometimes extranuclear DNA elements may contain important amplified genes (Beverley et al., 1984; Clark and Cross, 1987).

Extranuclear genomes, despite their inseparable relationship with their host cell, retain some features of their different origins. Most extranuclear DNA sequences have different mutation rates, codon usage and nucleotide bias to their respective nuclear genomes (Miyata et al., 1982; Moritz et al., 1987; Gray, 1989). Furthermore

extranuclear genomes usually possess their own replication mechanisms and employ their own polymerases and enzymes which usually differ from those of their nuclear counterparts (Sager, 1972; Fristrom and Clegg, 1988).

Features of extranuclear DNA have made it particularly useful in studies of the evolutionary history of organisms (Takahata and Slatkin, 1983; Horai et al., 1995; Laroche et al., 1995; Moore, 1995; Spicer, 1995). It often has higher overall mutation rates compared to those of nuclear DNA, but it also encodes a number of basic 'housekeeping' genes, whose sequences are closely conserved across a wide taxonomic range (Wilson, et al. 1985: Avise, 1994).

1.1.1 Inheritance of extranuclear DNA

In contrast to the typical Mendelian pattern of inheritance of nuclear DNA, the inheritance of extranuclear DNA displays a considerable variety of patterns ranging from strictly uniparental forms (through either the male or female line) to strictly biparental forms but including many modifed biparental forms often producing very unusual non-Mendelian ratios. Among eukaryotes, extranuclear DNA is commonly inherited uniparentally through the maternal parent (Grun, 1976; Gillham, 1978) but among prokaryotes and plants much more variation in pattern of inheritance has been observed (Yang et al., 1992; DeVerno et al., 1993).

The type of inheritance clearly influences the fate of the individual genes found in the extranuclear DNA. Uniparental inheritance, in general, results in the transmission of the extranuclear genome unchanged, except by mutation, from generation to generation. Bi-parental inheritance allows for the acquisition of mixed populations of extranuclear genomes in one organism, and the possibility of competition between them. During replication of mixed populations of extranuclear genomes there is the possibility of new forms of genes arising by recombination.

At the start of this project the inheritance pattern of the extranuclear DNA in malaria parasites was unknown.

1.2 The malaria parasite

The malaria parasite is the causative agent of what is still the most widespread vector-borne human disease in the world. A thorough knowledge of all aspects of the parasite's biology is thus an important goal in the efforts to control the disease. I hope to show in this thesis that the study of the inheritance of the extranuclear DNA of malaria parasites is of both academic and practical interest in this on-going process.

Malaria parasites are protozoa belonging to the phylum Apicomplexa. All members of this phylum are parasitic and capable of sexual as well as asexual reproduction. Malaria parasites are further classified in the sporozoite-producing class Sporozoea, sub-class Coccidia, order Eucoccidia and sub-order Haemosporina. All Haemosporina have two hosts in their life cycle. They produce sexual forms in the blood of a vertebrate host which are taken up by a blood-sucking insect where fertilisation and multiplication take place before the parasites are re-injected into the vertebrate host.

Within the Haemosporina, the family Plasmodiidae is represented by the single genus Plasmodium which includes all the species of malaria parasites. All Plasmodium species are characterised by cycles of asexual reproduction by schizogony, as well as by gametocyte production in a reptilian, avian or mammalian host. In mammalian malarias the sexual reproductive phase of the cycle occurs in mosquitoes of the genus Anopheles. In avian and reptilian forms this stage occurs in anopheline and some culicine species of mosquito and in the case of lizard malarias, also in sandflies (Kimsey, 1992).

1.2.1 The life-cycle in malaria parasites (with particular reference to P. falciparum)

The malaria parasite is haploid for the greater part of its life cycle. A diploid phase occurs briefly after fertilisation in the mosquito gut, but within a few hours meiotic division returns the parasites to a haploid state (Walliker et al., 1975; Sinden and Hartley, 1985) (Figure 1).

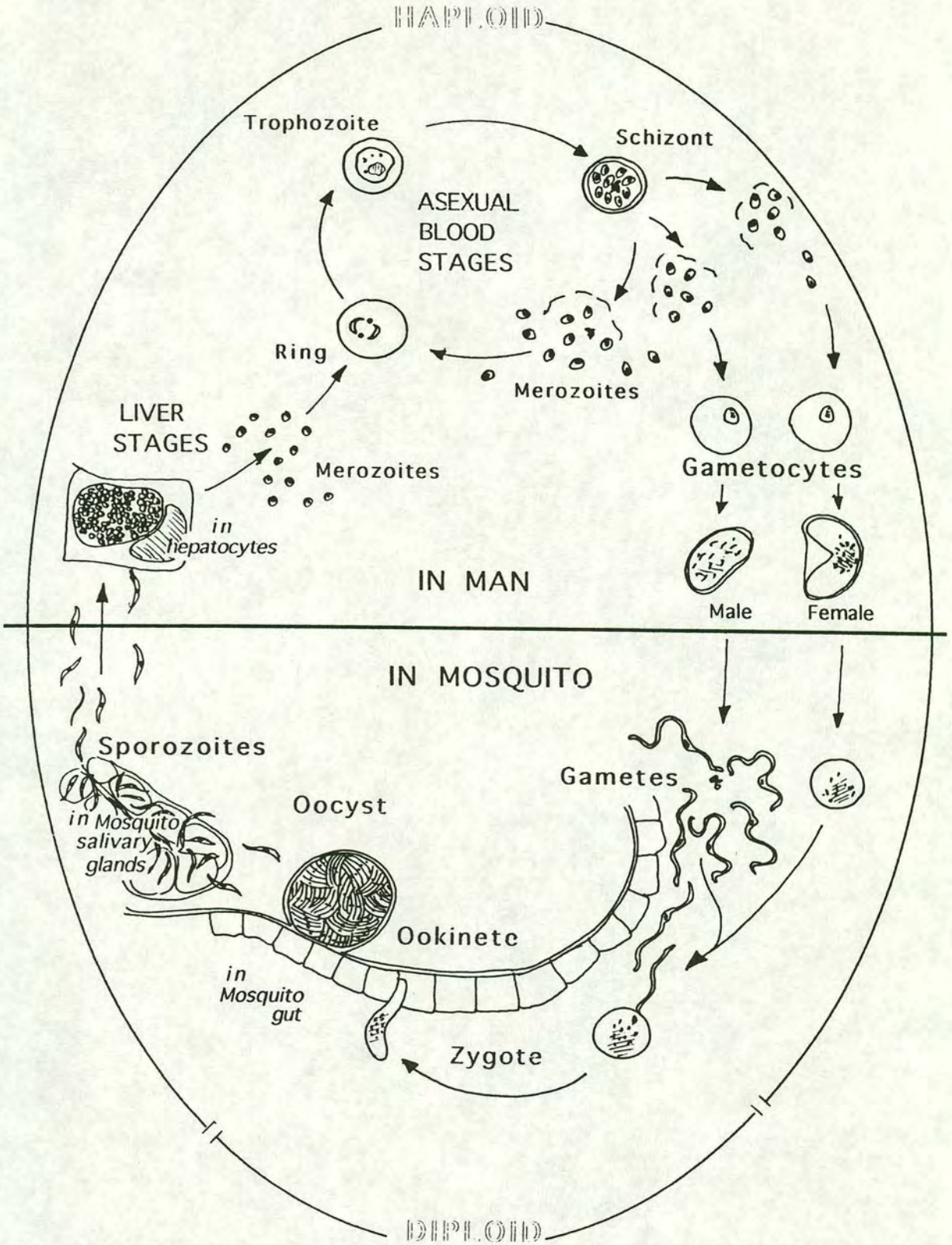
Figure 1. The malaria parasite life-cycle

Figure 1.

The malaria life cycle. The details and the morphology of the stages shown here are typical of Plasmodium falciparum, although the general form of the life cycle is common to all Plasmodium species.

Note that in the erythrocyte cycle, the schizont may proceed by one of three pathways : it may produce merozoites which re-invade unifected red blood cells (to repeat the asexual erythrocytic cycle again). or it may produce merozoites which develop into female gametocytes or merozoites which develp into male gametocytes. The commitment to one or other of these pathways occurs as early as the ring stage (Bruce et al., 1994). In a multiple infection, where a single red cell has been invaded by more than one merozoite, the resulting schizont will contain more than one population of merozoites each of which may be committed to a different pathway.

(Parasites not drawn to scale)


HAPLOID
Trophozoite
Schizont
ASEXUAL
BLOOD
STAGES
Ring
Merozoites
LIVER
STAGES
Merozoites
in
hepatocytes
Gametocytes
IN MAN
Male
Female
IN MOSQUITO
Sporozoites
Gametes
Oocyst
in Mosquito
salivary
glands
Ookinete
in
Mosquito
gut
Zygote
DIPLOID

a) Asexual stages

Sporozoites of P. falciparum injected by an anopheline mosquito into a human host migrate to the liver. They invade the hepatocytes, where each parasite undergoes schizogony to produce around 30 000 merozoites (Garnham, 1988). This part of the life-cycle is the exoerythrocytic stage. Five to six days after inoculation, the merozoites from the liver are released into the blood stream where they enter the erythrocytes. The parasite may then begin either the asexual erythrocytic cycle or the sexual cycle of multiplication. In the asexual cycle each merozoite within a red cell, develops through ring and trophozoite stages to the segmenting schizont which may contain up to 32 developing merozoites. When each schizont ruptures, the merozoites are released to invade uninfected red blood cells again. Within the human host the parasite population may proceed through any number of erythrocytic multiplication cycles (Garnham, 1988).

b) Sexual stages in erythrocytes

Within the human blood stream both exoerythrocytic and erythrocytic merozoites entering a red cell may develop into sexual forms, producing male or female gametocytes. This commitment to the asexual cycle or to gametocytogenesis has been demonstrated as early as the ring stage although the stimuli which cause the differentiation are complex and still not completely understood (Inselberg, 1983; Bruce et al., 1990). Similarly, nothing so far is known about the factors which determine the sex of an individual gametocyte although parasite clones grown from a single haploid parasite are capable of producing both male and female gametocytes. In P. falciparum clones grown in the laboratory, female gametocytes usually outnumber male gametocytes by about 5-10:1. (Ranford-Cartwright, 1992). The young gametocytes sequester in the deep circulation especially in the spleen and bone marrow (Garnham, 1935; Thomson and Robertson, 1935; Smalley et al., 1980), before emerging after 8-10 days into the peripheral circulation. Mature male and female gametocytes differ from each other in form, the male being cigar shaped with a larger nucleus and more diffuse distribution of pigment granules than the banana-shaped female. Both

gametocytes posses a single tubular branching mitochondrion loosely surrounding the nucleus (Sinden, 1982; Slomianny and Prensier, 1986; Kato et al., 1990).

c) Sexual stages in the mosquito

If mature male and female gametocytes are taken up by a mosquito, they emerge from their host red cells within the mosquito gut. The female becomes a single rounded macrogamete with a compact nucleus. The male undergoes an exflagellation process involving rapid mitotic division of the DNA into 6-8 genomes and the division of the cell into the same number of microgametes, arranged initially in a rosette pattern around a residual body (Carter and Graves, 1988). By electron microscopy each microgamete appears to consist of a nucleus, an axoneme and a highly motile tail all surrounded by a membrane (Aikawa, 1988). Within the mosquito gut the motile microgametes swim around energetically. The mechanism of finding a female gamete is not known, but fertilisation of the female gamete by the male takes place almost immediately. The male microgamete penetrates the macrogamete membrane and the nucleus and axoneme enter the cytoplasm leaving the membrane behind fused to the macrogamete membrane. The male nucleus fuses with the female nucleus to form a diploid zygote. The axoneme disintegrates (Carter and Miller, 1979).

The first stage of meiosis occurs within two and a half hours of zygote formation (Sinden and Hartley, 1985). The timing and the mechanism of extranuclear DNA replication through the sexual cycle is still largely unknown. During the next 18 hours, the zygote develops into a motile ookinete which then takes another 12 hours to travel through the mosquito gut wall to emerge as a young oocyst on the outer surface of the gut between the midgut basement membrane and the basal lamina, adjacent to the mosquito haemocoel. A considerable proportion of the ookinetes formed, however, never progress through the gut, so that in some species there are up to 60x less oocysts observed on the gut wall than zygotes formed within the gut (Vaughan, et al. 1994; Shahabuddin, et al. 1995). The reasons for this reduction are still unclear although refractory immune mechanisms of the mosquito host may play a major role in

this reduction. It would also be in the parasite's interest to maintain the gut infection level below that which would harm its host.

The exact timing of the second stage of meiosis is unknown, but by the young oocyst stage the haploid nuclei are undergoing many rounds of rapid mitotic division. The mature oocyst thus consists essentially of a bag of many thousands of haploid parasites each developing into a sporozoite. When the oocyst bursts, the sporozoites migrate to the salivary glands of the mosquito. These sporozoites are injected with the saliva when the mosquito next takes a blood meal from a human host.

1.3 The malaria parasite genomes

Early studies of malaria parasite DNA concerned the isolation and physical characterisation of total DNA, most of which was from the nucleus. However fractions of low density, around 1.677-1.679g/ml, which were distinct from the main bulk of nuclear DNA were also identified (Gutteridge et al., 1969; Chance et al., 1972). When this DNA was carefully liberated by osmotic lysis in P. lophurae, the duck malaria, it appeared as supercoiled molecules with a mean contour length of 10.3μm together with some smaller lengths thought to be broken or degraded fragments (Kilejian, 1975). Later in the simian malaria, P. knowlesi, covalently closed circles around 11.6μm in length showing a small cruciform structure were again identified (Williamson et al., 1985; Gardner et al., 1988). These circular molecules were assumed to represent an extranuclear mitochondrial genome, although later, they were shown to be a non-mitochondrial 35kb circular element with unexpected plastid-like features (Gardner et al., 1991; Wilson, et al., 1991).

Meanwhile molecular cloning techniques had identified another extranuclear DNA element in the rodent malaria, P. yoelii and in the avian malaria, P. gallinaceum. This appeared as fragments of 5.8 kilobases (kb) and 6.2kb respectively in parasite genomic libraries (Vaidya and Arasu, 1987; Joseph et al., 1989) and showed clear evidence of mitochondrial origin.

These early studies had thus established that the malaria parasite has three genomes one nuclear and two extranuclear (Wilson, et al., 1991) . The nuclear genome

consists of 14 chromosomes ranging in size between about 880 and 3 500 kb (Sinden and Strong, 1978; Prensier and Slomianny, 1986; Wellems et al., 1987; Kemp et al., 1987b). The two extranuclear genomes comprise a 6-kilobase (kb) linear mitochondrial element (Vaidya and Arasu, 1987; Aldritt et al., 1989; Joseph, et al., 1989; Vaidya et al., 1989; Vaidya, 1989; Feagin et al., 1991) and the plastid-like circular element of 35kb (Williamson, et al., 1985; Gardner, et al., 1988; Gardner, et al., 1991; Gardner et al., 1993)(Figure 2).

1.4 The nuclear genome

Aspects of the chromosome structure and organisation, as well as many of the genes of the nuclear genome have been well studied and are mentioned briefly below. This genome will not be discussed in detail in this study, except by comparison with the extranuclear genomes.

1.4.1 Organisation

The typical structure of the 14 nuclear chromosomes consists of a conserved central portion containing transcribed genes flanked on either side by domains composed of repetitive and non-transcribed sequences (Lanzer et al., 1994).

The length of homologous chromosomes varies between different clones of the same species and may even vary within a clone over time during in vitro culture (Kemp, 1985; Corcoran, et al., 1986). This may be due to translocations and to deletions, especially in the sub-telomeric regions (Lanzer, et al., 1994). The conventional naming of the chromosomes 1-14 consequently does not necessarily correspond to increasing size in every clone. The original numbers of the chromosomes were designated by reference to the first clone in which they were identified, namely in P. falciparum clone 3D7 of isolate NF54 (Figure 2).

1.4.2 Codon usage

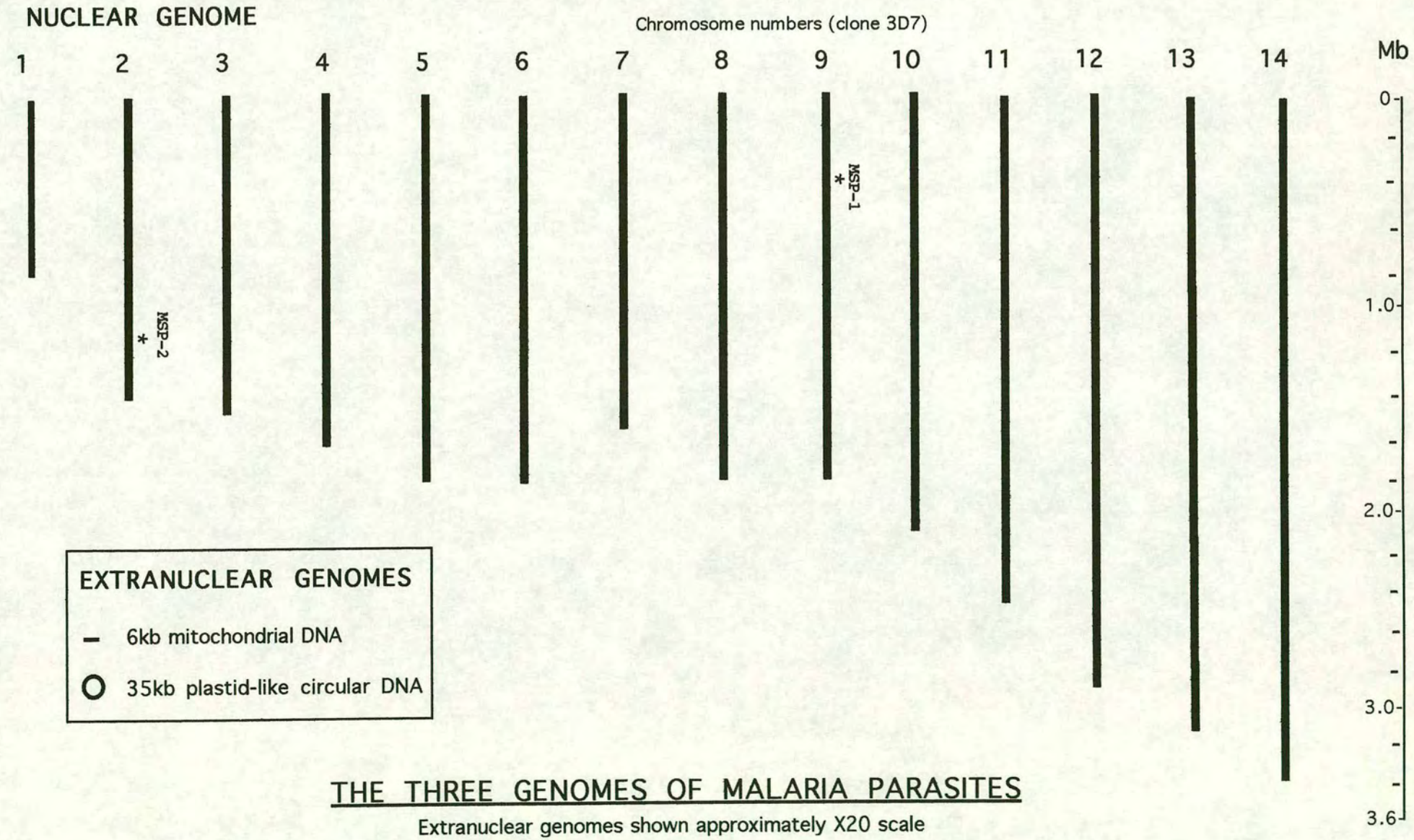
In most malaria species the nuclear genomes are rich in the nucleotide bases A+T. The P. falciparum nuclear genome, for example, is 81% A+T (Goman et al., 1982; Pollack et al., 1982). One of the consequences of such a high A+T content is that the codon bias is heavily skewed (Saul and Battistutta, 1988; Musto et al., 1995). Based

Figure 2. The three genomes of malaria parasites

Figure 2.

Diagrammatic representation of the three Plasmodium falciparum genomes showing the relative sizes of the 14 nuclear chromosomes and the two extrachromosomal genomes.

The positions of the nuclear genes MSP-1 and MSP-2 used in this project are indicated on chromosomes 9 and 2 respectively.
(adapted from Wellems et al. 1990)



THE THREE GENOMES OF MALARIA PARASITES

Extranuclear genomes shown approximately X20 scale

on an analysis of 175kb of coding sequence, no difference was detected in the A+T composition of the 'housekeeping' genes compared to antigen genes, indicating that this bias is a feature of the composition of the whole genome and not affected by function or level of expression (Musto, et al., 1995).

1.4.3 Repeat sequences

A striking feature of the malaria nuclear genome is the large number of repeat sequences found in many of the genes studied (Corcoran et al., 1986; Kemp et al., 1987). Most of the antigen genes whose sequences have been reported, include tandemly repeated sequences ranging in length from 3 base pairs (bp) to 243bp (Ravetch et al., 1984; Wahlgren et al., 1986; Wellems and Howard, 1986; Kemp, et al., 1987). Some of the genes have only one region of repeats while others may have two or three blocks of repeats. The total lengths of repeat regions may also vary from a few dozen nucleotides, in the CARP gene, for example (Wahlgren, et al., 1986) to about 13kb, in the pPF11.1 gene (Kahane et al., 1987).

Repeat sequences are also found in non-coding regions of the nuclear chromosomes. These include sequences named R-CG7, Rep 20, R-1A2. and R-FA3 (Patarapotikul and Langsley, 1988; deBruin et al., 1994). Deletion and re-arrangement of these sequences is thought to be a major source of the extensive chromosome polymorphism.

1.4.4 Variation of genes

Studies of genetic variation in human malaria parasites, especially in P. falciparum, have shown considerable allelic diversity in nuclear genes among natural infections. This is seen in the numerous alleles of genes encoding antigens (McBride et al., 1982; Conway and McBride, 1991), proteins (Fenton et al., 1985), and enzymes (Sanderson et al., 1981), as well as in the direct examination of sequences using molecular techniques (see review (Kemp et al., 1990)). Nuclear gene diversity has been most extensively studied among antigen genes where polymorphism might be expected (Weber, 1988). However polymorphism has also been reported for other types of nuclear genes including rRNA genes (Gunderson et al., 1987; Rogers et al., 1995).

The nuclear genome of Plasmodium species thus appears to tolerate a considerable degree of flexibility. The variations in repeat sequences the allelic sequence polymorphism and even the loss of certain genes appear to be accommodated quite easily without loss of viability (Patarapotikul and Langsley, 1988; deBruin, et al., 1994).

1.5 The extranuclear genomes

The extranuclear 6kb and 35kb elements have been identified in all malaria parasites examined to date. They display some interesting and unusual features which initially caused confusion about their identity.

The 35kb DNA element was at first thought to be the mitochondrial genome of the malaria parasite, since its size and circular structure is similar to mitochondrial genomes in some other organisms. The 6kb element was thought to be a mitochondrial satellite or episome, similar to those occurring in many fungi and higher plants (Rush and Misra, 1985). Much of the published work before 1991 which refers to the mitochondrial genome is actually describing the 35kb circle. This early uncertainty about the roles of the 6kb and 35kb elements has now been resolved.

The 6kb element has been identified as the mitochondrial genome of the parasite by its location in the mitochondrial fraction of the cell (Wilson et al., 1992) and by its possession of typical mitochondrial genes (Aldritt, et al., 1989; Vaidya, et al., 1989).

The 35kb element, despite having some sequences similar to mitochondrial RNAs (Gardner, et al., 1988), does not carry any typical mitochondrial features. Instead it shows certain prokaryote-like features and surprising similarities with chloroplast and plastid genomes. It has now become known as the 'malaria plastid-like DNA' (Gardner, et al., 1991).

1.6 The 6kb mitochondrial genome

The 6kb genome is so called because it consists of varying numbers of repeat sequences each being 6kb in length. In the literature it is most commonly referred to as the 6kb element : this term sometimes refers to a single 6kb sequence and sometimes to the total number of 6kb sequence copies within one cell.

The 6kb DNA element was first described in detail in P. yoelii where molecular clones containing the sequence were obtained by screening a genomic library with nick-translated total DNA (Vaidya and Arasu, 1987). It was later described in P. gallinaceum during a screen designed to find genes highly expressed in the zygote stage. Thirty-six out of 40 clones from a mung bean nuclease library screened with poly(A)- RNA isolated from purified zygotes were identified as being from the 6kb element (Joseph, et al., 1989). Subsequently the element was also described in detail in P. falciparum (Feagin, 1992a).

The 6kb element has been completely sequenced for all three species, P. yoelii, P. gallinaceum and P. falciparum, and homologous sequences of the element have also been shown to occur in P. chabaudi, P. berghei, P. knowlesi, P. lophurae and P. cynomolgi (Vaidya and Arasu, 1987; Joseph, et al., 1989; Wilson, et al., 1991). It appears to represent the entire mitochondrial genome of the parasite. As such, it is the smallest mitochondrial genome ever described for any organism, the next smallest, to date, being that of Ascaris suum which is 14.3kb long (Wolstenholme et al., 1987).

1.6.1 Organisation copy number and codon usage

The structure of the 6kb genome is mostly linear, and mainly arranged in directly-repeated head-to-tail arrays or concatemers (Vaidya and Arasu, 1987). The number of copies in each array appears to be quite small; probes of the 6kb element hybridised to undigested DNA show signals from 12-30kb (i.e. 2-5 copies), with the strongest hybridisation at 12kb indicating that the majority of sequences are in pairs (Joseph, et al., 1989). This tandem arrangement is not known to occur in any other mitochondrial DNA except in some of the 'petite' mutants of yeast which have a very defective mitochondrial genome (Faye, 1973; Nagley and Linnane, 1992).

A small proportion (1-2%) of the 6kb sequences, examined from asexual parasite DNA, appears to be present as circles (Williamson, 1992). These circles are thought to be a component of their replication mechanism (Preiser and al., 1995).

The number of copies of the 6kb sequence per cell varies among the three species examined. In P. yoelii approximately 150 copies of the sequence were detected by

comparing a probe of the 6kb sequence with a probe of a fragment of comparable size from a known single copy gene on Southern blot analysis of EcoR-I digested total P. yoelii DNA (Vaidya and Arasu, 1987). In this species, this represents almost 3% of the total DNA of the cell. P. gallinaceum and P. falciparum each contain around 20 copies per cell (Joseph, et al., 1989; Gardner et al., 1991) representing just under 0.5% of their total DNA.

Features of the 6kb sequences confirm their extranuclear location. They band at a greater density than nuclear DNA on caesium chloride gradients (Feagin et al., 1992b) and migrate ahead of nuclear DNA on pulsed-field gel gradients (Joseph, et al., 1989; Vaidya et al., 1993). The A+T richness of 68% in all three species of Plasmodium sequenced is lower than that recorded for their respective nuclear genomes. The genetic content of the element is very highly conserved between the three species being between 88-90% homologous at the nucleotide level (Feagin, 1992a).

1.6.2 Genes encoded by the 6kb sequence

The majority of the 6kb element consists of coding sequence, and specifies for three protein coding genes and at least 13 fragmentary rRNA genes (Suplick et al., 1988; Vaidya, et al., 1989; Feagin, 1992a) (Figure 3).

a) The protein coding genes

As shown in Figure 3, there are three open reading frames (ORFs) coding for cytochrome b (cytb) and subunits I and III of cytochrome oxidase (coxI and coxIII). The coxI and cytb genes lie on the same strand and are separated by less than 30 nucleotides. It is thought that they may be co-transcribed using the same promoter (Feagin, 1995b). The cox III gene is situated on the opposite DNA strand. All three proteins are components of the mitochondrial electron transport system.

i) Cytochrome b

Cytochrome b is one of the subunits of a large multimeric complex known as coenzyme QH_2-cytochrome c reductase and is thought to play a role in the haem-binding activity of the complex (Saraste, 1984; Widger et al., 1984).

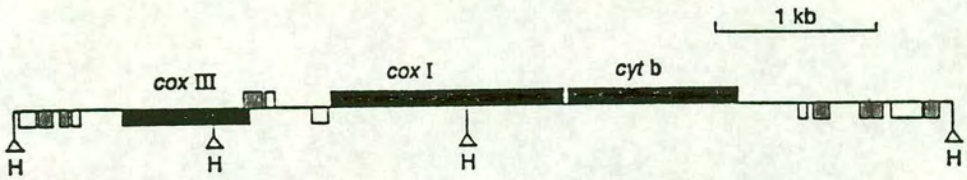


Figure 3. Schematic diagram of the 6kb mitochondrial DNA element of Plasmodium (as sequenced from Plasmodium falciparum). Genes shown above the line are transcribed left to right : those shown below the line are transcribed right to left. Small subunit (ssu) rRNA fragments are left white, large subunit (lsu) rRNA fragments are coloured grey, and protein coding genes black.

H = HindIII site.

In P. falciparum the predicted protein is 376 amino acids long and contains histidines which are in the same positions as those of cytochrome b in other organisms (Nobrega and Tzagaloff, 1980; Anderson et al., 1981; Waring et al., 1981).

However the amino acids around the histidines in P. falciparum differ from those in other organisms. This may be a significant factor in the differences between Plasmodium and mammalian species in their relative resistance to antibiotics such as myxothiozol and antimicin. Perhaps more significantly, they may play a part in the greater affinity of hydroxynapthoquinone and 8-amino-quinoline drugs to the Plasmodium cytochrome b bc_1 complex compared to that of its human host (Vaidya et al., 1993), (see also Section 1.9.1 a) and (Appendix II).

Sequence conservation of the cytochrome b gene between the Plasmodium species studied is extremely high, being 97% between P. falciparum and P. yoelii and 94% between P. falciparum and P. gallinaceum at the nucleotide level. It shares 43% amino acid similarity with the human cytochrome b (or up to 61% if conservative replacements are included) (Anderson et al., 1981; Feagin, 1992a).

ii) Cytochrome oxidase subunit I (cox I)

Cox I in P. falciparum is the most similar of the three protein coding genes to those of other organisms and its sequence shows 73% similarity to the corresponding human gene (Feagin, 1994). As with the cytochrome b, gene the histidine residues thought to be involved in the haem-binding function of the protein are invariant in the Plasmodium species examined and found in the expected locations within the gene.

iii) Cytochrome oxidase III (cox III)

In comparison to the cox I gene, the cox III gene in P. falciparum is less like the cox III of other organisms (showing only 31% similarity with human cox III at the amino acid level, for example) although still more than 95% conserved among the various Plasmodium species. In other organisms cox III is believed to be involved in the proton translocation function. A glutamic acid residue found in the protein binds to dicyclohexylcarbodimide, a compound that inhibits proton pumping. This residue is conserved in all Plasmodium cox III genes examined (Feagin, 1994).

b) Ribosomal RNA genes

At least 13 fragmentary ribosomal RNA genes, 30-200 nucleotides in length, have been identified on the 6kb element. Six of these show similarity to small subunit rRNAs (SSU rRNAs) and seven to large subunit rRNAs (LSU rRNAs). All were initially identified by 'homology'[1] searches and by secondary structure (Suplick, Akella et al., 1988; Aldritt, et al., 1989; Joseph, Aldritt et al., 1989; Vaidya, et al., 1989; Feagin, et al., 1992b). These rRNAs are only about one third of the size of the typical eubacterial rRNAs from which mitochondrial rRNAs are thought to have evolved (Iwabe et al., 1991). They are encoded out of order and are very fragmented. In this respect they are similar to the scrambled ribosomal RNA gene sequences described in Chlamydomonas reinhardtii (Boer and Gray, 1988). Despite this fragmentation there is evidence that in Plasmodium these genes are functional (Feagin, et al., 1992b). Recent work has identified several more gene fragments which have not yet been fully characterised (Feagin - personal communication).

1.6.3 Replication

Studies of the replication of the 6kb element are still in their early stages. Using P. falciparum DNA, a neutral/neutral 2-dimensional electrophoresis technique and electron microscopy has been applied at different stages of the erythrocytic cycle (Preiser et al., 1995). This study suggests that replication of these molecules involves massive and sometimes multiple recombination between all the linear copies in the cell in a system similar to that employed by the T4 bacteriophage (Mosig, 1987; Williamson, 1995). This system involves initial replication from a primary origin usually at the 3' end of the genome. The terminal 3' overhangs thus created invade other molecules at homologous sites and synthesis of DNA begins by addition of nucleotides onto the 3'ends of the invading strands. Thereafter a variety of replicative

[1]The terms 'homology' and 'similarity' in this thesis are generally used strictly according to the definitions suggested by Reeck et al. (Reeck et al., 1987). The term homology is used to imply a concept of quality and has the precise meaning of 'having a common evolutionary origin'. Similarity is used as a quantitative term or value when comparing two or more sequences and can be expressed as a percentage score or probability. The term homology as used in the papers referred to above would thus strictly be called similarity according to these definitions).

activities may take place to create the new complete double-stranded molecules. Other more complicated recombinational interactions between the replicating molecules are also possible (Mosig, 1983). The small numbers of circular molecules are thought to be products of this process and may undergo a rolling circle replicative mechanism overlaying the whole system.

1.6.4 Transcription

A variety of transcripts ranging in size from 300bp to 5kb has been reported from the 6kb element (Vaidya and Arasu, 1987; Aldritt, et al., 1989; Joseph, et al., 1989; Suplick et al., 1990; Feagin, et al., 1992b; Vaidya, et al., 1993). The transcripts for the protein coding genes are similar in size to their corresponding open reading frames suggesting that any untranslated regions or poly(A) tails must be very small. Other transcripts are much more variable in size and are thought to correspond to the fragments of rRNA. The larger transcripts may be precursor RNAs which require further processing (Vaidya, et al., 1993). Recent work shows that the abundance of organelle transcripts increases as the parasite develops from the ring stages to trophozoites and schizonts (Feagin and Drew, 1995a). Other recent reports indicate that there is co-transcription of the rRNA genes with the protein coding genes (Feagin, 1995b).

1.6.5 Cellular location of the 6kb genome within the cell

The 6kb element is assumed to lie within the malaria parasite mitochondrion although this has not yet been confirmed by in situ hybridisation. Sub-cellular fractionation experiments in P. yoelii demonstrated that the 6kb DNA (though not the 35kb DNA), separates with the mitochondrial fraction (Wilson, et al., 1992).

a) Mitochondria of malaria parasites

i) Structure

Early studies using electron microscopy revealed a varied array of organelles which were thought to be mitochondria. Some of these structures may have been artifacts of the technique used: for example, a branching structure sectioned in one plane may show a number of discrete objects that are actually part of the same organelle. Fragile

cristae, which in many species are not well-developed in the earlier stages of the parasite, may be damaged by sectioning procedures (Howells and Fullard, 1970b). However a review of the many studies carried out using different techniques and different species of Plasmodium make certain aspects clear.

Firstly, mitochondria from different species of malaria parasites have different morphology (Howells, 1970a; Howells and Fullard, 1970b; Langreth and Trager, 1973; Howells and Maxwell, 1973b; Aikawa, 1988). In a number of studies the same authors have examined the same parasite stage of different species under identical conditions thus confirming that these differences are real (Fry and Beesley, 1991).

Secondly, within each species the parasite mitochondrion undergoes changes in morphology through the various stages of its life-cycle (Ladda et al., 1966; Aikawa et al., 1969; Smith and Theakston, 1970; Langreth et al., 1978; Sinden and Strong, 1978; Aikawa, 1988). Within the different species and within the different stages of each species the size and complexity of the mitochondrion may reflect the degree of reliance on a functional electron transport system.

In P. falciparum, a careful three-dimensional modeling technique using serial electron micrograph sections of the trophozoite stages (Slomianny and Prensier, 1986) gives a clear picture of a single mitochondrion consisting of long tube-like branching structure loosely surrounding the nucleus. This becomes larger and more complex as the trophozoite ages. Vital staining with the cationic stain Rhodamine 123 in P. falciparum again shows a small tube-like structure in the ring stage which gradually develops branches and connections in the trophozoite stages and divides into each segmenter at the schizont stage, each merozoite showing a single structure. In the gametocyte Rhodamine 123 staining shows an intricate net or lattice-like structure surrounding the nucleus (Divo, et al., 1985; Kato, et al., 1990; personal observations) (Figure 4). More recently stains such as DiOC6 {3,3' dihexyloxacabo-cyanine} and JC-1 {5.5.6,6'-tetraethylbenzimidazolyl-carbocyanine iodide} give an even clearer view of this mitochondrial structure in P. falciparum parasites (Srivastava and Vaidya, 1995).

A. Position and structure of the single branched mitochondrion in a Plasmodium falciparum trophozoite. The parasite membrane and nuclear membrane are given in outline. (Drawn from 3-dimensioal reconstruction using electron micrographs of serial sections after Slomianny and Prensier 1986)

Rings

Trophozoites

Schizonts

B. The mitochondrion of erythrocytic stages of Plasmodium falciparum parasites stained with Rhodamine 123 and viewed by epifluorescence microscopy. The photographs show a small tube-like structure in the ring stages which beomes branched and net-like in the trophozoites and early schizonts and segregates into each segmenter in the mature schizonts. (after Divo et al., 1985)

Figure 4. The structure of the Plasmodium falciparum mitochondrion

i) Function - pyrimidine biosynthesis

Malaria parasites synthesise their own pyrimidines de novo, and there is evidence that mitochondria play an important part in this process (Gero, et al., 1984; Aldritt, et al., 1989; Feagin, 1994). Early work by Gutteridge et al. (Gutteridge et al., 1979) suggested that the electrons produced by the reduction of dihydroorotate to orotate are transferred via ubiquinone (or coenzyme Q) to the electron transport chain. The enzyme that catalyses this step, dihydroorotate dehydrogenase, is located in the mitochondrion in mammals and has been demonstrated in the particulate fraction of parasite lysate of P. berghei, indicating that it is probably mitochondrial in Plasmodium as well (Krungkrai et al., 1991). Indirect support for this argument is provided by the demonstration of increased mitochondrial transcription during the late trophozoite and schizont stages when pyrimidines would be most needed (Feagin and Drew, 1995a).

ii) Function - electron transport

In many Plasmodium species, intraerythrocytic parasites appear to derive most of their energy by metabolizing glucose to lactic acid via a conventional pathway of anaerobic glycolysis. Evidence for further processing of the products of glycolysis through the citric acid cycle and the electron transport chain to aerobic respiration is much less clear.

Biochemical studies: Early work in malaria parasites failed to find evidence of a fully functioning Krebs cycle in the erythrocytic stages of the life-cycle (Sherman, 1979). Subsequently, at least a portion of the electron transport system has been shown to be functional, and cytochrome oxidase activity detected (Scheibel, 1988). In the mosquito and liver stages there is evidence of more aerobic activity than in the erythrocytic stages (Howells and Maxwell, 1973a; Sherman, 1979; Scheibel, 1988).

Recent biochemical studies have found that the mitochondrial phosphate carrier (MPC) produces a message 2kb in size by Northern blot analysis at similar levels in both asexual and sexual stages of P. falciparum and is able to carry out oxidative phosphorylation continuously (Bhaduri-McIntosh and Vaidya, 1996).

In studying respiratory biochemistry there are technical difficulties in measuring the activity of an intracellular parasite. It is also difficult to compare results obtained by investigators using different techniques and different parasites at different stages of development.

Histocytochemical studies: Less prone to these problems are the histocytochemical studies which have identified cytochrome oxidase activity in most of the Plasmodium species studied (Howells, 1970a; Howells and Maxwell, 1973b) and cytochrome a-, b-, and c-, in both P. yoelii and P. falciparum (Fry and Beesley, 1991).

Mitochondrial inhibitors: Another way of determining the importance of the mitochondrial respiratory pathways in the parasite is to examine the effects of mitochondrial inhibitors on the live organism. Many investigators have shown that inhibitors of mitochondrial protein synthesis and the electron transport chain have antimalarial effects in vitro (Ginsburg et al., 1966; Thurnham et al., 1971 ; Geary et al., 1986; Fry et al., 1990). Studies in P. yoelii and P. falciparum erythrocytic stages (mainly trophozoites) using a variety of mitochondrial electron transport inhibitors indicate that oxidation of alpha-glycerophosphate and succinate proceed through a 'classical' respiratory chain (Fry and Beesley, 1991). However the concentrations at which these inhibitors are effective are, in most cases, much higher than those needed to inhibit mammalian mitochondria (Thierbach and Reichenbach, 1981), indicating that the parasite electron transport chain must be considerably different from its mammalian equivalent.

The respiratory pathway of the malaria parasite is an attractive target for the construction of antiparasitic drugs, especially where this pathway differs from that of human cells. Recent work on the respiratory coenzyme, coenzyme Q has demonstrated that the parasite utilises the Q_8 form of the coenzyme and not the Q_{10} form which is used in human respiration (Ellis, 1994).

1.6.6 Evolution of the mitochondrion

The mitochondrial genetic system is thought to have arisen by endosymbiosis of primitive archaebacteria within the evolving eukaryotic cell. The mitochondrion remains semi-autonomous in that it retains a distinctive genome that is replicated and expressed but it is incapable of independent existence. This implies that during evolution there was a transfer to the nucleus and/or loss of genetic information from the endosymbiont genome. The malaria mitochondrial genome is one of the most extreme examples of this reduction: many genes which are usually found in mitochondrial DNA in other organisms are absent (their functions presumably having been taken over by the nuclear genome), and even the genes that have been retained appear to have been reduced to the lowest functional unit.

1.7 The 35kb circular genome

The 35kb circular DNA was first described for the duck malaria parasite, Plasmodium lophurae (Kilejian, 1975). Similar molecules were later found in P. berghei and P. knowlesi (Dore et al., 1983) and in P. falciparum (Gardner, et al., 1988). At the time of writing the 35kb circle in P. falciparum has been cloned and completely sequenced (R.J.M. Wilson, D.Williamson - persona; communication) and parts of this sequence are available on gene databases.

1.7.1 Organisation codon usage and copy number

The most striking organisational feature of the 35kb circle is a large inverted repeat sequence occupying about a third of the molecule. Each copy of the repeat encodes one small subunit (SSU) rRNA and one large subunit (LSU) rRNA transcribed in opposite directions (Gardner, et al., 1991) as shown in Figure 5. The repeats explain the cruciform appearance of the molecule and also the fact that when denatured and allowed to snap back the molecule migrates much faster than its full size would predict (Feagin, 1994). A similar inverted repeat pattern is also found in many chloroplast genomes. The 35kb circle also carries an RNA polymerase, another feature shared with many chloroplast genomes (Gardner, et al., 1991; Birky, 1994).

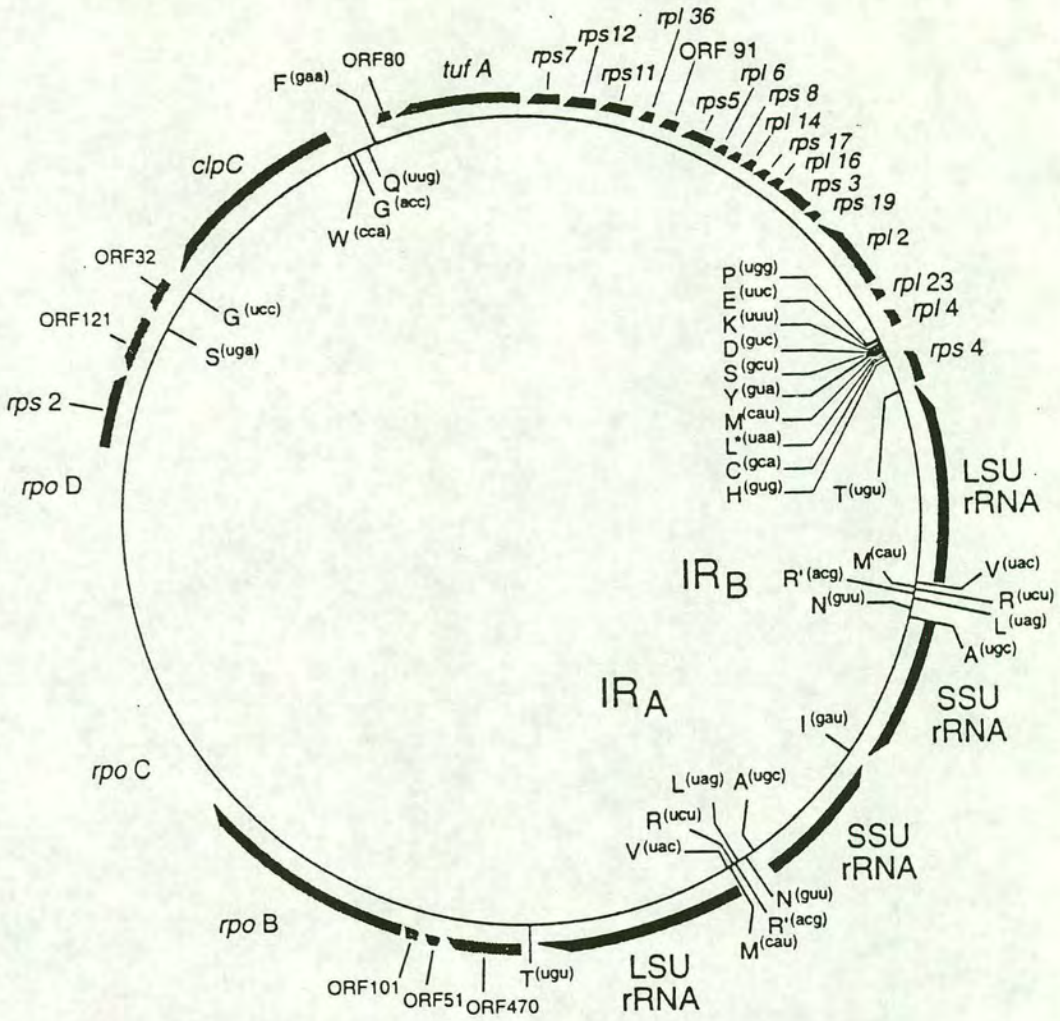


Figure 5. Schematic diagram of the 35kb circular DNA element of Plasmodium (as sequenced from Plasmodium falciparum clone BWC10). The inverted repeat sequences are labelled IR_A and IR_B. Genes are arrowed in the direction of transcription. tRNA genes are identified by their single letter code with the anticodon sequence given in brackets. Those transcribed clockwise are written outside the circle, while those transcribed anti-clockwise are shown inside the circle.

(This diagram includes unpublished sequence from Wilson and colleagues, N.I.M.R. Mill Hill London). Adapted from Wilson et al 1995.

In P. falciparum the density of the 35kb DNA on caesium chloride gradients is slightly less than that of nuclear DNA, indicating that it is more A+T rich than the nuclear genome - probably around 89%-90% (Williamson, et al., 1985; Gardner, et al., 1988). Each ring-stage cell appears to have between 1-3 copies of the element as assessed by dot-blot analysis (Feagin, 1994).

1.7.2 Genes encoded by the 35kb DNA element

The 35kb DNA encodes around 60 genes, most of them concerned with gene expression including rRNAs, tRNAs, several ribosomal proteins and the subunits of the RNA polymerase (Figure 5).

a) RNA Polymerase

Three of the protein coding genes identified on the 35kb circle show similarity to subunits of an RNA polymerase (Gardner et al., 1991a).. These resemble the rpoB rpoC and rpoD genes of E.coli (Ovchinnikov et al., 1981; Ovchinnikov et al., 1982).

b) Other protein coding genes

Several other open reading frames (ORFs) show similarity to ribosomal protein genes. One of these (ORF470), shares 50% similarity with a partially sequenced ORF of the red alga Antithamnion (Maid and Zetsche, 1991; Williamson et al., 1994; Wilson et al., 1994).

c) Ribosomal genes

Unlike the fragmented rRNA genes of the 6kb element, the RNAs on the 35kb element are encoded as continuous sequences. They are similar in size to eubacterial RNAs and appear to have secondary structures quite similar to those of E. coli. Regions within the rRNAs which tend to be conserved in other organisms are also conserved in malaria parasites (Gardner, et al., 1988; 1991b and 1993). Twenty-five tRNAs identified by sequence analysis are all transcribed and are sufficient to decode all the protein genes present on the circle (Preiser and al., 1995).

1.7.3 Replication

Very little is known at present about the replication process employed by the 35kb circle. Electron microscopy in P. knowlesi has shown a few molecules with fully

double-stranded replication bubbles (Williamson, et al., 1985). This may indicate that replication takes place bi-directionally from a single site.

1.7.4 Cellular location

The physical location of the 35kb genome within the Plasmodium cell has not been established, although in situ hybridisation studies are in progress (R.J.M. Wilson - personal communication). A number of workers have speculated that this DNA may be located in the so-called spherical body, also known as the Golgi-like apparatus or hohlzylinder in malaria parasites (Kilejian, 1991; Wilson, et al., 1991; Siddal, 1992). Recently, this has been shown to be the location of a similar genome in Toxoplasma gondii (McFadden, et al., 1996).

1.7.5 Evolution of the 35kb circle

Phylogenetic analysis of nuclear-encoded SSU rRNA sequences suggests that Apicompexa are monophyletic and distantly related to the ciliates and dinoflagellates (Barta et al., 1991). Both groups of organisms have a tendency to incorporate other cells as secondary endosymbionts. It has therefore been suggested that a dinoflagellate or related ancestor of the Apicomplexa may have acquired a plastid genome in this way in their evolution, and that the 35kb element is its reduced remnant (Wilson, et al., 1994).

Unpublished reports on the entire gene sequence of the 35kb element indicate that the majority of its genes specify components required for gene expression but that it lacks all genes required for photosynthesis (R.J.M. Wilson - personal communication). This supports earlier suggestions that it bears superficial resemblance to the plastid genomes of the achlorophyllous plant Epifagus (dePamphilis and Palmer, 1990), the non-photosynthetic euglenoids (Howe, 1992), and the red alga, Antithamnion (Williamson, et al., 1994; Wilson, et al., 1994). [In this context it is surprising to note that even a nuclear gene, the enolase gene, in P. falciparum shows some plant-like features (Read et al., 1994)].

In phylogenetic analyses the unusually high A+T content of the 35kb genome tends to complicate interpretation of relatedness. The similarity shown to the red alga

Antithamnion has recently been challenged by workers taking advantage of the LogDet transformation programme (Lockhart et al., 1994), which minimises nucleotide bias (Egea and Lang-Unnasch, 1995). Using the available Plasmodium sequences together with sequences from the organellar ribosomal rRNA genes of Toxoplasma gondii and Babesia bovis, this new analysis suggests that the Apicomplexan plastid-like DNAs is more similar to the plastid DNAs of euglenoids than to those of the rhodophytes (Figure 6).

1.8 Extranuclear elements in other Apicomplexa

Both the 6kb element and the 35kb circle in Plasmodium species show close homology to similar sized elements in other Apicomplexa although none of these organisms have been studied as extensively as the malaria parasites.

Sequences from the Plasmodium 6kb extranuclear genome cross-hybridise with DNA from Toxoplasma gondii, Babesia microti and Theileria parva (Joseph, et al., 1989) T. annulata (Megson et al., 1991) and Babesia bovis (Jasmer et al., 1990).

In Theileria species this DNA has been identified as linear mitochondrial elements of 7.1kb in T.parva (Kairo et al., 1994) and 6.3kb in T. annulata (Hall et al., 1990; Megson, et al., 1991). These code for the same three protein genes found in malaria species, and the mitochondrial rRNA genes in Theileria like those of Plasmodium are extremely fragmented. At least three of these rRNAs correspond closely to four Plasmodium fragments. Most of the sequence of the T. parva element has now been published (Kairo, et al., 1994).

A molecule similar to the 35kb circle has been found in Toxoplasma gondii (Borst et al., 1984; Egea and Lang-Unnasch, 1995) and in Babesia bovis (Gozar and Bagnara, 1993 and 1995). suggestsing that this molecule was present in the ancestral progenitor of the Apicomplexa and probably essential to the survival of the members of this phylum.

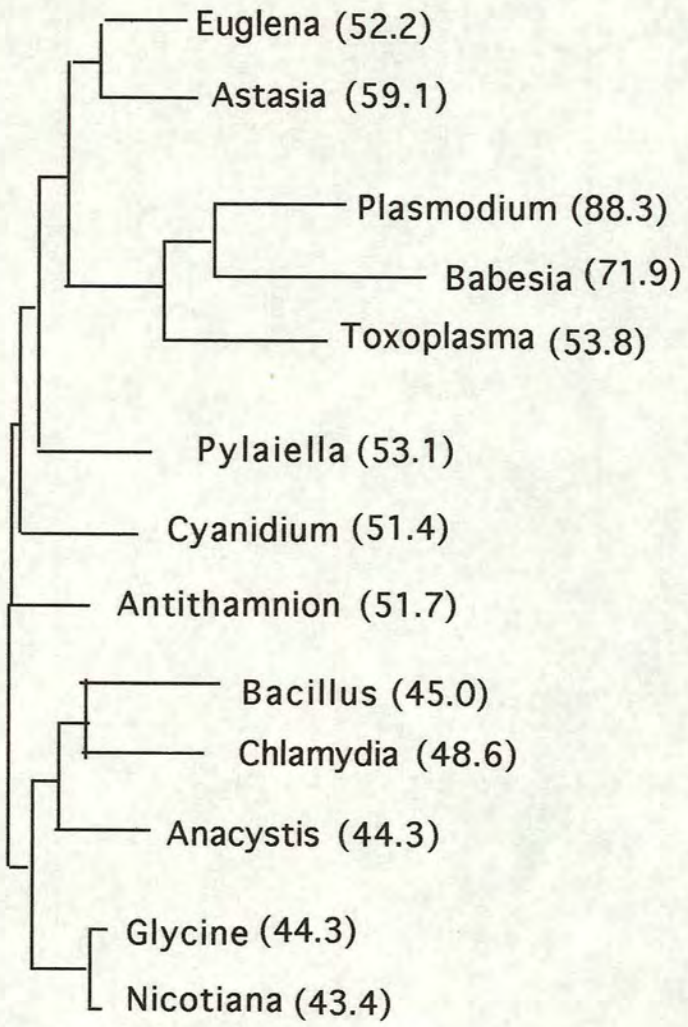


Figure 6. A phenogram showing the position of Plasmodium 35kb circle sequences in relation to some other bacterial, plastid and organellar DNA sequences as determined by the LogDet transformation followed by neighbour-joining techniques. The nucleotide bias in % A+T of each sequence is indicated in brackets after the genus name. (Adapted from Egea and Lang-Unnasch, 1995)

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Anacystis nidulans (Tomoioka & Sugiura, 1983), Astasia longa (Siemeister & Hachtel,1990), Antithamnion sp. (Maid & Zetsche, 1991), Babesia bovis (Egea & Lang-Unnasch, 1995), Bacillus subtillis (Ash, 1991), Chlamydia psittaci (Weisburg et al., 1986), Cyanidium caldarium (Maid & Zetsche,1991), Euglena gracilis (Roux et al.,1983), Glycine max (VonAllmen & Stutz 1988), Nicotiana tabacum (Tohdoh & Sugiura,1982), Plasmodium sp. (Gardner et al.,1991), Pylaiella littoralis (Markowitcz et al.,1988), Toxoplasma gondii (Egea & Lang-Unnasch, 1995).

1.9 Effect of antimalarial drugs on extranuclear elements

1.9.1 Effect of drugs on mitochondria

Several drugs presently used in antimalarial therapy are known to act principally on the mitochondria.

a) 8-aminoquinolines and napthoquinones

8-aminoquinolines (the most common of which is primaquine) and the quinone-quinolone derivatives, such as menoctone, appear to cause swelling and thickening of mitochondria as well as duplication of mitochondrial membranes in trophozoites. This has been clearly demonstrated in experimental studies in P. fallax (Beadouin and Aikawa, 1968; Aikawa and Beadouin, 1970), P.yoelii (Boulard et al., 1983) and P. berghei (Howells et al., 1970b). Lines of P. berghei which were selected for resistance to primaquine were described as having synthesised new mitochondria apparently to compensate for the action of the drug (Warhurst, 1984). The presence of a large and well-developed mitochondrial structure in the gametocyte may explain why primaquine is one of the most effective drugs for the clearance of gametocytes in the treatment of human malarias (Lanners, 1991).

More recently, another hydroxynapthoquinone, Atovaquone, (Wellcome 566C80) has shown very potent inhibition of Plasmodium respiration (Davies et al., 1993; Fowler et al., 1994; Fowler et al., 1995), its site of action being the cytochrome bc_1 complex (Fry and Pudney, 1992) (and Appendix II). The distinctive secondary structure of the haem-binding sites reported in the P. falciparum cytochrome b (Vaidya, et al., 1993) and (see Section 1.6.2.a) i)) may account for the deleterious effect of the 8-aminoquinolines and napthoquinones and other coenzyme Q homologues on the parasite while the mitochondrial function of the host remains unaffected.

Unfortunately from the clinical point of view use of Atovaquone has been shown both in vitro and in vivo to result in rapid development of resistance (Haille and Flaherty, 1993; Olliaro and Trigg, 1995). A recent study in Thailand using pre- and post-treatment parasites from Atovaquone-treated patients identified two distinct forms of resistance to Atovaquone (Wilson et al., 1996). Some recrudescent parasites showed

IC_{50} levels from 35-200-fold greater than those of the pre-treatment parasites and were termed low-level resistant parasites. Other recrudescent parasites showed IC_{50} levels 5 000-30 000-fold greater than their respective pre-treatment forms and were termed high-level resistant parasites. The authors claim that the high-level resistance, (though not the low-level), is associated with a single point mutation in the cytochrome b gene near the region associated with myxothiozol resistance in yeast. The high level resistant isolates also had a decreased sensitivity to myxothiozol but neither group had altered sensitivity to antimycin A.

b) Qinghaosu (artemesinin)

Qinghaosu, a crystalline extract of the herb qinghao (Artemesia annua), has been used for over 2000 years in China for the treatment of malaria. (Jiang, et al., 1982). Its low toxicity, rapid action and effectiveness against both P. vivax and P. falciparum make it a very attractive drug, especially since it has been shown to be effective against P. falciparum that is resistant to chloroquine pyrimethamine and pyrimethamine/ sulfadoxine (Thaithong and Beale, 1985: Chawira and Warhurst, 1987).

In studies of P. inui from splenectomised monkeys (Macaca assamensis) treated with 50-200mg/kg of sodium qinghaosu, mitochondria showed marked disruption in all blood stages two hours after treatment. Degenerative morphological changes to other parts of the cell did not appear until 6-8 hours after treatment. However in treated parasites only about 30% of the mitochondria observed exhibited these changes (Jiang et al., 1985). Similar changes were reported in P. berghei and P. falciparum following artemesinin treatment (Ellis et al., 1985). In P. falciparum, radio-labeled artemesinin was concentrated almost exclusively in 70% of the food vacuoles and 40% of the mitochondria 1-4 hours post-treatment (Maeno et al., 1993). Significantly no morphological changes were seen in the host cells or their mitochondria after treatment.

At least two modes of action are proposed for artemisinin. The morphological effects are consistent with observations that it reacts specifically with intraparasitic haeme (Meshnick et al., 1991). Malaria parasites are also at risk from oxidative damage

(Golenser et al., 1991). Qinghaosu is a sesquiterpene lactone containing a peroxide group which may produce the membranous changes observed in the parasite. (WHO Scientific Working Group on Chemotherapy of Malaria 1981 unpublished document; The development of Qinghaosu and its derivatives as malaria drugs. TDR/CHEMAL-SWG(4)/(QHS)/81.31981).

c) Antibiotics

A number of antibiotics that act principally on mitochondrial protein synthesis and membrane-bound oxidases are also effective antimalarials. Antibiotics such as erythromycin, tetracycline, chloramphenicol, clindamycin, rifampicin, penicillin and streptomycin all of which act on mitochondria in other organisms act with varying levels of inhibition in malaria parasites in vitro (Geary and Jensen, 1983b; Kiatffuengfoo et al., 1989). Tetracyclines in particular have been shown to be effective against the primary exoerythrocytic forms of P. falciparum and are thought to be curative for up to 4 days after inoculation with sporozoites.

In clinical use antibiotics have generally been found to act rather slowly making them unsuitable for treatment on their own. However, when combined with other quick-acting antimalarials they are very useful for treatment of malaria, especially in areas, such as Thailand, where multi-drug-resistance is common. Clindimycin, one of the most potent antimalarial antibiotics is limited because of its adverse side-effects on the gastrointestinal tract (Bruce-Chwatt, 1986).

1.9.2 Effect of drugs on genes of the 35 kb element

The presence of a prokaryotic-type polymerase on the 35kb circle raises the question of whether antibiotics which are effective specifically in the inhibition of these genes are acting at this location in malaria parasites. Mutations of the ß subunit of the rpoB gene in E. coli are known to confer resistance to rifampicin.

In malaria parasites rifampicin has a definite antimalarial effect (Geary and Jensen, 1983b; Pukrittayakamee et al., 1994) although it is not as potent as some other inhibitors of bacteria-like RNA polymerases (Strath et al., 1993). However the mutations responsible for rifampicin-resistance in the bacteria polymerases were not

found in rifampicin-resistant lines of P. falciparum (Lang-Unasch, 1994). The antimalarial action of this antibiotic must therefore rest, at least partly, in other mechanisms.

The possible use of drugs targeted against the 6kb and 35kb DNA makes it important to have a good understanding of the genetic characteristics of these elements, including knowledge of how they are inherited in natural populations.

1.10 The genetics of the malaria parasite

The main requirements for genetic analysis of parasites are;-

1. A knowledge of the DNA replication process in the parasite's life cycle.
2. Genetically pure lines or clones which differ from each other in some clearly defined and quantitatively measurable characters .
3. Methods for making crosses between two clones.
4. Methods for analysing the progeny of such crosses.

1.10.1 DNA replication in the malaria life-cycle.

As described in Section 1.1.1 the malaria parasite is haploid for the greater part of its life cycle, becoming diploid only briefly after fertilisation. Fertilisation is followed rapidly by meiosis. However the various stages of the life cycle individually vary considerably in their DNA content according to the processes of mitotic division, position in the cycle relative to actual genome replication and, possibly, gene amplification in certain stages (Figure 7). This process has been studied by a number of methods including buoyant density measurements and DNA melting temperatures (Tm points) carried out in P. falciparum (Pollack, et al., 1982), detailed electron microscopy carried out in P. berghei (Sinden and Hartley, 1985), by radio-labeling of DNA synthesis precursors also carried out in P. berghei (Janse et al., 1986), by flow-cytometry and microfluorometry (Janse. et al., 1987) and by H.P.M.P.A (S)-9-(3-hydroxy-2-phosphonyl-methoxypropyl) adenine arrest (Smeijsters, 1994).

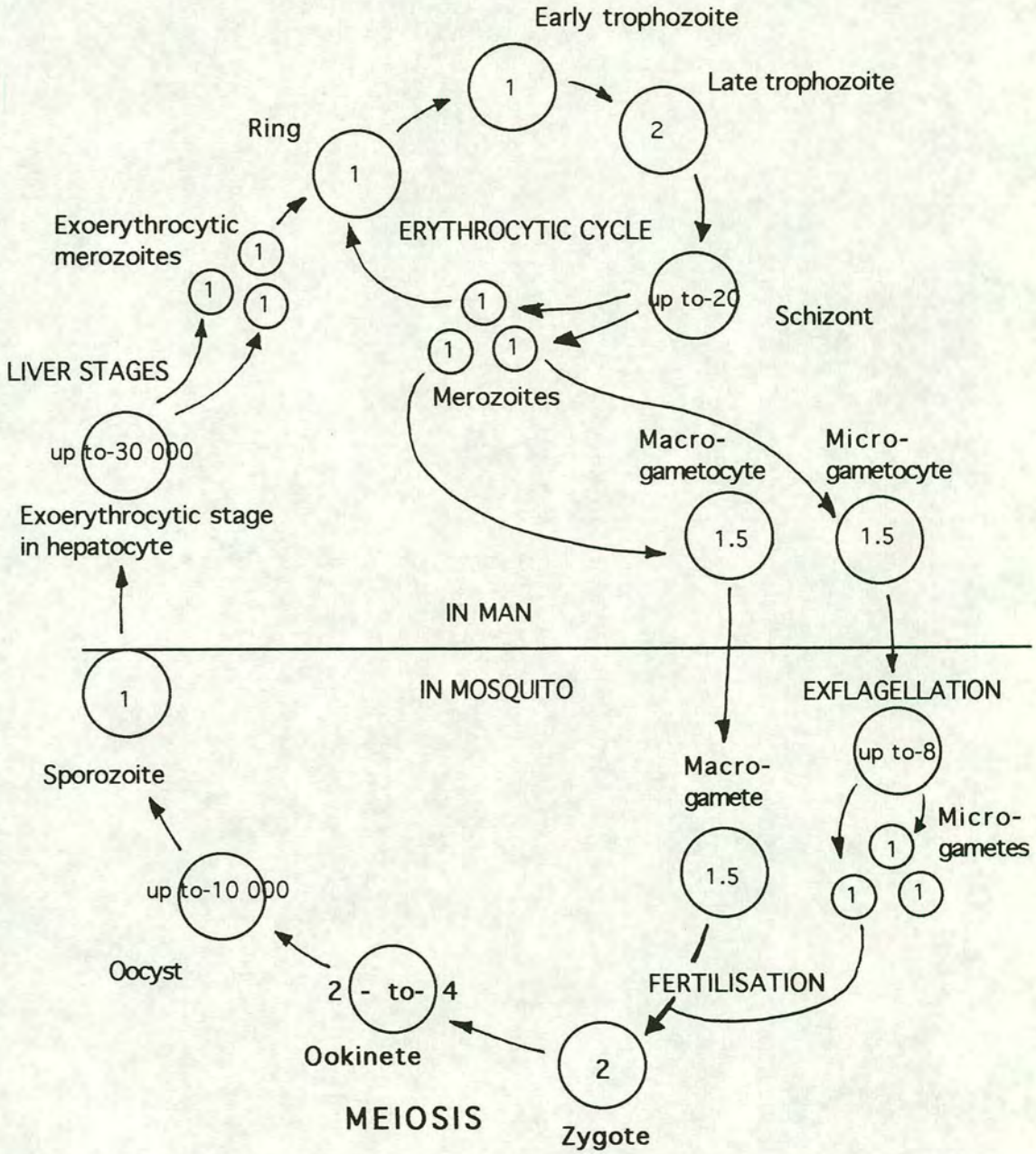


Figure 7. Schematic representation of DNA replication in the malaria parasite life cycle during mitosis and meiosis. The DNA content of each stage is shown relative to the haploid number.
(Diploidy and meiotic replication during the zygote and ookinete stages is shown in bold)

a) Ring Forms

The ring form of the asexual cycle contains the smallest amount of DNA and so is considered to represent the haploid complement of the parasite. In the radio-labeling studies the ring stage amount is taken as the standard against which the DNA content of the other stages is measured.

The mitochondrial 6kb DNA element has been shown to be present in about 20 copies and the 35kb element between 1-3 copies per cell in P. falciparum (Feagin, 1994). This represents roughly 0.5% of the total DNA. The timing of its replication relative to the replication of the nuclear DNA is not precisely known at present.

b) Trophozoites

Young trophozoites show no DNA synthesis but as they mature their DNA content rises to the diploid value even in trophozoites that apparently still have only a single nucleus. Extranuclear DNA is also reported to begin replication at this stage roughly two hours before the first signs of nuclear division (Smeijsters, 1994; Preiser et al., 1995).

c) Schizonts

At the schizont stage the DNA content rises in line with the number of nuclei visible, typically up to around 20x the haploid number. Each individual nucleus in a schizont contains up to but not more than the diploid quantity implying that DNA replication and genome segregation are alternating events (Read et al., 1993). Presumably extranuclear DNA multiplication and segregation must mirror nuclear division closely for each merozoite to receive the correct complement of extranuclear DNA. This must be particularly important in the case of the few copies of the 35kb element.

d) Sexual stages

In the development of the sexual stages the young gametocytes, both male and female, show a sudden rise of around 50% in DNA content (Janse,et al., 1986). The exact nature of this rise is unclear. Studies in P. falciparum have suggested that this represents the start of genome replication based on the fact that these stages are sensitive to mitomycin-c, which prevents DNA replication, and that intranuclear

microtubules are present (Sinden, 1982; Sinden et al., 1984). Other studies in rodent malaria suggest that selective gene amplification may account for this rise (Cornelissen et al., 1985; Janse, et al., 1986).

At gamete formation the macrogamete DNA remains at roughly the same level between the haploid and diploid quantity. The nuclear DNA of the microgametocyte during the exflagellation process, however, undergoes amazingly rapid replication which produces up to 8 microgametes within a few minutes. The few individual microgametes that have been examined show a haploid DNA quantity similar to that of the ring-stage. The rapid increase in nuclear DNA required to produce the microgametes represents a replication time of only about 3.2 minutes and almost certainly implies at least 1300 origins of replication (Janse, et al., 1986). Such rapid replication might be a source of errors. There is some evidence from early electron microscope studies using P. yoelii nigeriensis that, morphologically deformed microgametes may be found at this stage and that possibly up to 60% of microgametes produced may in fact be anucleate (Sinden, 1975; Sinden, 1978; Sinden, 1982). This will obviously affect the ratio of viable male to female gametes available for fertilisation. As the results of the present study will demonstrate, neither of the extranuclear DNA elements appears to be present in the microgametes.

e) Fertilisation

At fertilisation the macrogamete and microgamete nuclei fuse (Sinden and Hartley, 1984). Coinciding with the start of meiosis, the resulting zygote synthesises DNA to almost the tetraploid number within 2-3 hours, and this level persists throughout the ookinete stage. Detailed examination of the developing zygote by electron microscopy has been carried out (Howells and Davies, 1971; Sinden and Strong, 1978; Sinden and Hartley, 1985) and it has been suggested that the zygote nucleus probably follows the conventional 2C to 4C replication prior to the first meiotic division. Broadly, meiosis progresses in a typical fashion except that the synaptonemal complexes do not show the nucleating centre thought to be responsible for chiasmata formation, but persist into a classical metaphase with no diplotene or diakinesis. Metaphase and

anaphase are completed rapidly and by telophase no chromosomes are visible on the kinetochores which are now clustered around the retracted spindle poles. The first meiotic division takes place entirely in the ookinete. It is not clear when the second meiotic division occurs as it is not possible to distinguish it from mitotic divisions in the young oocyst. The fate of the extranuclear DNA during meiosis is not clear. Each sporozoite however appears to contain a haploid genome similar in size and content to that of the erythrocytic ring stage.

1.10.2 Clones of malaria parasites

Malaria parasite clones have been made in a number of laboratories, either by limiting dilution techniques (Rosario, 1981) or by micro-manipulation (Trager et al., 1981; Thaithong and Beale, 1992), so that populations of genetically identical parasites are produced from one asexual stage parasite. Because of the widespread genetic diversity of the parasite in natural populations, (see Section 1.4.4), inevitably most of these clones differ from each other for allelic markers of various nuclear genes as well as for characters for which the genetic control is unknown.

1.10.3 Parasite crosses

Crosses of parasite clones are made by culturing gametocytes of two parental clones, mixing them together, and feeding them to mosquitoes. Each parasite clone produces both male and female gametocytes so that within the mosquito gut, male microgametes of each clone may fertilise female macrogametes of their own clone (self-fertilisation) or macrogametes of the other parental clone (cross-fertilisation), producing four different kinds of zygote (Figure 8). If there are equal quantities of the gametes of each clone present and if mating occurs randomly between the gametes, it is expected that twice the number of heterozygotes will be produced for each kind of homozygote, the typical 1:2:1 ratio as illustrated.

1.10.4 Analysis of parasite crosses

In the first two crosses carried out using P. falciparum clones the sporozoites produced by the cross in the mosquitoes were transmitted to a chimpanzee host, and the resultant infection allowed to develop to the erythrocytic stage. Infected blood

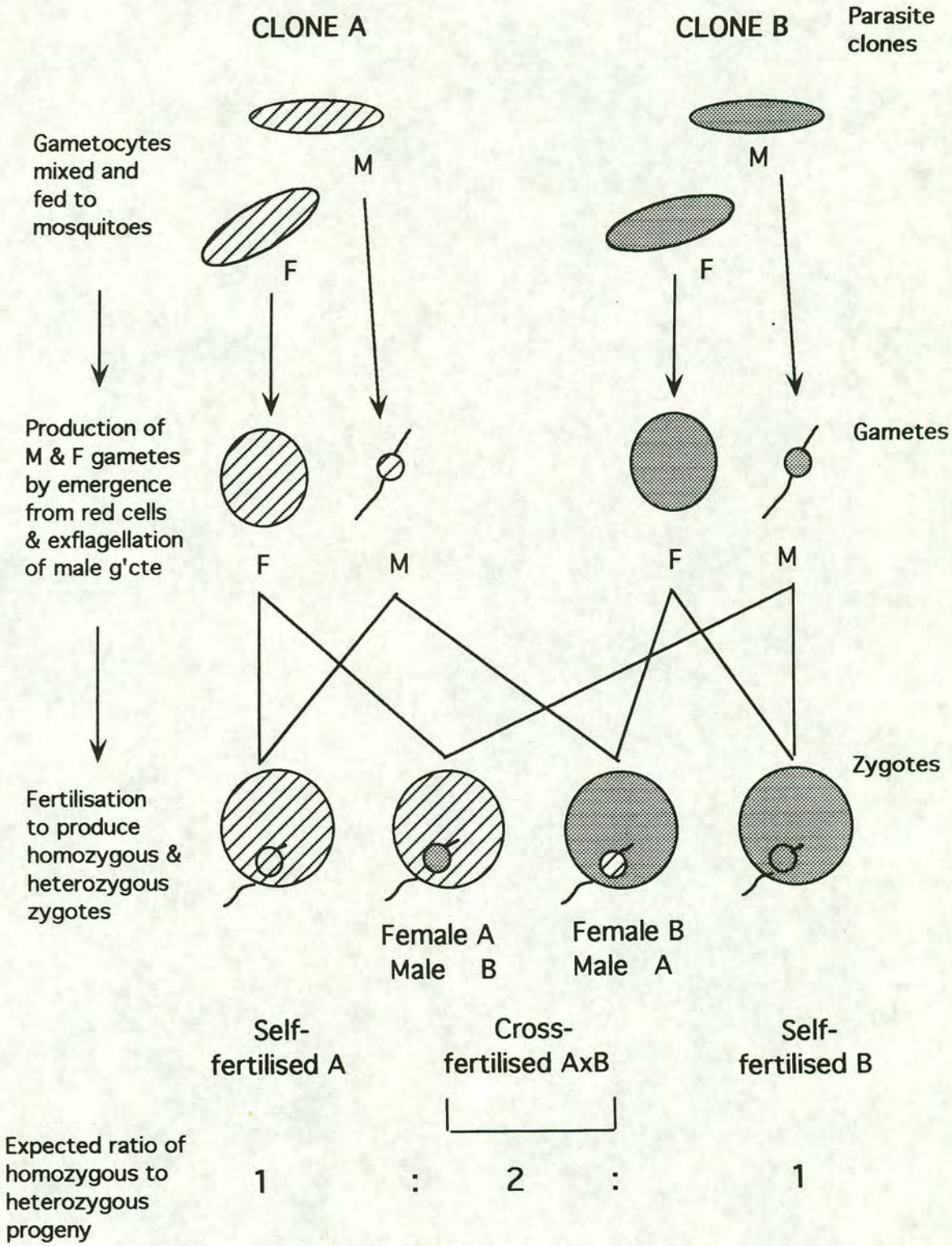


Figure 8. Diagram of a cross between two different clones of Plasmodium falciparum showing the expected frequencies of homozygotes resulting from self-fertilisation and heterozygotes resulting from cross-fertilisation among the progeny.

was drawn from the chimpanzee and the parasites cultured and cloned in vitro. Examination of such progeny clones has been very important both for demonstrating that crosses produce progeny with novel combinations of the parental genes (Walliker et al., 1987) and for investigation of possible linkage between genetic markers and specific phenotypes, such as drug sensitivity (Wellems et al., 1990). For the study of the actual mating behaviour of the parasite, however, the transmission of a human parasite through an unnatural (chimpanzee) host as well as the subsequent in vitro culturing and cloning procedures may introduce very artificial selection processes.

To address this problem a number of subsequent crosses have been analysed by a different set of techniques. In these crosses, the mixture of gametocytes from two different parasite clones is fed to the mosquitoes as before. The products of the cross are allowed to develop to the oocyst stage in the mosquito and single oocysts dissected from the mosquito gut tissue. DNA extracted from individual oocysts may then be used as a template for PCR amplification. The products of amplification allow various kinds of analysis including subsequent molecular manipulations such as DNA sequencing and restriction digestion. Since each oocyst is the product of a single zygote these techniques make it possible to examine directly the products from a single fertilisation event.

1.10.5 Inheritance of nuclear DNA in malaria parasites

Crosses between different clones of P. falciparum, as well as earlier crosses between clones of rodent species of Plasmodium, have shown that nuclear genes of malaria parasites are inherited in a typical Mendelian manner. Chromosomes segregate randomly during meiosis and lead to frequent recombination events resulting in independent assortment of unlinked genes, crossing-over events between linked genes and intragenic recombination (Walliker et al., 1973; Walliker, et al., 1975; Walliker, et al., 1987; Sinnis and Wellems, 1988; Wellems, et al., 1990; Walker-Jonah et al., 1992; Kerr et al., 1994).

The phenotypic results of such recombination events have also been well studied by showing that combinations of enzymes, antigens, protein and drug responses occur in

the progeny which were unlike those of either parent (Walliker, et al., 1987; Corcoran, Thompson et al., 1988; Walliker et al., 1988; Fenton and Walliker, 1990). High recombination rates ensure the rich variety of haploid combinations observed in parasite populations (Creasey, et al., 1990, Babiker, et al., 1991a and 1991b)

1.11 The strategy for determining the inheritance of the extranuclear DNA elements in malaria parasites (Figure 9)

The first task was to identify polymorphic allelic markers in the extranuclear DNA elements of two different P. falciparum clones. These clones had to be ones which were capable of producing gametocytes infective to mosquitoes so that mixtures of their gametocytes could be fed to mosquitoes in a genetic cross. Self-fertilised oocysts of each clone as well as hybrid oocysts resulting from the crossing of the two clones were expected.

Oocysts from the cross were isolated individually by dissection from the gut of the infected mosquitoes. DNA from each oocyst was extracted and used as a template in a PCR reaction. Polymorphic regions of the nuclear genes MSP-1 and MSP-2 which differ in size and sequence between the two parent clones were examined and hybrid oocysts identified by their possession of both allelic forms of the nuclear gene markers (Ranford-Cartwright et al., 1991b). The same DNA from each hybrid oocyst was further analysed for the variant forms of the extranuclear marker. In this way it was possible to find out whether a hybrid oocyst was receiving extranuclear genes from one parent or both parents and if only one, then from which parent it was derived.

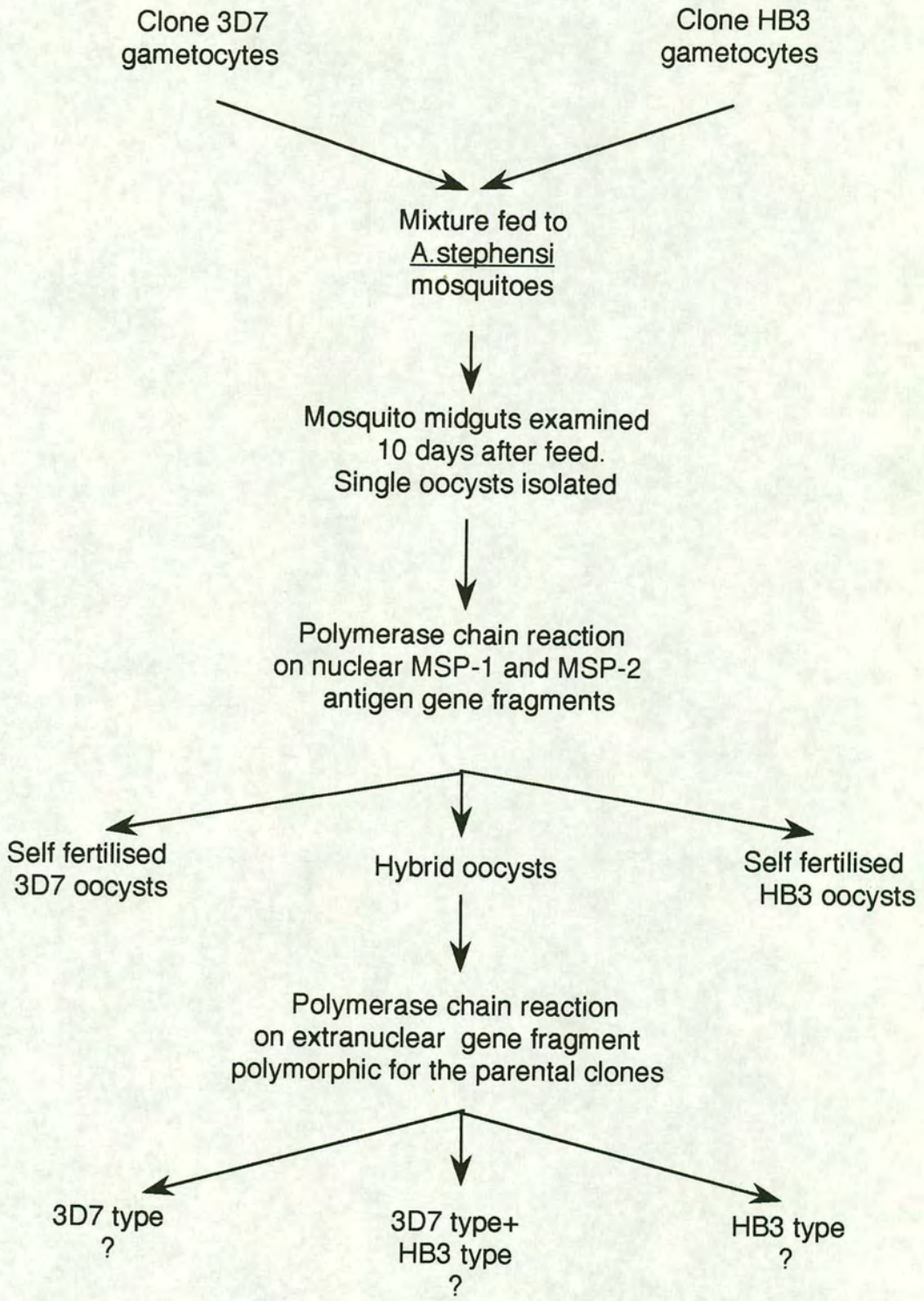


Figure 9. Diagram of the strategy for determining the inheritance pattern of the extranuclear DNA of malaria parasites using Plasmodium falciparum clones 3D7 and HB3 in Anopheles stephensi mosquitoes.

Chapter 2 MATERIALS AND METHODS

2.1 Parasites used

Most of the work in this project involved cloned lines of the human malaria parasite Plasmodium falciparum. DNA of the avian parasite Plasmodium gallinaceum was used in certain experiments to determine the presence of extranuclear DNA in parasite gametes.

2.1.1 P. falciparum parasites

All the clones and isolates used were from the World Health Organisation Registry of Standard Strains of Malaria Parasites kept at the University of Edinburgh. Clones 3D7 and HB3 on which a large part of the study was based have both been used extensively in laboratory work.

Clone 3D7 was cloned in 1985 by limiting dilution (Walliker, et al., 1987) from the isolate NF54, cultured in 1978 from a patient in the Netherlands. The geographical source of the isolate is unknown. From the case history of the patient and from certain of its protein characteristics and sensitivity to drugs (Fenton, et al., 1985; Walliker, et al., 1987), it seems likely that this isolate may be of African, possibly West African origin. It is well adapted to in vitro culture and produces infective gametocytes (Ponnudurai et al., 1982b).

Clone HB3 was derived by microscopic selection of single parasites (Trager, et al., 1981) from the isolate H1, originally taken from a patient in Honduras. The clone used in this work was acquired by the National Institutes of Health, Bethesda in 1984 and received in Edinburgh in 1986. It too was known to produce infective gametocytes (Bhasin and Trager, 1984).

These two clones were used as the parents of the first P. falciparum cross to be performed in the laboratory using chimpanzees as the mammalian host (Walliker, et al., 1987). More recently, crosses of these two clones were used for analysis of individual oocysts from the mosquito gut (Ranford-Cartwright, et al., 1991b). The clones 3D7 and HB3 were chosen for this work for several reasons:

1. They are well characterised and known to differ for a number of nuclear gene allelic markers.

2. Being of different geographical origin they might be expected to have a greater chance of displaying extranuclear gene polymorphism than clones from the same area.

3. They produce infective gametocytes as confirmed by the successful laboratory crosses already carried out.

4. Although isolated from patients many years ago, the cloning procedures for both clones were carried out from frozen stabilates which were close enough to their date of isolation from the patient to be producing abundant infective gametocytes (see Ponnudurai et al., 1982b). The material used in this project was from frozen stabilates which were as close as possible to this original cloning.

Clone C10 for which much of the sequence of the extranuclear elements is available, was derived from the isolate BW (Burroughs-Wellcome) originally obtained from a patient in the Gambia and adapted to Aotus monkeys before being cultured long-term in vitro (Hempelmann et al., 1981). It is hereafter referred to as BWC10.

Other clones and isolates cultured and screened briefly in this project are listed together with their geographic origin in Table I (Results Section 3.2.3).

2.1.2 P. gallinaceum parasites (see Section 2.6.1)

2.2 Parasite Culturing techniques

2.2.1 Culture of asexual forms of P. falciparum

Asexual cultures of all the clones and isolates were grown to obtain genomic DNA for PCR templates. In addition, asexual parasite cultures of clones 3D7 and HB3 were retrieved from liquid nitrogen stabilates every 2-3 months and maintained as stocks for the culture of infectious gametocytes.

a) Culture medium

The basic medium used for manipulations such as washing of parasites and red cells comprised RPMI 1640 medium (Gibco) supplemented with 25mM HEPES buffer

(5.94g l^{-1}) containing 50mg ml^{-1} of hypoxanthine. This was sterilised by filtration through a 0.22micron filter, stored at 4ºC and used within one month. This medium is hereafter termed 'incomplete medium'.

For growing the parasites 10% human serum (each batch pooled from 6-10 different donors) and 0.2% (w/v) sterile sodium hydrogen carbonate ($NaHCO_3$) was added to the incomplete medium. This is termed 'complete medium'. Complete medium was kept at 37ºC and used within one week.

b) Erythrocytes

Erythrocytes used in the cultures were obtained from O^+ human blood collected into citrate phosphate dextrose (16mM citric acid monohydrate, 89mM sodium citrate, 16mM sodium acid phosphate, 258mM dextrose, 2mM adenine). The cells were washed three times by centrifugation at 1500**g** for 5 minutes in incomplete medium using a volume equal to the original blood volume. The washed pellet was re-suspended to the required haematocrit using complete medium.

c) Retrieval of parasites from liquid nitrogen

Deep-frozen stabilates from liquid nitrogen storage were thawed using the technique of Aley (Aley et al., 1984), and established in 25ml flasks (J. Bibby Science Products Ltd.), according to standard techniques (Trager and Jensen, 1976). If necessary a drop or two of fresh washed O^+erythrocytes (at 50% haematocrit in complete medium) was added to the newly thawed culture in order to achieve a final haematocrit of 5% in a 3-5ml volume of complete medium.

d) Routine maintenance

Cultures were gassed with a mixture of 1% oxygen, 3% carbon dioxide and 96% nitrogen and kept at 37ºC with daily medium changes. The progress of each culture was monitored by microscopic examination of a Giemsa-stained thin-smear of the culture. Cultures were diluted every 2-3 days using fresh washed O^+erythrocytes according to the parasitaemia and morphology of the parasites.

2.2.2 Culture of gametocytes

15ml cultures were set up at 6% haematocrit and 0.5-0.7% parasitaemia in 75ml culture flasks, according to established methods (Carter and Miller, 1979; Ifediba and Vanderberg, 1981). To ensure good production of gametocytes the initial cultures used were always less than 2-3 months from the date of their removal from liquid nitrogen, and were growing healthily at a high parasitaemia as asexual cultures. Erythrocytes used for initial dilution were always less than one week old to ensure that they would survive for the duration of the gametocyte culture. Cultures were gassed and incubated at 37ºC, as for asexual cultures, with daily medium changes using complete medium pre-warmed to 37ºC. At the point when cultures had a high parasitaemia and the parasite morphology was starting to show signs of stress (normally 3-4 days after set-up), the volume of each culture was increased to 25 ml, to provide adequate nutrition for the young developing gametocytes. This reduced the final haematocrit of the culture to 3.6%. From this point onwards the cultures were kept in as stable a state as possible in order to prevent premature triggering of the developmental cycle (Carter and Graves, 1988). The medium changing area was kept warm, cultures were changed as far as possible at the same time each day and each culture was kept out of the incubator for the minimum length of time during medium changes. Cultures were monitored by microscopic examination at intervals for the next 10-14 days and harvested either on Day 14 or Day 17 for feeding to mosquitoes. On the day of the feed the gametocytaemia and the proportion of mature male to female gametocytes was recorded.

2.3 Mosquito Infection

2.3.1 Preparation of infectious bloodmeals

Fresh O^+ erythrocytes (less than one week old) were washed three times in incomplete medium and re-suspended to 40% haematocrit with heat-inactivated pooled human serum and kept at 37ºC in a water-bath for subsequent dilution of cultured material. Gametocyte cultures were centrifuged at 1500g for 5 minutes, the medium removed and the pellet re-suspended with the pre-warmed serum to a haematocrit of 40%. At

this stage, according to the design of the experiment, appropriate dilutions and mixtures of the cultures were made. In the case of crossing experiments between 3D7 and HB3, the cultures were mixed to give a 1:1 ratio of mature gametocytes for each clone. On each occasion, control feeds of the individual cultures 3D7 and HB3 were also carried out.

For the easiest and most accurate dissection of single oocysts from a mosquito gut the optimum mosquito infection rate should produce from one to no more than 5 oocysts per gut. Accordingly the prepared cultures were diluted, usually about 3-5 times, using the 50% fresh erythrocytes in the serum prepared previously, to try to obtain this kind of infection rate. This proportion of fresh erythrocytes in each feed is also necessary to encourage the mosquitoes to feed successfully. One-two ml of this suspension was pipetted into the pre-warmed mosquito membrane feeders. Care was taken at every stage to maintain the bloodmeal at 37ºC to ensure good viability and prevent premature exflagellation of the microgametes.

2.3.2 Preparation of unfertilised female gametes

For certain experiments, preparations of viable female gametes free of male gametes were required. For this purpose, Mosquito Exflagellation Factor (MEF) was prepared as follows. Approximately 100 pupae of Anopheles stephensi mosquitoes were rinsed well in distilled water, and added to 20ml incomplete medium. The suspension was homogenised manually with a teflon pestle and centrifuged at 20 000**g** for 10 minutes at 4ºC. The clear supernatant lying beneath the thick fatty layer was carefully removed and filtered through a 0.22micron filter. The filtered extract was diluted approximately 1:50 and adjusted to pH 8.7 with 1M NaOH (Nijhout and Carter, 1978).

For preparation of the female gametes, gametocyte cultures were grown normally as described in Section 2.2.2 above. On Day 17, the culture was centrifuged at 1500**g** for 5 minutes and the supernatant removed leaving a pellet comprising red cells and gametocytes. Approximately 10x the volume of the pellet (usually about 5 ml) of Incomplete medium containing Mosquito Exflagellation Factor (MEF) at a 1:10 dilution was added to the pellet, mixed well and incubated at room temperature for 1

minute. The sample was spun at 1500g for 2 minutes, the supernatant removed and the pellet re-suspended in approximately 400ml of incomplete medium at pH 8.2, again mixed well and left at room temperature for 1 hour to allow all the male gametocytes to exflagellate. Although both MEF and a high pH were used in this experiment to ensure male exflagellation, recent experiments in P. berghei have shown that either MEF or high pH is sufficient to trigger the mechanism (O. Billker - unpublished observations) and this may also be the case with P. falciparum. At the same time the female gametes are expected to emerge from their erythrocytes (Nijhout, 1979). Under these dilute conditions it is anticipated that female gametes will still be viable but male gametes will die without having fertilised any female gametes (Carter et al., 1979). The preparation was then centrifuged at 1500g for 5 minutes and the supernatant removed (Figure 10). The pellet containing the unfertilised female gametes was diluted as required for feeding to mosquitoes.

2.3.3 Membrane feeding

Adult female A. stephensi mosquitoes 3-5 days old were collected into nylon mesh-topped cartons and kept on a diet of water for two days prior to feeding. On the day of the feed, glass membrane feeders jacketed with water at 37ºC and fitted with a Baudruche membrane were placed on the feeding cartons and the gametocyte-infected blood added to the feeder (Vanderberg and Gwadz, 1980; Ponnudurai, et al., 1982b) (Figure 11). Mosquitoes were allowed to feed for half an hour or until all were engorged with blood. A drop of each feeding culture was placed under a glass coverslip and examined for exflagellation. The blood-fed mosquitoes were maintained on a diet of 10% glucose in 0.05% p-aminobenzoic acid (PABA) {Sigma} until dissection.

2.3.4 Oocyst dissection

10-11 days after the bloodmeal, mosquitoes were killed using chloroform. Each dead mosquito was dipped briefly in 70% alcohol and kept moist in phosphate buffered saline (PBS) pH 7.4. Mosquitoes were dissected individually using 26*g* syringe needles under a 40x dissecting microscope. Midguts were removed to a clean drop of

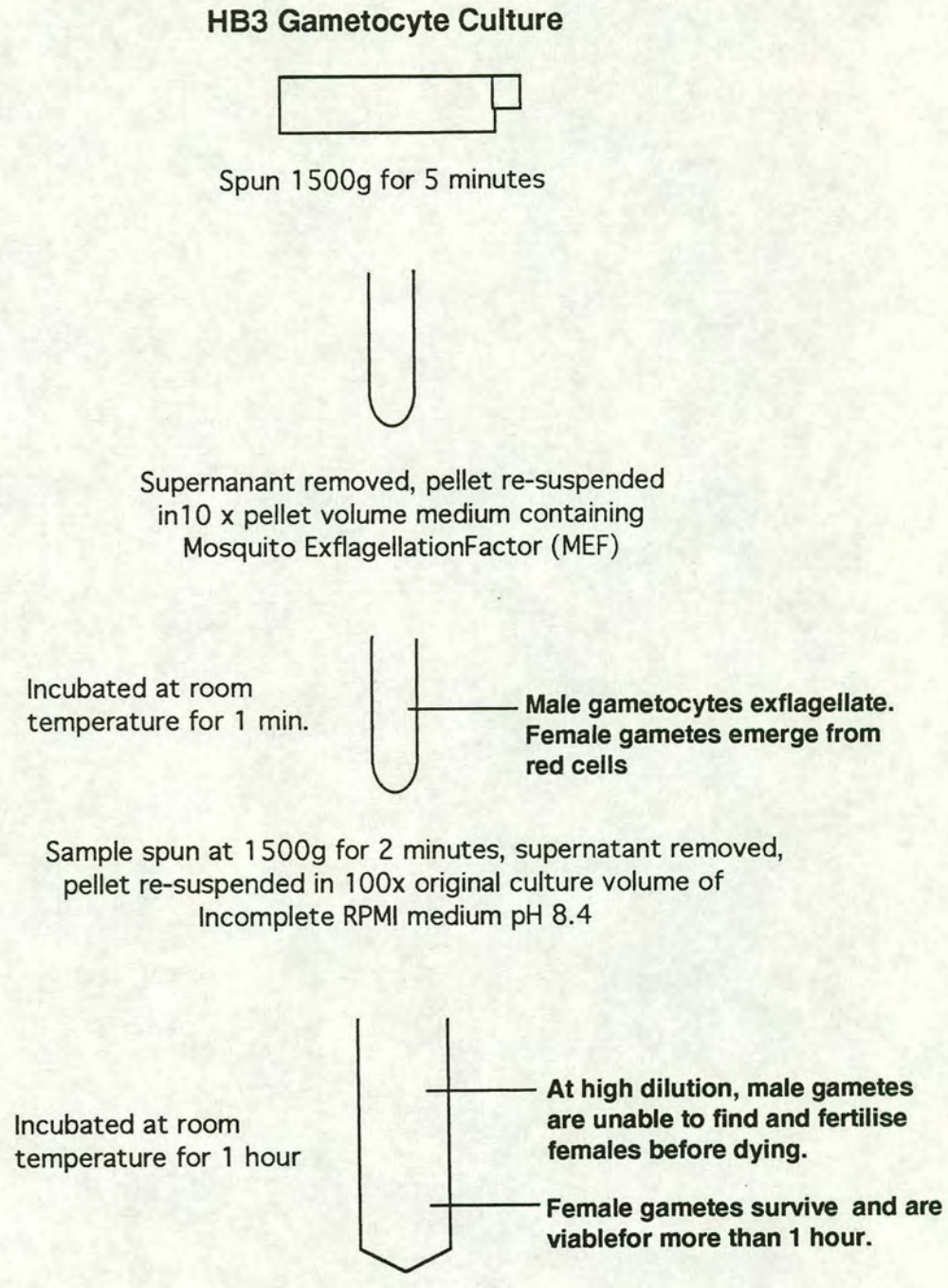


Figure 10. Preparation of HB3 gametes for an "Artificially Controlled Cross". The diagram illustrates the procedure for obtaining unfertilised HB3 female gamete preparations without male gametes.

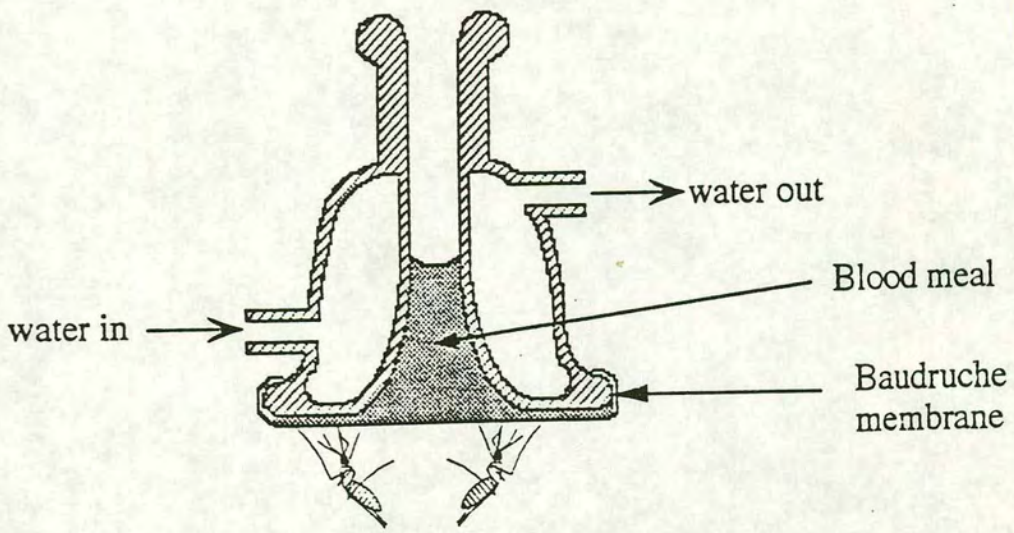


Figure 11. Diagram of the glass, water-jacketed, mosquito feeder. Water at 37ºC is circlated around the central chamber to maintain the temperature of the blood meal during the feed. The feeder is laid onto the net of the pot containing the mosquitoes and the mosquitoes allowed to feed through the membrane.

PBS and teased apart using disposable microneedles made by heating and pulling out 2µl micropipettes (Camlab). Single oocysts were isolated by gently cutting them from the gut tissue using the micropipettes and transferring them to a microfuge tube containing 50µl of lysis buffer (100mM NaCl, 25mM EDTA (pH8.0), 10mM Tris-HCl (pH 8.8), 0.5% sarkosyl and $1mg^{-1}$ Proteinase K (Boehringer)) (Ranford-Cartwright et al., 1991a). A fresh pair of microneedles was used for each oocyst.

2.4 Preparation of DNA Fragments

2.4.1 Lysis of oocysts and extraction of oocyst DNA

Each oocyst dissected from the mosquito gut tissue was incubated in lysis buffer for 1 hour at 56ºC or overnight at 37ºC (Ranford-Cartwright, 1991b). DNA was either extracted immediately following incubation or samples were frozen at -20ºC for later extraction.

1µg of salmon sperm DNA was added as a carrier to the lysed oocyst. DNA was extracted once using an equal volume (50µl) of phenol (equilibrated with Tris.Cl, pH 8.0), once using a 1:1 mixture of phenol and chloroform and once using 100% chloroform (adapted from (Maniatis et al., 1982)). The DNA was precipitated with isopropranol (propan-2-ol) at -20ºC for 2 hours and the pellet washed twice with 70% ethanol and left on the bench to dry for 10 minutes. The dried pellet was re-suspended in 10µl of sterile distilled water and boiled for 7 minutes. The prepared DNA was stored at -20ºC.

2.4.2 Extraction of genomic DNA

Cultures of asynchronous parasites were grown in 5ml volumes in 25ml flasks. Parasites were harvested when the cultures had reached 6-10% parasitaemia with at least half the parasites at the schizont stage. The medium was removed by centrifugation and the red-cells resuspended to the original culture volume with a 0.15% solution of saponin in 1x PBS at pH 7.4 to lyse the red cells. This was mixed, incubated at 37ºC for 20 minutes and centrifuged at 3 000**g** for 10 minutes. The supernatant was removed and the parasite pellet washed three times in incomplete medium (Sanderson, et al., 1981). The pellet was resuspended in 1ml EDTA buffer

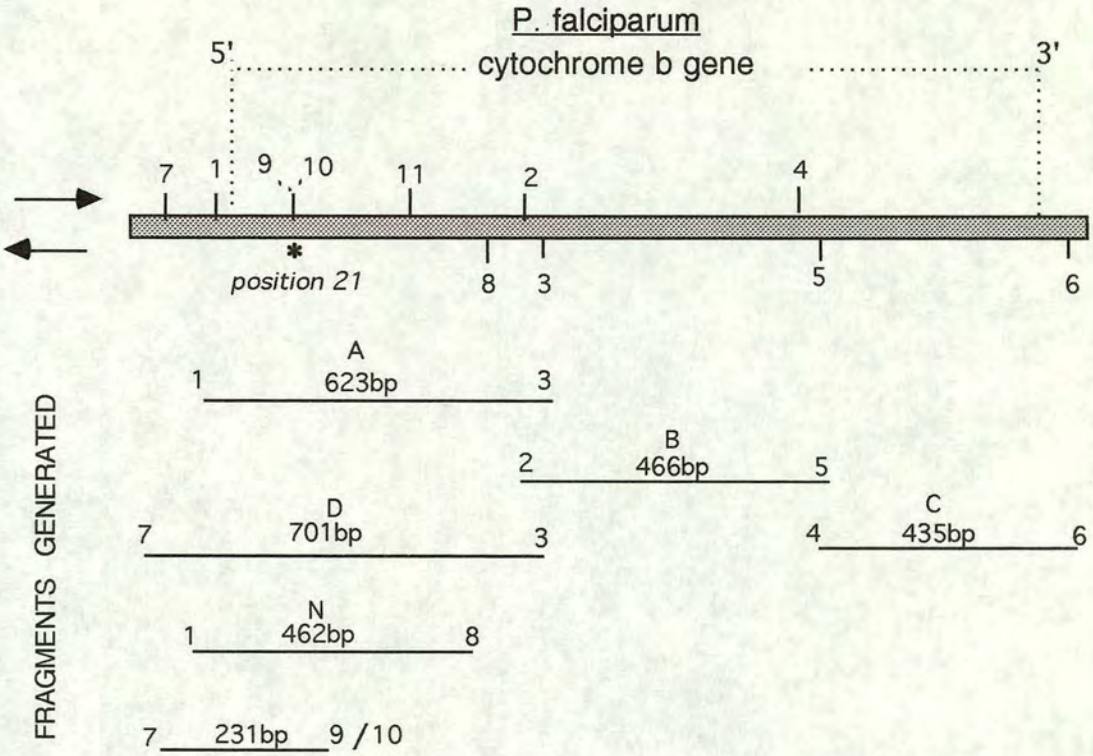
(150mM NaCl, 25mM EDTA) in a 1.5ml microfuge tube, and centrifuged at 10 000g for five minutes. The supernatant was removed and the EDTA buffer wash repeated twice. After the last wash, the pellet was resuspended in 400µl of the EDTA buffer to which was added 10µl of a 10% SDS solution and approximately 50µg Proteinase K (Boehringer). The pellet was mixed well and left at 56ºC for 2 hours or at 37ºC overnight.

Following incubation, standard phenol chloroform extraction was carried out once with phenol, three times with a 1:1 phenol and chloroform mixture and once with chloroform (Maniatis, et al., 1982). The extracted DNA was precipitated with two volumes (800µl) of ice-cold ethanol containing 0.3 volumes (120µl) of 3M sodium acetate solution (pH 5.2) at -20ºC for 30 minutes. The tube was centrifuged at 10,000g for 10 minutes at 4ºC, and the precipitate washed twice with 70% ethanol. The pellet was dried for 10 minutes in a speedvac (Savant IEC) and re-suspended in either 1x Tris-EDTA buffer (TE) or distilled water and incubated for 10 minutes at 37ºC. The DNA was tested for purity and amount in a spectrophotometer (OD260/OD280) and/or by running 2µl on a 1% agarose gel. The DNA was used immediately or stored at -20ºC.

2.4.3 Design of P. falciparum primers

All the primers used in this study were designed from known DNA sequences some of which have been published and are obtainable from the University of Wisconsin Genetics Computer Group gene database package (version 7) (UWGCG) (Devereux et al., 1984), and some of which were obtained from R.J.M. Wilson and D.H. Williamson (National Institute of Medical Research (N.I.M.R.), Mill Hill Laboratories, London). All the primers were synthesised by Oswel DNA Service (Chemistry Department, University of Edinburgh {now located at Medical and Biological Sciences Building, University of Southampton}).

For the P. falciparum cytochrome b gene (6kb element) a total of 11 primers were designed from the known sequence of the BWC10 clone (Figure 12).



PRIMERS

1	5′	TCAACAATGACTTTATTTGG	3′	Forward
2	5′	CAAATGAGTTATTGGGGTG	3′	Forward
3	5′	TGCTGTATCATACCCTAAAG	3′	Reverse
4	5′	TACCATTTTATGCAATGTTA	3′	Forward
5	5′	TTTGTTCTGCTAATAAGAAT	3′	Reverse
6	5′	TATATATCGAAGCATCCAT	3′	Reverse
7	5′	GCCTAGACGTATTCCTG	3′	Forward
8	5′	GACATAACCAACGAAAGCAG	3′	Reverse
9	5′	GAACTTTACTCTATTAATT	3′	Forward
10	5′	GAACTTTACTCTATTAATC	3′	Forward
11	5′	GCAAGTCGATATACACCAG	3′	Forward

Figure 12. Sketch map of the cytochrome b gene of the 6kb element of Plasmodium falciparum showing the positions of the primers used in this study and the sizes of the fragments which they amplify.

Primers 1-3, 2-5 and 4-6 respectively amplified three fragments, A - 623bp, B - 466bp and C - 435bp which together covered the entire gene.

Primers 7-3 were used as outer primers and Primers 1-8 as inner primers for a nested PCR covering the polymorphism at position 21 in the gene. They amplified fragments D - 701bp, and N - 462bp respectively.

Primers 9 and 10 were both forward primers whose sequences were identical except for the last 3' base which incorporated the T form of the polymorphism and the C form of the polymorphism at position 21 on the gene respectively.

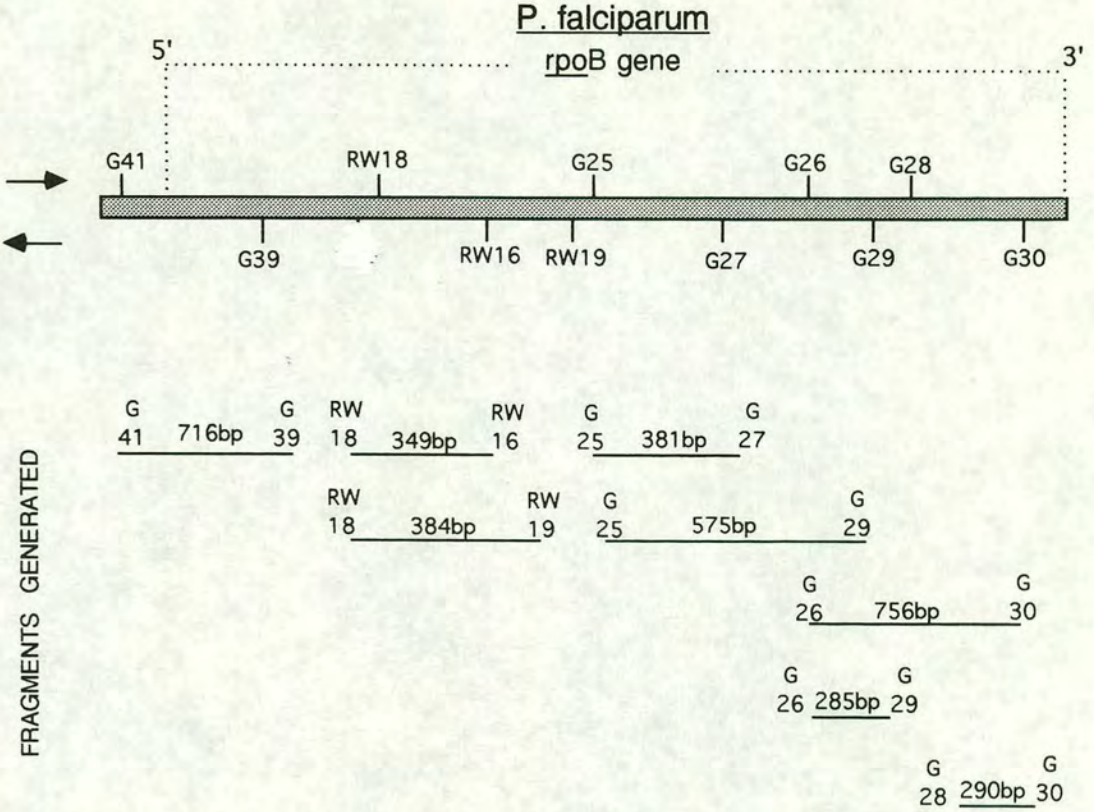
Primer 11 was designed 5' to Primer 3 to facilitate easy sequencing of the central section of Fragment A.

For the P. falciparum rpoB gene (35kb circle)

A total of 12 primers were synthesised covering this entire gene plus 523 (check) bases upstream of the 5' end. Most of these were designed by the malaria group at N.I.M.R. Mill Hill and are unpublished except for Primers G39 and G41 (Creasey et al., 1994). The sizes of the fragments which can be amplified with these primers and the approximate positions of each primer are shown in Figure 13.

2.4.4 Amplification of fragments by Polymerase Chain Reaction.

Polymerase Chain Reaction (PCR) amplification was performed according to standard methods (Saiki et al., 1985; Saiki et al., 1988), and carried out on a Biometra TRIO thermocycling machine. Each PCR was carried out in either 20µl or 50µl volumes in a 0.5ml microfuge tube. The reaction mix containing buffer, nucleotides, primers and enzyme was overlaid with 30-50µl of sterile mineral oil before the addition of the target DNA. Pipettes used for dispensing reaction mix solutions were kept solely for this purpose to avoid the risk of contamination with any solution containing extraneous DNA. A positive control comprised the addition of a DNA sample that had worked successfully on previous occasions. Two negative controls were included, one consisting of 1µg salmon sperm added to the reaction mix and the other of 1µl of distilled water added to the reaction mix.



PRIMERS

RW16	5′	CCCTAATATATTTCTAGGTAATTC	3′	Reverse
RW18	5′	CAATAAAATTAAAATTTTTCATATTATTCTG	3′	Forward
RW19	5′	CTAAACCACAAGTTAATCCTTCAT	3′	Reverse
G25	5′	CGTAATATATTATAAAC	3′	Forward
G26	5′	GGTCAGATTTTAGC	3′	Forward
G27	5′	CCTTCTTGATATG	3′	Reverse
G28	5′	GGTACATATATATTAGC	3′	Forward
G29	5′	CTACATATTTCTGG	3′	Reverse
G30	5′	CTTTATGTCCATG	3′	Reverse
G39	5′	AATTTTAAAGAAATTAATATATTTAAAT	3′	Reverse
G41	5′	AATAATTGAATACATGTTTTATATAATC	3′	Forward

Figure 13. Sketch map of the rpoB gene on the 35kb circle of Plasmodium falciparum showing the positions of the primers used in this study and the sizes of the fragments which they amplify.

a) Reaction mix

The buffer used consisted of 50mM KCl (Analar, BDH), 10mM Tris-HCl pH 8.8 (Aristar, BDH), 2.5mM $MgCl_2$ (AR, Fisons) made up as a 10x solution. A 100x stock solution of the four nucleotides dGTP, dATP, dTTP and dCTP each at 7.5mM was made from 100mM solutions (BCL) aliquotted into amounts suitable for 4-5 runs and stored at -20ºC. The enzyme used was Taq Polymerase extracted from Thermus aquaticus, and was obtained from various sources according to the cost at the time of ordering. These suppliers included Boehringer (Taq), Cetus (Amplitaq), Promega (Taq), and Gibco (Taq). All produced good amplification under the appropriate conditions and with slight changes to the routine buffer used. Since Taq-polymerase used for PCR amplification is estimated to incorporate a wrong nucleotide with a frequency of 2.1 $x10^{-4}$ (Keohavong , P. and Thilly W.G., 1989) all PCR reactions in this project were carried out at least twice and usually more than twice to account for this possibility

b) DNA concentrations

DNA was used at approximately 100ng-1µg per reaction depending on the particular primers being used.

c) Reaction conditions

The denaturing annealing and extension times and temperatures for the PCR reactions carried out in this project were as follows:-

For the 6kb cytochrome b gene

Fragments A, B, D, N : Denaturation 95ºC for 30 seconds, annealing 42ºC for 30 seconds, extension 72ºC for 2 minutes

Fragment C : Denaturation 95ºC for 30 seconds, annealing 46ºC for 30 seconds extension 72ºC for 2 minutes

For the 35kb (rpoB gene) for all fragments : Denaturation 95ºC for 30 seconds annealing 42ºC for 30 seconds, extension 72ºC for 2 minutes

2.4.5 Sequence-specific PCR

Primers 9 and 10 which were identical except for the last 3' base were designed to enable a sequence specific PCR reaction to distinguish between the two forms of the gene (Zolg et al., 1990). It was expected that primer 9 which incorporated the T form of the polymorphism at the 3' end would amplify a fragment from 3D7 DNA but not from HB3 DNA. Similarly primer 10 which incorporated the C form of the polymorphism at the 3' end was expected to amplify a fragment from the HB3 DNA but not the 3D7 DNA. PCR reactions were set up using each primer and a range of dilutions of each DNA template. The annealing temperature of the routine PCR conditions was carefully adjusted over a range between 45ºC and 52ºC with 0.5ºC increments to try to achieve this specificity. Where necessary, a range of different reaction times from 20 seconds to 60 seconds were used for the annealing step.

2.4.6 Oocyst PCR

The PCR technique described for genomic DNA in Section 2.4.4. above was modified for amplification from oocyst DNA. Ten micromoles of the dGTP in the oligonucleotide mix was replaced by its base analogue 7-deaza-2-deoxyguanosine (C7 dGTP) (McConlogue et al., 1988; Ranford-Cartwright, 1991b). This helped to inhibit the production of secondary structure. All reactions were carried out in 20µl or 50µl volumes using 1-2µl of the oocyst DNA template. A 'nested' PCR technique was used (Simmonds et al., 1990). One microlitre of PCR product from the initial round of PCR amplification was used as a template for a second round of amplification using primers internal to the first pair. The temperatures and times for the reactions were the same as those used for amplification using genomic DNA templates.

2.4.7 Analysis of PCR fragments

The success of the PCR amplification was ascertained by running a small aliquot (3-5µl) of each reaction on an ethidium bromide-stained agarose gel. 1.5% agarose was used as this produced a good separation for all the fragment lengths being used in this study (Maniatis, Fritsch et al., 1982). The gel was prepared in 1 x TBE (0.59M Tris-borate, 0.09M Boric acid, 0.002M EDTA) with the addition of 0.5µg ml^{-1} of the DNA

stain ethidium bromide (3,8-diamino-6-ethyl-5-phenyl-phenanthridium bromide) (LePecq and Paoletti, 1966).

Five microlitres of each PCR was mixed with 0.5µl of 10x Gel Loading buffer (0.25% bromophenol blue, 25% Ficoll, 10mMTris-Cl, 1mM EDTA) (Maniatis, Fritsch et al., 1982) and loaded into 0.25mm wells on the gel. Suitable markers from the Boehringer range of DNA markers were diluted 10: 1 with gel loading buffer and run in tracks on each side of the samples. The gel was run submerged in 1 x TBE buffer at 5V cm^{-1}. The PCR-amplified DNA fragments were visualised by UV transillumination and photographed.

2.4.8 Purification of DNA fragments

40µl of the PCR-amplified fragment were extracted from beneath the oil overlay of the PCR reaction and placed in a clean 0.5ml microfuge tube and purified using either the Geneclean (Bio-101) or Wizard (Promega) methods.

a) Geneclean II method (Bio-101)

Geneclean DNA purification was carried out essentially according to the manufacturer's instructions. To each PCR reaction sample three volumes (120µl) of 6M sodium iodide solution was added followed by 5µl of silica matrix (Glassmilk). The mixture was vortexed gently and intermittently to keep the silica in suspension for 5 minutes at room temperature. The sample was centrifuged briefly at 10 000g to pellet the silica with the bound DNA. The matrix was washed three times with 500µl of ice-cold NEW™ wash containing sodium chloride and EDTA dissolved in ethanol. The DNA was eluted by resuspension of the pellet in 10µl of TE buffer (10mM Tris-HCl pH 7.6, 1mM EDTA) and incubation at 55°C for 5 minutes. The tube was centrifuged at 10 000g for 30 seconds and the supernatant containing the DNA removed to another tube. The elution step was repeated and the two supernatants pooled to give a final volume of 20µl of DNA solution.

b) Wizard DNA Clean-Up system (Promega)

The protocol followed was essentially that supplied by the manufacturer.

One millilitre of the Wizard DNA Clean-Up Resin was placed in a 1.5ml microfuge tube with the DNA sample (usually between 50-150µl) and the suspension mixed by inverting the tube several times. The barrel of a 3ml. syringe was attached to the luer-lock top of the Promega Wizard Minicolumn. The Clean-Up Resin containing the bound DNA was pipetted gently into the syringe barrel, the syringe plunger inserted and the slurry slowly pushed through the column. The syringe was detached from the Minicolumn, the plunger removed and the barrel re-attached to the column again. Two ml of 80% ice-cold isopropanol was pipetted into the syringe barrel and pushed gently through the column to wash the resin- bound DNA. The column was placed over a 1.5ml microfuge tube and centrifuged for 20 seconds at 12 000**g** to dry the resin. The Minicolumn was transferred to a clean tube and 50µl of TE buffer, pre-warmed to 37ºC, was added to the column. The column was left at room temperature for one minute before it was placed over a sterile tube and then centrifuged for 20 seconds at 10 000**g** to elute the purified DNA.

2.5 Examining DNA fragments for sequence polymorphism

2.5.1 Temperature Gradient Gel Electrophoresis (TGGE)

The Temperature Gradient Gel Electrophoresis (TGGE) system was used to identify small sequence differences between complementary fragments of DNA from different clones of P. falciparum parasites.

a) The principle of TGGE

The TGGE system works on the principle that a fragment of DNA exposed to increasing temperature undergoes conformational changes. In the case of double stranded DNA, hairpin structures melt and the double strands eventually become single stranded (Reisner et al., 1989).

Different DNA sequences melt at different temperatures and so display different conformations at the same temperature. The electrophoretic mobility of DNA fragments is influenced by changes in their conformation and they migrate at different rates through an acrylamide gel. Even single base substitutions usually cause sufficient difference in mobility to be detected visually on the gel.

b) The TGGE apparatus

The TGGE apparatus is shown in Figure 14. The central element of the system is an aluminium plate measuring 190 X 210mm coated with a heat-resistant epoxide coating. Beneath each of the shorter edges of the plate is a chamber accessed by an inlet and outlet pipe through which water may be circulated. A linear temperature gradient across the plate is established by circulating water of a different temperature down each respective side. A polyacrylamide gel supported by a thin acetate film laid across the plate develops a similar temperature gradient to that of the plate. In the present study the apparatus was used in the so-called 'Perpendicular' orientation so that the current was made to flow at right angles to the temperature gradient.

c) Detection of polymorphism by TGGE

DNA fragments were amplified from the two clones 3D7 and HB3 using the same pair of primers. (Such fragments are hereafter referred to as 'complementary' fragments). These complementary fragments were mixed together, denatured and then renatured again in the expectation that some of the single strands would renature with strands from the opposite clone to form heteroduplexes between the two clones. If there were a sequence difference between the two clones, then the reannealed sample would contain four different types of molecule namely two homoduplex molecules, one from each clone, and two heteroduplex molecules comprising one strand from each clone as illustrated in Figure 15. The DNA sample thus obtained was placed in a long slot at the cathode end of the gel and run at right angles to a temperature gradient of 20ºC-60ºC. Since the Taq-polymerase used for the PCR reaction is estimated to incorporate a wrong nucleotide with a frequency of 2.1 $x10^{-4}$ (Keeohavong , P. and Thilly W.G., 1989), where there was evidence of polymorphism duplicate PCR reactions were carried out on the relevant fragments.

d) DNA preparation, purification and heteroduplex formation

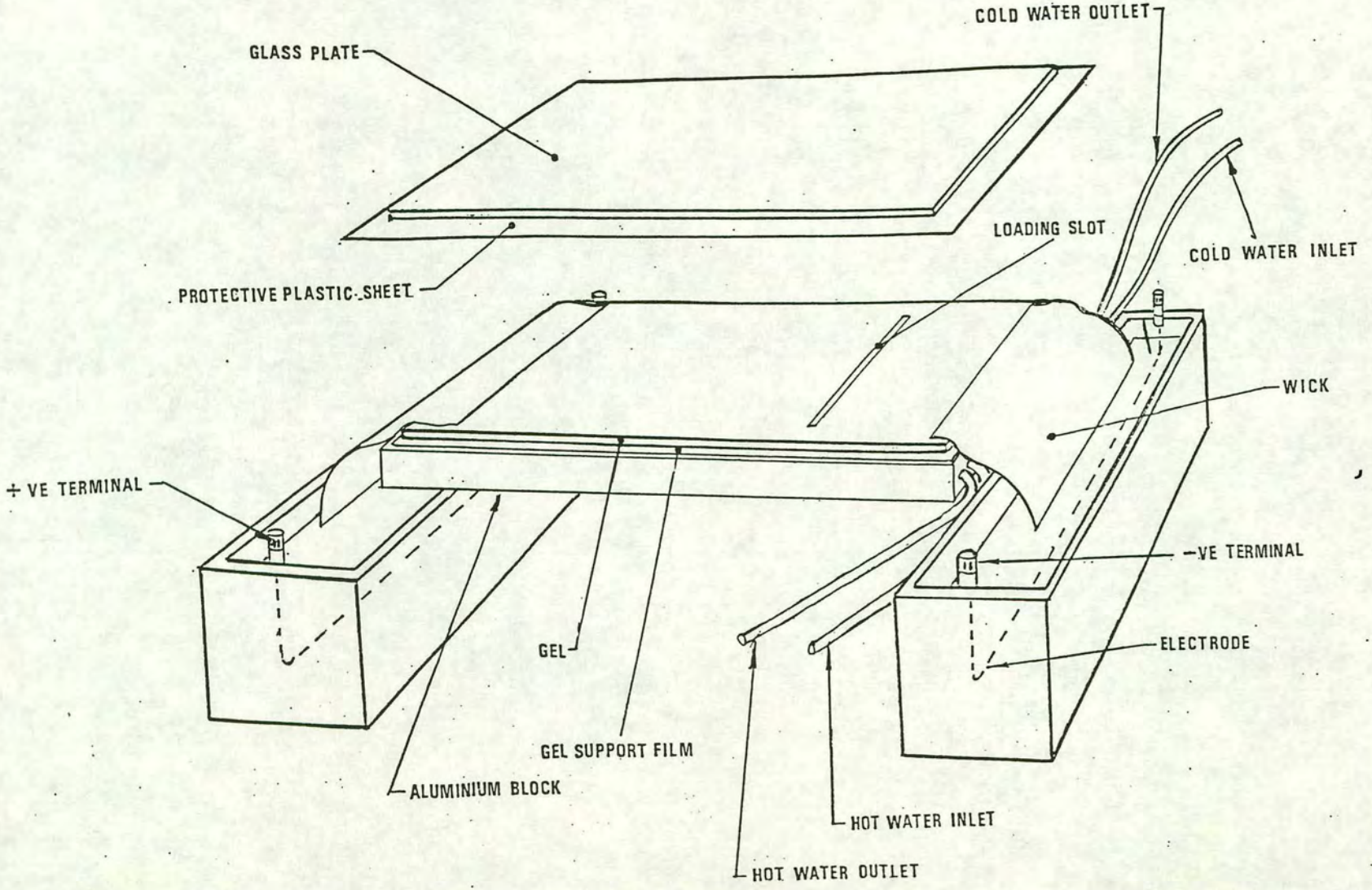
Fragments of the cytochrome b gene from the two clones 3D7 and HB3, amplified using the conditions outlined in Section 2.4.4 above. were purified using Geneclean II (Section 2.4.8 a). Approximately 500ng of DNA of each of the complementary

Figure 14. Temperature gradient gel electrophoresis (TGGE) apparatus

Figure 14.

Sketch of the Temperature Gradient Gel Electrophoresis (TGGE) apparatus in the 'Perpendicular' orientation.

The sample to be tested is run into the gel for a short distance. The current is removed and a temperature gradient established across the aluminium plate (and the gel), by means of the hot and cold water channels in the plate. The current is re-applied and the sample run until the dye front reaches the bottom of the gel.


COLD WATER OUTLET
GLASS PLATE
LOADING SLOT
COLD WATER INLET
PROTECTIVE PLASTIC SHEET
WICK
+ VE TERMINAL
-VE TERMINAL
GEL
ELECTRODE
GEL SUPPORT FILM
ALUMINIUM BLOCK
HOT WATER INLET
HOT WATER OUTLET

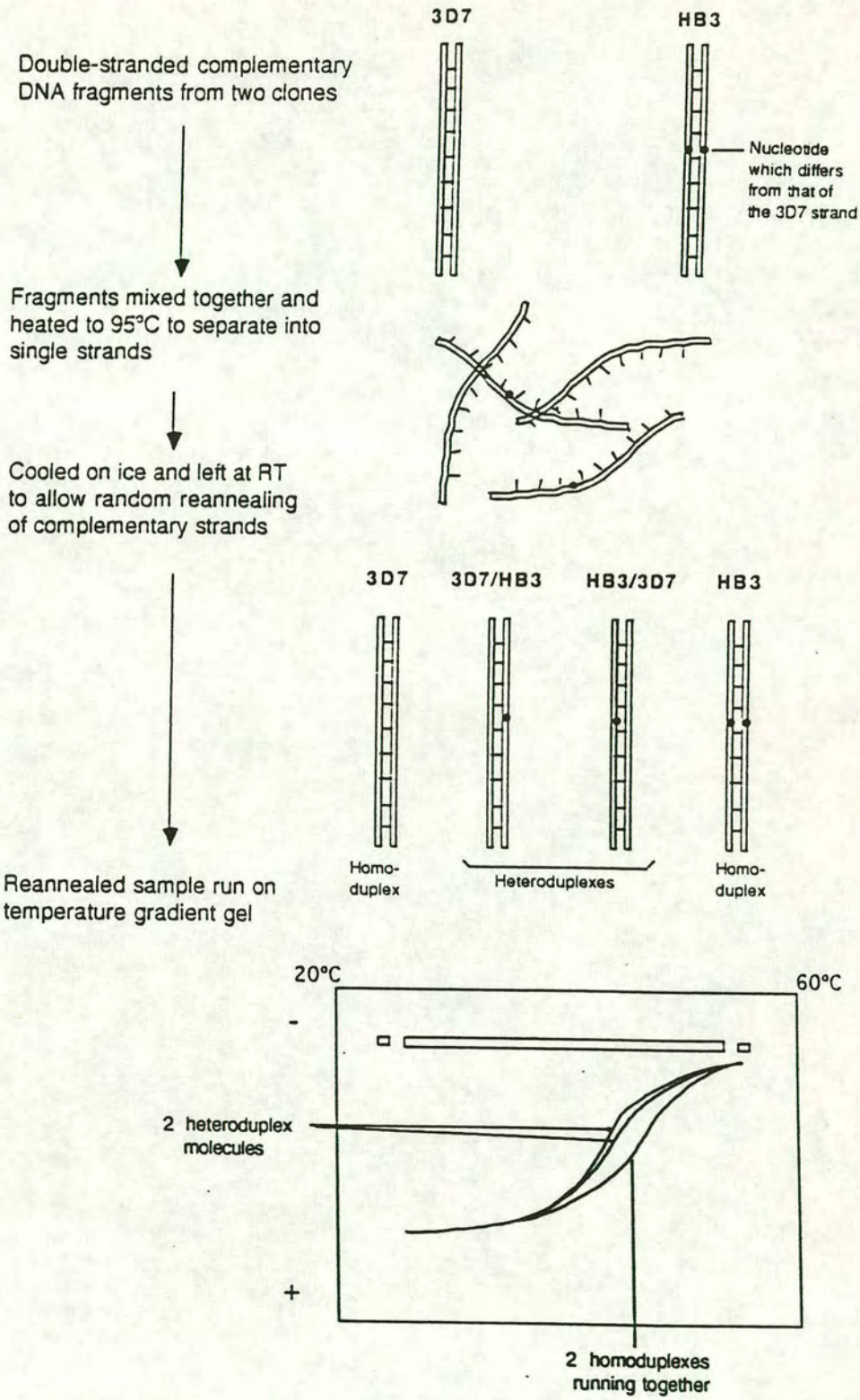


Figure 15. The principle of temperature gradient gel electrophoresis (TGGE) for identifying polymorphisms in double stranded DNA.

fragments from each clone were mixed together and made up to 300µl with distilled water. This sample was heated to 95ºC for 5 minutes, cooled on ice for two minutes and then left at room temperature for one hour to reanneal.

e) Preparation of the TGGE gel

The polyacrylamide gel was poured according to the manufacturer's instructions (Diagen). The optimum percentages of the acrylamide used varied between 5%-8% according to the length of the fragment being examined. An 8% gel was used for fragments of 100-300bp, a 6.5% gel for fragments of 300-500bp and a 5% gel for fragments of 600-800bp. Gels were made up in a modified 1 xTBE buffer (8.9mM Tris, 8.9mM boric acid and 0.25mM EDTA). Immediately prior to use 0.3ml of a 4% ammonium persulphate solution and 75µl of TEMED (N,N,N,',N'-tetramethylethylene-diamine) (Sigma) were added to initiate polymerisation. The gel was poured onto the hydrophilic side of a cellulose acetate sheet held in a mould consisting of two glass plates. When set, the glass plates were removed and the gel adhering to the acetate sheet was placed on the heating block of the gel tank.

f) Running the gel

The renatured DNA sample prepared as in Section b) above was loaded into the long moulded slot extending across the top of the gel. TGGE-modified 1x TBE was added to the two tanks containing the electrodes, the wicks applied to the top and bottom edges of the gel and held in place firmly with one of the glass plates. Current at 300V was applied and the sample run into the gel for 1 hour. The current was removed and the gel protected against dehydration with a sheet of plastic. Two temperature baths, adjusted respectively to 20ºC and 60ºC, were attached to the aluminium plate. A temperature gradient was allowed to establish over the plate and gel for half an hour. The current was then re-applied and the gel run overnight at 15V for 16 hours. The gel was removed and soaked in TGGE-modified 1xTBE buffer containing ethidium bromide at 0.5µg ml^{-1} for ten minutes, inverted onto a UV transilluminator and photographed.

g) Modifications to the standard TGGE technique for Plasmodium DNA analysis

The A+T richness of the Plasmodium genomes (between 68-92%A+T) created specific problems in the application of the TGGE technique. Initial work resulted in the DNA fragments denaturing very rapidly and springing apart to become single stranded molecules without a visible melting phase on the gel. Several strategies were employed to try to resolve this problem. These included the addition of a G+C clamp to the primers to try to slow down the melting process (Sheffield et al., 1989), the lowering of the steepness of the temperature gradient and various modifications of the polyacrylamide gel mix. Eventual success was obtained by reducing the denaturing urea in the gel mix from the recommended 4M to 2M or 1M or by omitting it altogether. In addition the gels were routinely run overnight at a low current rather than at the higher current and shorter times recommended. This kept the temperature caused by the current consistently low during the run, and also produced clear resolution of the bands.

h) Controls for the detection of polymorphisn by TGGE analysis

The conditions under which each set of complementary fragments denatures are specific for that particular sequence (Reisner et al., 1989). Controls therefore involve subjecting each of the two complementary fragments singly to the same de-naturation/re-naturation procedure and gel conditions as the experimental gel. This would confirm that each of the fragments mixed in the experiment consists of a single population of fragments with the same conformation. An example of this control is shown in Figure (Results Section).

2.5.2 Direct sequencing of PCR fragments

Where there was an indication on TGGE that there might be a polymorphism between two fragments of the chosen clones, direct sequencing of the fragment in question was carried out using the technique of Sanger et al (Sanger et al., 1977).

Forty microlitres of the PCR amplified fragment was purified using Geneclean II (Bio-101). The sequencing reaction was carried out using the Sequenase^{R} Version 2.0 enzyme and reagents (United States Biochemical Corp., Cleveland, Ohio, USA). The

standard protocol was modified by the addition of 10% dimethyl sulphoxide (DMSO) (Amersham) to the reaction, to reduce non-specific background and to reduce the formation of secondary structure (Winship, 1989).

a) Annealing the template

Six microlitres of the purified template DNA was combined in a sterile microfuge tube with 1µl primer (diluted to 10ng/µl in distilled water), 1µl DMSO and 2µl of the Sequenase Reaction Buffer (40mM Tris-Cl pH 7.5, 20mM $MgCl_2$, 50mM NaCl). The mixture was boiled for 3 minutes to denature the template and snap-cooled on ice to prevent renaturation. The annealed template was used within 4 hours.

b) Labelling the reaction

To the cooled annealed template was added 1µl each of DTT (DL-dithiothreitol), α-^{35}-S dATP, Sequenase Labelling mix (diluted 1:5 or 1:15 with distilled water) and Sequenase enzyme diluted 1:6 with Sequenase Enzyme Dilution Buffer (10mM Tris-Cl pH7.5, 5mM DTT, and 0.5mg-ml^{-1} bovine serum albumin {BSA}). The reaction was incubated at room temperature for 3 minutes.

c) Terminating the reaction

After the labelling reaction the sample (14µl) was divided into four and 3.5µl aliquots dispensed into four microfuge tubes each one containing 2µl of one of the prepared termination mixes, ddGTP, ddATP, ddTTP and ddCTP (supplied as 80µM of the deoxy-form and 8µM of the dideoxy-form for each mix). These termination reactions were allowed to proceed for 3 minutes at 37ºC when 4 µl of Stop Solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to stop further reaction. Each tube was centrifuged briefly and the samples were either loaded immediately onto a sequencing gel or stored for up to one week at -20ºC before use.

d) Sequencing Gels

Sequencing gels were run on the Baserunner (International Biotechnologies Inc.), Sequi-Gen (Bio-Rad) or Anachem Sequencing systems depending on the availability of the apparatus. The glass plates were thoroughly cleaned prior to use and the top

plate in each system coated with a siliconiser (Sigmacote™, Sigma) to prevent the gel adhering to this plate.

A 6% w/v polyacrylamide gel solution was prepared (8M urea, 6% acrylamide, 0.15% methyl-bis-acrylamide in 1 xTBE), filtered through a 0.4micron filter and de-gassed. Immediately prior to pouring, 0.7µl/ml of a 25% solution of ammonium persulphate and 0.7µl/ml TEMED (N,N,N,',N'-tetramethylethylene-diamine) were added to initiate polymerisation. For the Sequi-Gen system the gel was poured according to the manufacturer's instructions. For the Baserunner and Anachem systems the gel was poured onto the bottom sequencing plate and the top plate slid into place taking care not to trap bubbles. When set, the gel was pre-run for 30 minutes at 50W to reach a temperature of 56ºC. Three microlitres of each sample was loaded onto the warm gel and run at 50W for 2-5 hours. The gel was dried on a gel drier (Hoeffer Scientific Instruments) at 70ºC for two hours and exposed to Kodak X-OMAT AR film at room temperature overnight. Film was developed using the Compact X2 automatic developer (X-Ograph Ltd., Malmesbury).

2.5.3 Restriction digestion

Endonuclease restriction digestion of certain PCR amplified DNA fragments was carried out in three areas of this project.

1. Restriction endonucleases Acc I, Alu I, Bcc I, Dra I, Fok I, Hinc II, Sau3AI and Taq I (New England Biolabs. Ltd.) were used to screen 10 clones for restriction fragment length polymorphisms (RFLPs) within the cytochrome b gene in an attempt to find a marker which could be followed through a cross in mosquitoes. These clones originated from different geographical areas - from Brazil clones 7G8, IEC514/1, from Thailand T994, T996 and T998, from Indochina Dd2, from Sudan Sud105/9, Sud 123/5, from Honduras HB3 and clone 3D7 whose origin is discussed in Section 2.1.1.

2. The two forms of a polymorphism identified between clones 3D7 and HB3, could be distinguished using the enzyme Bfa-I, which cuts the HB3 form but not the 3D7 form at the polymorphism site. Endonuclease restriction digestion with Bfa-I was

used to screen 18 other clones and isolates for this polymorphism. The names and origins of parasites screened are shown in Table I. (Chapter 3. Section 3.2.3)

For the experiments in a) and b) above, 10µl of purified DNA was diluted with 36µl of distilled water, 4µl of a 10x solution of the appropriate NEB buffer 4 (50mM potassium acetate, 20mM Tris-acetate, 10mM magnesium acetate, 1mM dithiothreitol {pH7.9}) was added and 1-2 units of the enzyme.

3. When screening oocyst DNA for the polymorphism a smaller scale digest was routinely used in which 2µl of DNA was added to 7µl of water, 1µl of enzyme buffer and 0.5unit of enzyme.

All the digestions were incubated at 37ºC overnight and the products run on a 1% agarose gel stained with 0.5µg ml^{-1} ethidium bromide. The DNA fragments on the gel were visualised by UV transillumination and photographed.

2.6 Work using P. gallinaceum

2.6.1 P. gallinaceum parasites

Because of the fragile nature of the P. falciparum male microgametes (R. Carter, unpublished observations), it was not possible to purify them for use in experiments to determine whether the male gametes carried cytoplasmic DNA. Consequently this investigation was carried out using the avian (chicken) malaria, P. gallinaceum, in which microgametes are more robust. The P. gallinaceum line used was originally isolated from a chicken in Ceylon (Sri Lanka) (Brumpt, 1949).

2.6.2. Preparation of gametes of P.gallinaceum

Two types of gamete preparation were derived from the blood of chickens infected with P. gallinaceum. One consisted of purified male gametes and the other was a mixture of female gametes and fertilised zygotes, hereafter referred to as the female gamete preparation. Preparations of male and female gametes of P. gallinaceum, were carried out by Professor Kamini Mendis of the University of Colombo, Sri Lanka, working in the Laboratory of Professor Dyann Wirth at the Harvard School of Medicine Boston, Massachusetts.

10 ml of chicken blood containing gametocytes of P. gallinaceum was added to 50ml Tris bicarbonate saline buffer (10mM Tris, 30mM $NaHCO_3$, 166mM NaCl, 10mM glucose) containing 10% foetal calf serum at a pH of 8.2. These conditions triggered emergence of gametocytes from the red cells and exflagellation of the male gametocytes. After 30 minutes incubation at room temperature the cells were centrifuged at 2,000**g** for 5 minutes. The pellet, which contained host cells and fertilised and unfertilized female gametes, was resuspended in Tris buffered saline (166mM NaCl, 10mM Tris, 10mM glucose, pH 7.4) and the female gametes and zygotes harvested and purified as previously described (Kaushal and Carter, 1984). The supernatant from the 2,000**g** centrifugation containing free male gametes as well as cell membranes and debris, was centrifuged at 30,000**g**. All except 100 µl of the clear supernatant was quickly removed and the tube was placed at an angle for five minutes. This allowed the active male gametes to swim into the supernatant which was then harvested as the male gamete preparation (Figure 16). Both male gamete and female gamete preparations were approximately 98% pure, with respect to other contaminating cell material, as assessed by microscopic examination.

2.6.2 Analysis of male and female gamete preparations

a) Preparation of DNA

DNA was extracted from both male and female gamete preparations by conventional phenol/chloroform extraction techniques as described for the extraction of DNA from asexual parasites in Section 2.4.2 (Maniatis, et al., 1982).

The DNA concentrations were estimated following electrophoresis of 2µl of each sample on a 1.5% agarose gel containing ethidium bromide. Since the concentration of the DNA in the male preparation was very low, this sample was not diluted. The female DNA preparation was diluted to give three samples estimated as covering the same range of concentration as that of the male. The three diluted samples and the male sample were digested at 37ºC overnight, with HincII (Boehringer) according to the protocol in Section 2.5.3.

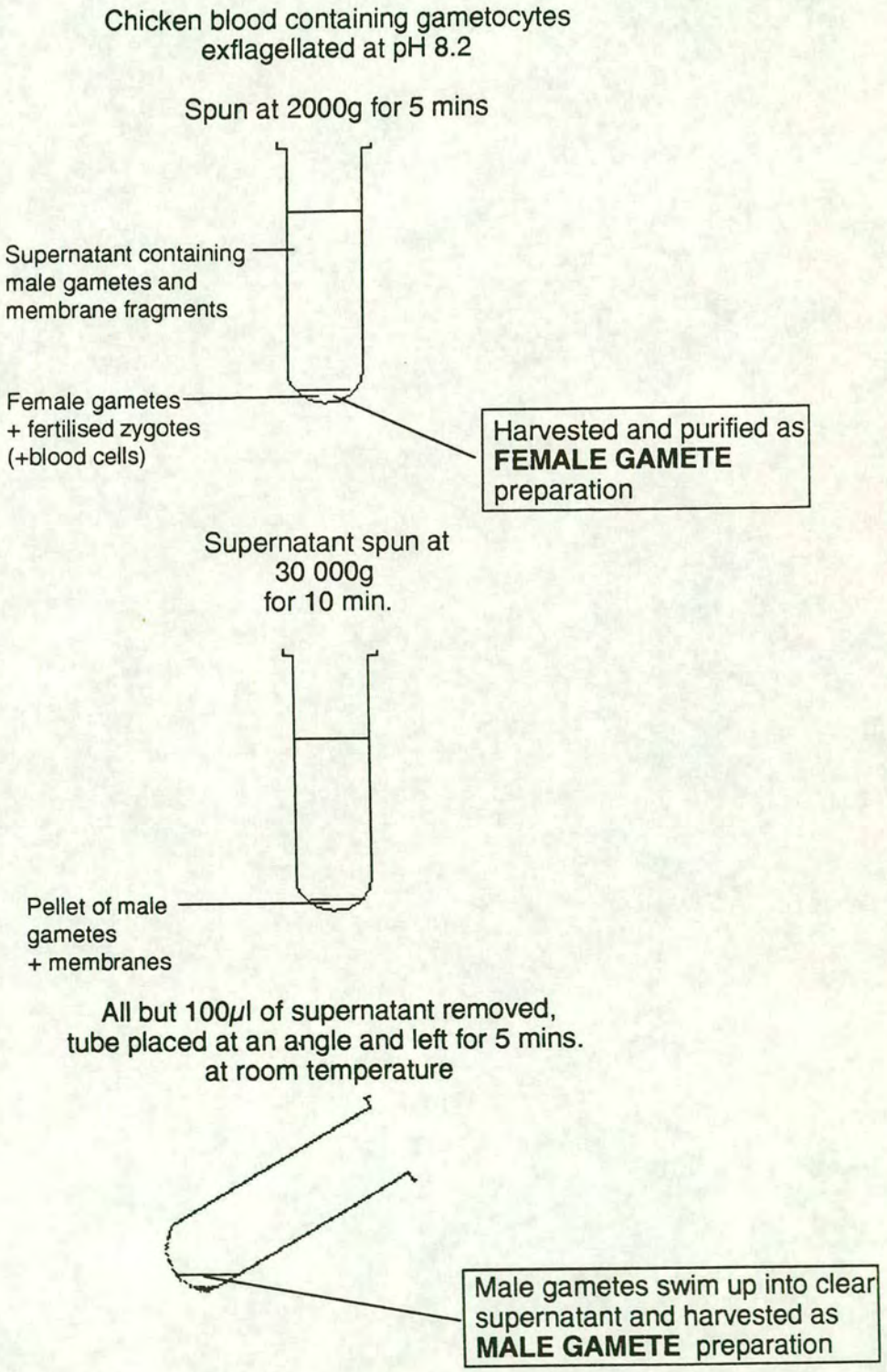


Figure 16. Preparation of male and female gametes of Plasmodium gallinaceum

b) Preparation of blot

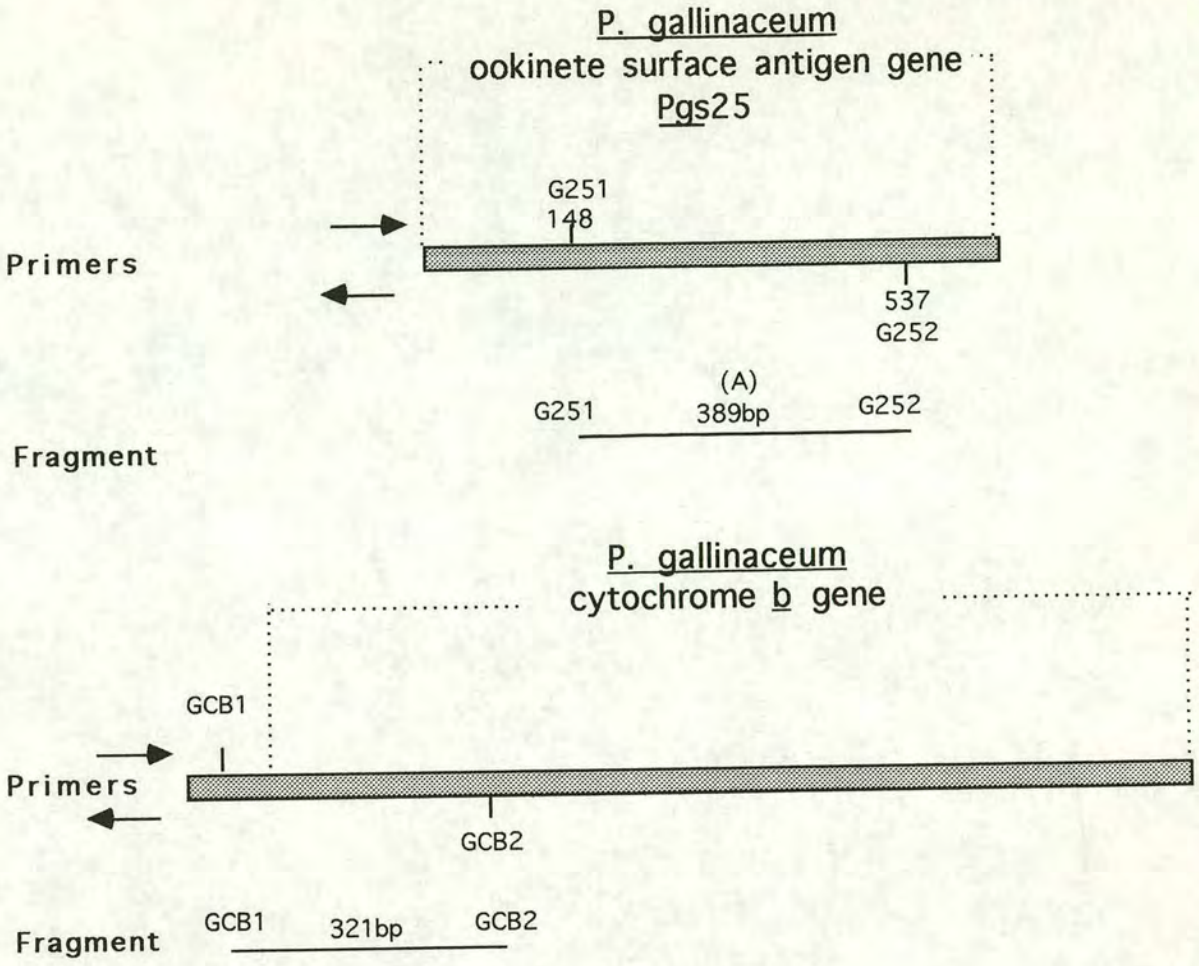
Each DNA sample with gel loading buffer (0.25% bromophenol blue, 0.05% xylene cyanol FF, 25% Ficoll, 10mM Tris-Cl, 1mM EDTA) at 1x, was run at 70V on a 1% agarose gel until the dye front reached the bottom of the gel. After electrophoresis the gel was soaked in 0.25M HCl until the colour of the dyes changed and then left an additional 10 minutes. The gel was soaked in distilled water while a capillary blotting tank was set up with 0.4M NaOH. The gel was blotted overnight onto nylon membrane (Hybond N+, Amersham) by alkaline transfer, essentially using the principle of Southern (Maniatis, et al., 1982).

c) DNA probes

i) Nuclear gene probe ; To determine the relative DNA concentrations in the preparations of male and female P. gallinaceum gametes, a fragment of a single copy nuclear gene, Pgs25 (Kaslow, et al., 1989), which encodes an ookinete surface protein, was amplified by the polymerase chain reaction (PCR) technique using a genomic P. gallinaceum DNA template. Primers 15 and 16 (Figure 17) were designed from the sequence found on the UWGCG database Accession No. J04008 and amplified a 389bp fragment. The conditions of the PCR are outlined in Figure 17.

ii) Mitochondrial cytochrome b gene probe; To determine the relative concentrations of the mitochondrial 6kb element in the male and female gamete preparations, a fragment of the P. gallinaceum cytochrome b gene (Aldritt, et al., 1989) was amplified using a genomic DNA template. Primers 13 and 14 (Figure 17) were designed from the published sequence (Aldritt, et al., 1989) and produced a fragment of 321bp at the 5' end of the gene. The PCR conditions used are shown in Figure 17.

iii) Probe for 35kb element; In the absence of any sequence information on the 35kb element in P. gallinaceum, primers G39 and G41 for the 35kb element in P. falciparum (see Figure 12) were used to amplify a fragment overlapping the 5' end of the rpoB gene using genomic DNA from the P. falciparum clone 3D7 as a template. An attempt to amplify this fragment using a P. gallinaceum template failed to give



Fragment	Primers	PCR conditions
Nuclear Pgs25 (A)	5' GTA CTA ACA TCT GAA AGT ACC TG 3" 5' CTT CCT TAT CGA AAG TGT AAC C 3'	95°C 30 sec 50°C 1 min 70°C 2 min (30 cycles)
6kb cytb (B)	5' TCA ACA ATG ACT TTA TTG G 3' 5' TTT GTT CTG CTA ATA G 3'	95°C 30 sec 45°C 30 sec 72°C 1 min (35 cycles)
35kb rpo B (C)	5' AAT AAT TGA ATA CAT GTT TTA TAT AAT C 3' 5' AAT TTT AAA GAA ATT AAT ATA TTT AAA T 3'	95°C 30 sec 42°C 30 sec 72°C 2 min (35 cycles)

Figure 17. Sketch maps of Plasmodium gallinaceum nuclear Pgs25 gene and 6kb cytochrome b gene showing the fragments used as probes of the male and female gamete DNA preparations and the primers and PCR conditions used to amplify them.

reliable results. The primers (Creasey, et al., 1994) and PCR conditions used are shown in Figure 17.

Samples from both the nuclear and the two extrachromosomal fragments were labelled with ^{32}P dATP by random-priming (Feinberg and Vogelstein, 1983), using a Boehringer Mannheim kit. Depending on the amount of DNA in each sample, between 2-5µl of DNA was placed in a 1.5ml microfuge tube together with 5µl of ^{32}PdATP, 2µl of the reaction mix (Boehringer Mannheim), and 1µl of Klenow enzyme and the mixture made up to 20µl with distilled water. The tubes were centrifuged briefly to bring all the liquid to the base of the tube before being incubated at 37ºC overnight. The probes were cleaned using Chroma Spin-30 columns (Clontech) according to the manufacturer's instructions. The matrix of each column was re-suspended by gentle inversion of the sealed column before the top was removed and the fluid was allowed to drip through the column into a 1.5ml microfuge tube for five minutes before spinning it at 1500**g** for 3 minutes. The waste fluid was discarded and the column centrifuged again to dry the matrix. After placing a clean tube on the base of the column, approximately 1µg of herring sperm carrier DNA was added to the restriction digest sample which was then carefully pipetted into the centre of the column. The column was centrifuged at 1500**g** for 5 minutes and the purified sample collected from the bottom of the microfuge tube.

d) Hybridisation of probes

The Southern blots of male and female gamete DNA preparations were hybridised successively with the three prepared probes

The blot was placed in a hybridisation tube and probed first with the nuclear Pgs25 probe on the rotisserie of a hybridisation oven (Hybaid) at 65ºC overnight. The blot was washed three times with 0.5xSSC pH 7.0 (3M NaCl, 0.3M Na_2 citrate,) +0.1%SDS, twice for five minutes each and then once for 30 minutes, all three washes being carried out at 65ºC. The washed blot was exposed to Kodak X-omat AR film at -70ºC for four hours before examination.

The blot was stripped between each probing exercise using two washes of 100mM NaOH, 15 minutes each, at room temperature, followed by one rinse in distilled water and two more 15 minute washes in 2x SSC. The blot was exposed to X-omat film overnight and checked to confirm that it was clear before proceeding with the next probing exercise.

The hybridisation procedure was repeated as before using the 6kb element cytochrome b gene probe. For this probe the blot was exposed to film at -70ºC for four hours, eight hours and overnight.

For the 35kb probe the blot was hybridised at a lower stringency of 50ºC overnight and washed with 2xSSC+0.1%SDS. before being exposed to film for four hours, overnight, and for six days. All autoradiographs were processed using a Compact X2 developer system (X-Ograph Ltd., Malmesbury). The autoradiographs were examined visually but where appropriate the density of the bands was read using a densotometer (Joyce Loebl) and a Phosphorimager (Molecular Dynamics Ltd.).

Chapter 3 RESULTS

3.1 The Search for an extranuclear DNA marker

3.1.1 The choice of extranuclear element and gene

To determine the inheritance pattern of malaria extranuclear DNA elements it was necessary to search for a polymorphic marker in the sequences of either the 6kb or the 35kb DNA elements between two P. falciparum clones which were infective to mosquitoes. The cytochrome b gene of the 6kb element was chosen for beginning this search for for several reasons : -

1. Published reports based on hybridisation and on limited sequence comparison suggested that there was a high degree of conservation of both extranuclear elements in Plasmodium spp.(Vaidya and Arasu, 1987; Joseph, et al., 1989; Wilson, et al., 1991; Wilson, et al., 1992). However these reports also suggested that the 6kb DNA element was slightly less conserved than the 35kb DNA element (Vaidya, 1989).

2. The cytochrome b gene of one clone of P. falciparum (clone BWC10) was published and available on the University of Wisconsin Genetics Computer Group (UWGCG) gene database.

3. The higher G+C content and the greater copy number of the 6kb element in the cell would facilitate PCR amplification and direct sequencing procedures

3.2 Polymorphism in the cytochrome b gene of the 6kb element

3.2.1 Restriction digestion of P. falciparum clones

The restriction digestion map of the P. falciparum cytochrome b gene obtained from the UWGCG gene database identified the enzymes Acc I, Alu I, Bcc I, Dra I, Fok I, Hinc II, Sau3A I and Taq I as suitable for restriction digestion of the Fragments A, B and C (Figure 12) covering the cytochrome b gene. Restriction digestion using these enzymes revealed no obvious polymorphism in the cytochrome b gene among ten different clones of P. falciparum. These clones were 7G8, and IEC514/1 from Brazil, K1, T994 and T996, from Thailand, Dd2 from Indochina, Sud105/9, and Sud 124/3 from Sudan, HB3 from Honduras, and 3D7 (possibly from W. Africa).

While many other isolates could have been examined in this way, most would need to be cloned before they could be used in a cross and their ability to produce infective gametocytes was largely unknown. It was decided, therefore, to seek for polymorphic markers by a more detailed examination of the cytochrome b sequences in the two well-characterised P. falciparum clones, 3D7 and HB3, which were already known to be suitable for crossing work in mosquitoes (Walliker, et al., 1987; Ranford-Cartwright, et al., 1991b).

3.2.2 Examination of clones 3D7 and HB3

a) Analysis of the cytochrome b gene by TGGE

Corresponding fragments of the cytochrome b gene from clones 3D7 and HB3 were mixed together, subjected to denaturation and renaturation and run on perpendicular temperature gradient gels as described in Section 2.5.1. Samples of Fragment A from 3D7 and HB3, amplified by primers 1 and 3 (Figure 12), showed a typical multiple-band pattern on the temperature gradient gel indicating that this sample contained molecules which denatured at slightly different temperatures from each other. This was in contrast to the samples B and C which each produced a single band indicating that all the molecules in the sample were denaturing uniformly at the same temperature (Figure 18). These results were repeatable and consistent when using PCR fragments amplified on three different occasions from three different DNA stocks.

It was concluded that a mismatch between the corresponding fragments of the two clones 3D7 and HB3 within Fragment A could be causing heteroduplex molecules and homoduplex molecules to denature at different positions along the temperature gradient. Fragment A was therefore sequenced to see if such a mismatch were present.

b) Sequencing of fragment A

Fragment A of clones 3D7 and HB3 was sequenced as described in Section 2.5.2. These sequences revealed a single base-pair transition at position 21 of the predicted cytochrome b open reading frame, a T (tyrosine) in 3D7 being replaced by a C (cytosine) in HB3 (Figure 19). This changed the sequence TTA to CTA at this

Figure18. Temperature gradient gel analysis showing polymorphism in the Plasmodium falciparum cytochrome b gene

Figure 18.

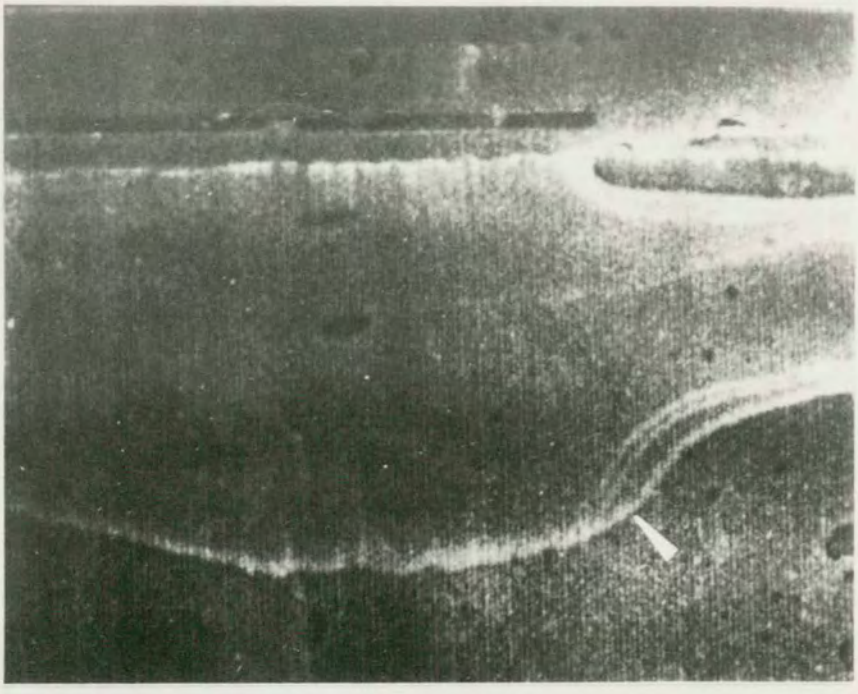
Temperature gradient gel electrophoresis analysis (TGGE) of a 623bp fragment of the cytochrome b gene (Fragment A) from Plasmodium falciparum clones 3D7 and HB3. The fragment was amplified using Primers 1 and 3 (see Figure 12).

Gel I The Experimental Gel.
The amplified products (Fragment A) from clone 3D7 and from clone HB3 were mixed together, denatured and renatured before TGGE analysis. The bands on the gel show the double-stranded DNA being denatured with increasing temperature across a gradient between 20ºC and 60ºC. The homoduplex molecules of 3D7 and HB3 appear almost together as the fastest running band (arrowed). The two heteroduplexes of 3D7 and HB3, being less stable, denature at lower temperatures and appear as the two slower running bands.

Gel II and Gel III (overleaf) Control Gels.
Amplified products from clone 3D7 (GelII) and clone HB3 (Gel III) were each subjected to the same denaturation and renaturation protocol and gel conditions as the mixed experimental sample. The single band on each gel indicates that separately these products are denaturing at the same temperature. This suggests that the population of DNA fragments is probably identical within each clone and it is only when mixed together and allowed to form heteroduplrxes that the differences between the fragments become detectable using the TGGE technique.

20°C 60°C

Origin

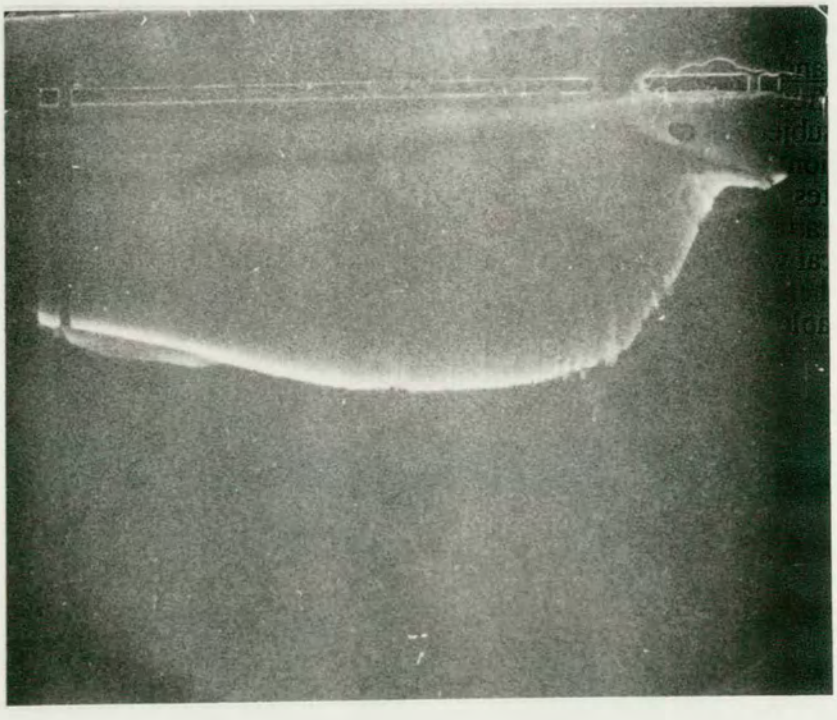


-

+

20°C 60°C

Origin

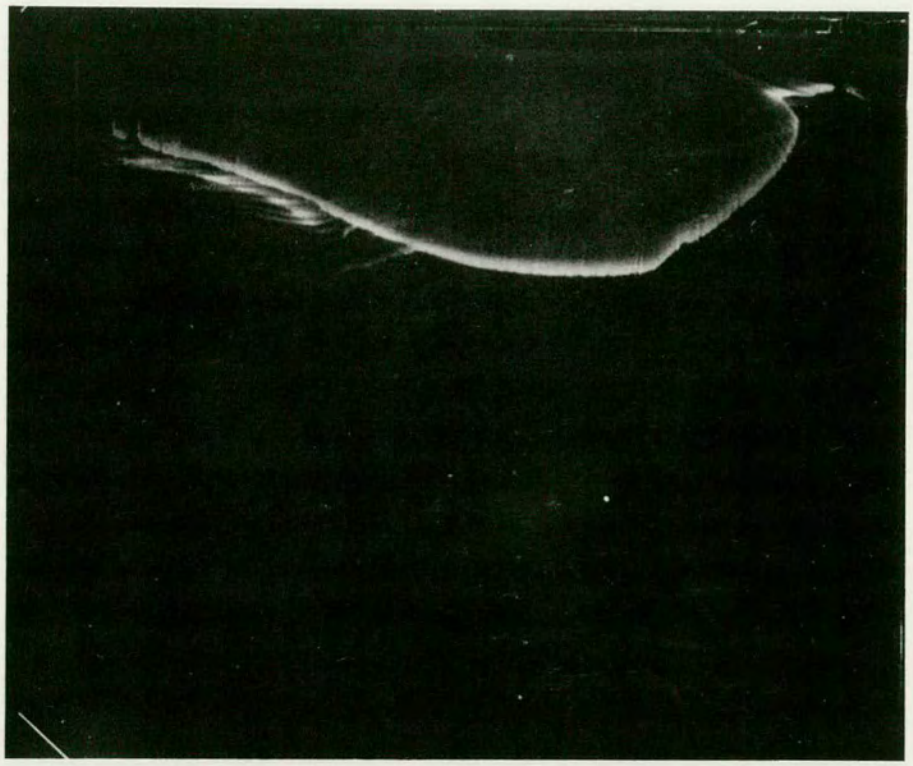


-

+

20°C 60°C

Origin



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\+

Figure 19. Sequence polymorphism in the Plasmodium falciparum cytochrome b gene

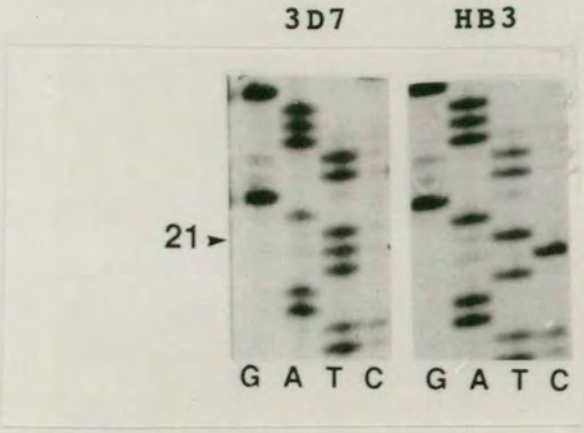
Figure 19

A) Sequencing gels of DNA fragments covering the site of the polymorphism at position 21 of the cytochrome b gene between clones 3D7 and HB3.

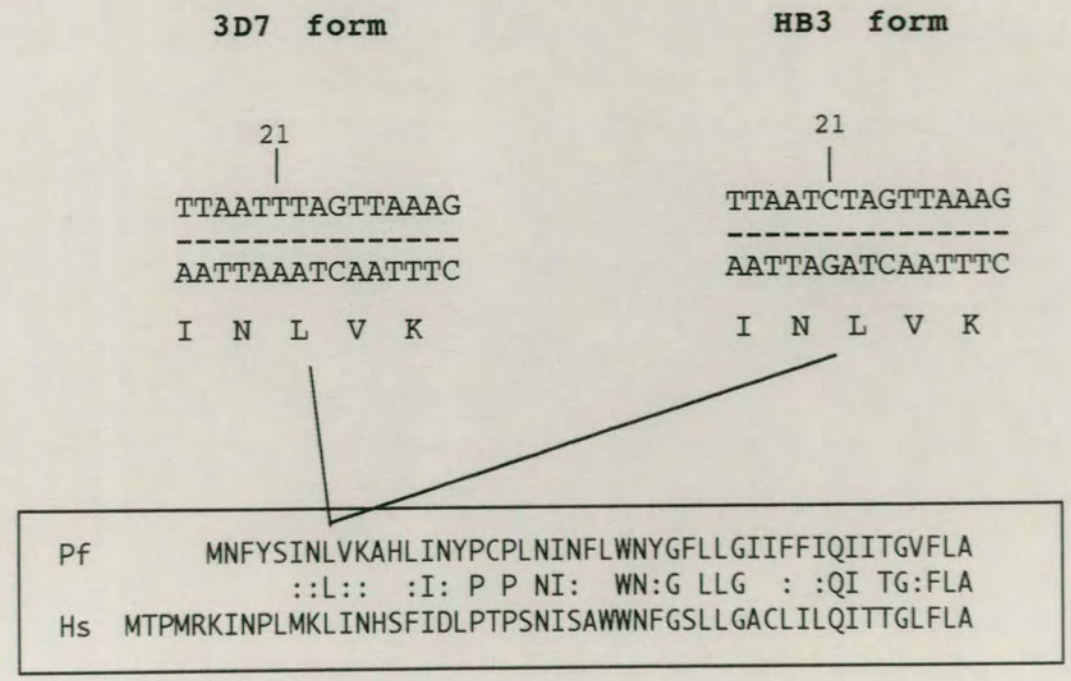
Lane 1 - 3D7 sequence
Lane 2 - HB3 sequence

B) Diagrammatic representation of the position of the polymorphism in the Plasmodium falciparum cytochrome b gene and the corresponding segment of the human cytochrome b gene.

A



B



Pf = Plasmodium falciparum

Hs = Homo sapiens

position but did not alter the predicted amino acid, leucine. Five different preparations of DNA from the two clones were sequenced in order to eliminate the possibility that this base change could be a Taq error in the PCR or an artifact of the sequencing procedure. Direct sequencing was also carried out on Fragments B and C (which had not shown the multiple band pattern on TGGE analysis) to confirm the absence of polymorphisms, as well as to check the sensitivity of the TGGE technique. No other differences were found between 3D7 and HB3 in the 75% of the gene that was sequenced.

c) Confirmation of polymorphism at position 21 by restriction digestion

The results of the sequencing of Fragment A from clones 3D7 and HB3 were fed into the UWGCG gene database SEQED programme (Devereux, et al., 1984) and, using the MAP command, an enzyme restriction map of the region covering the polymorphism was obtained (Figure 20A). This showed that it would be possible to distinguish between the two clones, using the restriction endonuclease enzyme Bfa-I. The enzyme was predicted to cut the HB3 clone, which includes the target sequence ATC|TAG, but not the 3D7 clone at the polymorphism site. No suitable enzyme was available which would cut the 3D7 sequence but not the HB3 sequence at this position. The enzyme T509-I, which cuts the 3D7 sequence, but not the HB3 sequence, at this site has the target sequence |AATT which occurs frequently throughout Fragment A and so would produce fragments too small to be resolved on an agarose gel.

Digestion of Fragment A from the two clones, using Bfa-I, confirmed the results obtained by direct sequencing ; the 3D7 product remained uncut and the HB3 product was cut into two fragments of the predicted sizes (351bp and 81bp; Figure 20 B).

d) Sequence-specific PCR

The two sequence specific primers, Primer 9 (containing the 3D7 form of the cytochrome b gene marker as the last base at the 3' end), and Primer 10 (containing the HB3 form of the marker as the last base at the 3' end), were used to see if a PCR reaction alone could distinguish between 3D7 and HB3.

Figure 20.

A. Endonuclease restriction map of the portion of the Plasmodium falciparum cytochrome b gene covering the polymorphism between clones 3D7 and HB3 at position 21. The target sequences of those enzymes which cut specifically at this site together with those which cut nearby are given below:-

Bfa I	5' ACT|TAG 3'		Ase I	5' AT|TAAT 3'	
T590 -I	5' |AATT 3'		Mse I	5' T|TAA 3'	

B. Agarose gel showing the restriction digestion products of Fragment A of the cytochrome b gene of clones 3D7 and HB3 using the restriction enzyme Bfa-I.

Lane 1 - 3D7 product
Lane 2 - HB3 product

A

Clone 3D7

Ase-I
Mse-I
T590-I
Mse-I

CTCTATTAATTTAGTTAAAGC

GAGATAATTAAATCAATTTCG

Restriction site Tsp590-I - AATT

Clone HB3

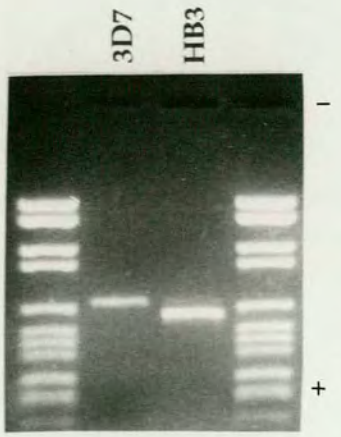
Ase-I
Mse-I
Bfa-I
Mse-I

CTCTATTAATCTAGTTAAAGC

GAGATAATTATATCAATTTCG

Restriction site Bfa-1 - ATCTAG

B



DNA marker fragment sizes :
2176, 1766,1230, 1033, 653, 517, 453, 394, 298, 234, 220

Primer 9 (with reverse Primer 3) used in a reaction with a denaturation temperature of 95ºC for 30 seconds, an annealing temperature of 49.5ºC for 30 seconds, and an extension time of 2 minutes for 35 cycles, consistently amplified a band from a 3D7 DNA template but not from an HB3 DNA template.

Primer 10 (with reverse Primer 3) was expected to amplify a fragment from HB3 DNA but not 3D7 DNA. This primer failed to give consistent specific results even when a range of annealing temperatures and PCR conditions was tried.

3.2.3 Polymorphism in other isolates

P. falciparum clones and isolates from various geographical areas were examined for the presence of the transition at position 21 of the cytochrome b gene. Eighteen clones/isolates examined all showed the 3D7 type marker at position 21 of the cytochrome b gene. The clones and isolates examined and their geographic origin are listed in Table I.

3.3 Search for polymorphism in the rpoB gene of the 35kb circle

A polymorphic marker was sought in the 35 kb element so that its inheritance could be followed in the hybrids already analysed for the inheritance of the 6kb marker. Fragments covering approximately 70% of the rpoB gene were amplified by PCR using the primers shown in Figure 12. Complementary fragments from clones 3D7 and HB3 were subjected to TGGE for the detection of polymorphism as described in Section 2.5.1 e).

While this work was in progress a polymorphism in this gene was reported between the clones Dd2 and HB3 (Vaidya, et al., 1993) at position 128 in the rpoB gene sequence where a valine ATT in Dd2 changes to a leucine TTT in HB3. This marker of the 35kb element followed the same inheritance pattern as the marker for the cytochrome b gene of the 6kb element in the cross examined.

TABLE I

Plasmodium falciparum clones and isolates screened for the polymorphism at position 21 of the cytochrome b gene.

Name	Clone/Isolate	Origin	3D7 type at position 21/ HB3 type at position 21
H1	isolate	Honduras	HB3
M23	isolate	Honduras	3D7
7G8	clone	Brazil	3D7
ITO	isolate	Brazil	3D7
51/84	isolate	Brazil	3D7
52/84	isolate	Brazil	3D7
565	isolate	Brazil	3D7
FPV-1	isolate	Brazil	3D7
FCB-1	isolate	Colombia	3D7
Dd2	clone	Indochina	3D7
K1	isolate	Thailand	3D7
T994	clone	Thailand	3D7
T996	clone	Thailand	3D7
Mad20	isolate	Papua New Guinea	3D7
FCR-3	isolate	Gambia	3D7
PA17	isolate	Uganda	3D7
WL	isolate	Nigeria	3D7
Ro33	isolate	Ghana	3D7

PCR amplification of Fragment A using primers 1-3 (Figure 12), was followed by restriction digestion with Bfa-I which cuts the HB3 form but not the 3D7 for of the polymorphism.

The high A+T content of the 35kb gene sequence made the PCR and sequencing exercises more difficult than for those of the 6kb cytochrome b gene. The cytochrome b gene marker, rather than an rpoB gene marker, was therefore used routinely to follow the inheritance of the extranuclear DNA in the P. falciparum cross between 3D7 and HB3.

3.4 Inheritance of the cytochrome b gene marker in a P. falciparum cross

The 3D7 x HB3 parasite cross in A. stephensi mosquitoes was performed by Ranford-Cartwright et al. and full details of this cross and analysis of the MSP-1 and MSP-2 nuclear gene alleles have been published (Ranford-Cartwright et al., 1993).

3.4.1 Gametocyte production

Clone 3D7 produced a greater number of mature gametocytes per 1000 red blood cells (rbc) than clone HB3 giving an average of 12.3/1000 rbc for 3D7 and 6.8/1000 rbc for HB3. The standard deviation was also greater in HB3 (Table II). There was a consistent difference between the proportion of males to females in the 3D7 and HB3 cultures, the HB3 producing fewer females to males than the 3D7 (Table III). This difference between the two clones is significant as assessed by the Student's t test (t_S = 5.04) (Ranford-Cartwright, et al., 1993).

3.4.2 Mosquito infection and oocyst harvest

Gametocytes from clones 3D7 and HB3 which were mixed together (1:1) and fed to Anopheles stephensi mosquitoes as described in Section 2.3.1 resulted in 9 successful feeds. In these feeds the 3D7/HB3 mixtures and both parental control feeds produced infections at a density suitable for isolation by micro-dissection of single oocysts (Ranford-Cartwright, 1991b). A total of 110 single oocysts from the mixed 3D7/HB3 feeds were analysed in this project.

3.4.3 PCR analysis of nuclear genes MSP-1 and MSP-2

Since each parental clone produced both male and female gametes, zygotes resulting from both self fertilisation and cross fertilisation events were expected. Single oocysts were analysed using the nested PCR technique described in Section 2.4.6. and

TABLE II

GAMETOCYTE PRODUCTION

Clone	Mean no. mature gametocytes/per 1000 rbcs
3D7	6.1 (4.4)
HB3	4.8 (3.1)

The numbers of stage V gametocytes in each culture flask were estimated from the smears taken on day 14 or 17 of culture and prior to feeding. They are expressed as the mean number of gametocytes per 1000 red blood cells. for the 9 successful feeds that produced the 110 oocysts analysed in this study. Standard deviations are shown in brackets.

TABLE III

GAMETOCYTE SEX RATIOS

Clone	No. of slides examined	Mean % of mature females	Standard deviation SD	Variance
3D7	33	91.7	± 5.98	35.8
HB3	18	82.1	± 7.37	54.3

The sex ratio data was taken from the culture smears taken on the day of the feed. The proportions of male and female gametocytes in each culture for the 9 feeds were estimated and expressed as the mean number of female gametocytes as a percentage of the total number of mature gametocytes of both sexes (Carter, et al., 1979).

(Data reproduced with kind permission - Ranford- Cartwright et al. 1993).

(Ranford-Cartwright, et al., 1993). Fragments of the polymorphic regions of the MSP-1 and MSP-2 genes showed that the alleles of each gene were readily identified as different-sized products on ethidium bromide stained gels :-

The MSP-1 gene primers produced fragments of:-

645bp (565bp nested) for clone 3D7

552bp (472bp nested) for clone HB3

The MSP-2 gene primers produced fragments of

726bp (534bp nested) for clone 3D7

678bp (486bp nested) for clone HB3.

Of the 110 oocysts, 60 oocysts were typed for both nuclear genes MSP-1 and MSP-2. Fifty oocysts were typed for either the MSP-1 gene or the MSP- 2 gene only : these samples typed successfully with the set of primers for one of the genes but failed to give a result with the set for the other gene. The reasons for this difference are not known. It is thought that differences in quality of the DNA from individual oocysts and/or different binding capacities of the respective primers may influence these results. In summary, approximately 19% of the oocysts typed successfully for the MSP-1 gene were not typed for the MSP-2 gene and 23% of the oocysts typed successfully for the MSP-2 gene were not typed for the MSP-1 gene.

For the purposes of the present analysis, demonstration of both parental bands for either of the nuclear genes was sufficient for defining a hybrid oocyst.

The 110 oocysts typed from the cross comprised;-

3D7 x 3D7 type homozygotes	37
HB3 x HB3 type homozygotes	14
3D7 x HB3 heterozygotes	59
Total	110

These proportions, of homozygote to heterozygote oocysts, are not significantly different from those which are expected for random mating between the gametes (assessed by the chi^2 value at the 0.05 level) as shown overleaf:-

Gamete (or allele frequency) ;-

$$3D7 \quad \frac{(37+37+59)}{220} = 0.60454$$

$$HB3 \quad \frac{(14+14+59)}{220} = 0.39545$$

If 3D7 = p and HB3 = q the Hardy-Weinberg expectations for random mating are :-

$$p^2;2pq;q^2 = 0.36547;0.47813;0.15638$$

and the expected number of homozygotes and heterozygotes:-

3D7 x 3D7 homozygotes = 0.36547 x 110 = 40.2017

HB3 x HB3 homozygotes = 0.15368 x 110 = 16.9048

3D7/HB3 heterozygotes = 0.47813 x 110 = 52.5943

	Observed	Expected(E)	Deviation(D)	$\frac{D^2}{E}$
3D7 homozygous	37	40.2017	-3.2017	0.25499
HB3 homozygous	14	16.9048	-2.9048	0.60270
3D7/HB3 heterozygous	59	52.5943	+6.4057	0.78018
			x^2 =	1.63787

At the 5% probability level $p< 0.05 = 3.84$, therefore the observed proportions of homozygotes to heterozygotes are not significantly different from those predicted during random mating.

These results are consistent with analyses already published for this same cross. These were analyses of 98 oocysts typed for the MSP-1 alleles, and 87 oocysts typed for the MSP-2 alleles (Ranford-Cartwright, et al., 1993) and 60 oocysts typed for both the MSP-1 and MSP-2 alleles (Ranford-Cartwright, 1994).

3.4.3 Inheritance of the cytochrome b marker in the hybrid oocysts

The 59 oocysts from the cross found to be hybrid by their possession of the two alleles of the nuclear genes were examined for the cytochrome b gene markers which distinguish between clones 3D7 and HB3 (Section 3.2.2).

a) Sequencing

Direct sequencing of Fragment A of the cytochrome b gene (which contains the polymorphic marker) was carried out on DNA from each of the 59 hybrid oocysts using the protocol described in Section 2.5.2.

All 59 oocysts showed only one form (and never both forms) of the parental cytochrome b marker. Fifty eight oocysts possessed the 3D7 form TTA at position 21, and one oocyst (oocyst number 33) possessed the HB3 form CTA (Figure 21, Lanes 1 and 2).

As a control, DNA taken from self-fertilised oocysts of both parental types were mixed together (1:1) amplified and sequenced and the results showed that both forms of the gene were detectable when both forms were present (Figure 21, Lane 3).

b) Restriction digestion

The results obtained by direct sequencing were compared with those obtained by digestion of the 59 oocyst PCR products using Bfa-I. Only the one product (from oocyst number 33) was cut by the enzyme this being the same one identified as possessing the HB3 marker at position 21 of the sequence.

c) Summary

The results of the sequencing and the restriction digestion of the polymorphic marker in the 59 hybrid oocysts suggested that the inheritance of the cytochrome b gene in P. falciparum, is uniparental (Creasey et al., 1993). As this was the first time uniparental inheritance of a malaria extranuclear gene had been shown experimentally, considerable effort was made to check these results before publication. The fragment from each oocyst DNA was sequenced at least twice, and a set of the PCR products sent to the National Institute for Medical Research (N.I.M.R) Laboratories, Mill Hill in London where the sequencing was repeated again (by D.J. Moore in the laboratories of Drs R.J.M. Wilson and D.H. Williamson), and these results confirmed. A summary of the results is illustrated in Figure 22.

3.4.5 Inheritance of the extranuclear marker in cloned progeny of a 3D7xHB3 cross

Twenty clones which were the progeny of the 3D7 x HB3 cross through chimpanzees (Walliker, et al., 1987) were examined by restriction digestion with Bfa-I. All 20 clones showed the 3D7 type marker at position 21 of the cytochrome b gene. This result was consistent with the dramatic bias towards the one form of heterozygote which was seen in the oocyst results of the 3D7 x HB3 cross in the present study.

Figure 21. Sequence of polymorphism at position 21 of the cytochrome b gene between Plasmodium falciparum clones 3D7 and HB3

Figure 21.

Sequencing gels of DNA from hybrid oocysts from the cross between clones 3D7 and HB3. The area shown covers the site of the base change in the cytochrome b gene which was used as a marker.

Lane 1 DNA from a hybrid oocyst (oocyst no. 12) showing only the 3D7 form at position 21

Lane 2 DNA from a hybrid oocyst (oocyst no. 33) showing only the HB3 form at position 21

Lane 3 DNA from self-fertilised oocysts from each of the parental clones 3D7 and HB3, mixed together before amplification and sequencing

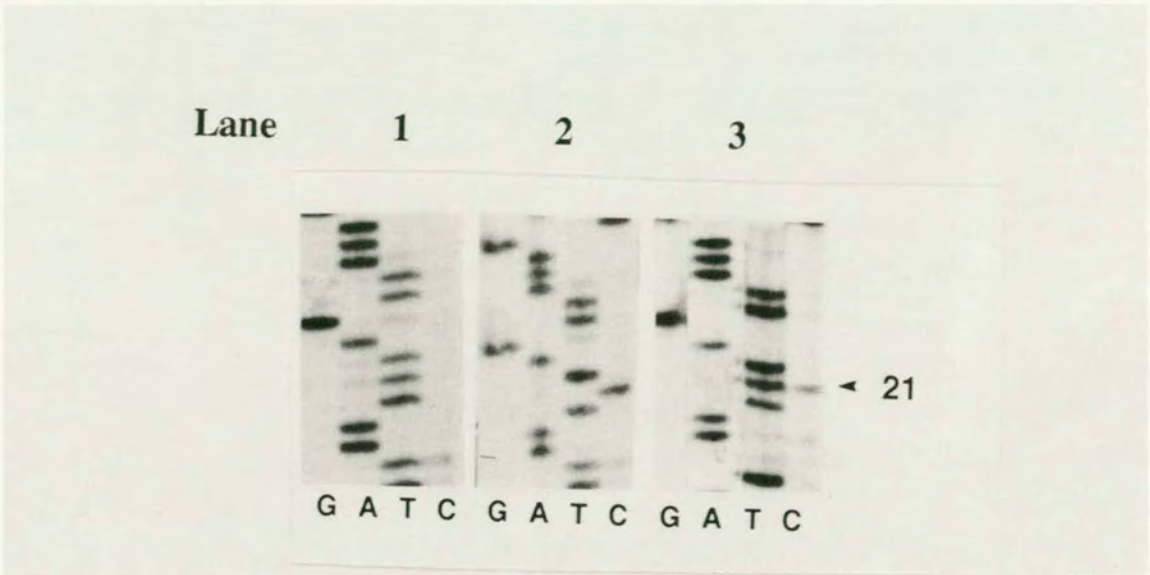

Lane
1
2
3
21
G A T C G A T C G A T C

Figure 22. Summary of the results of oocyst DNA analysis from the Plasmodium falciparum cross between clones 3D7 and HB3

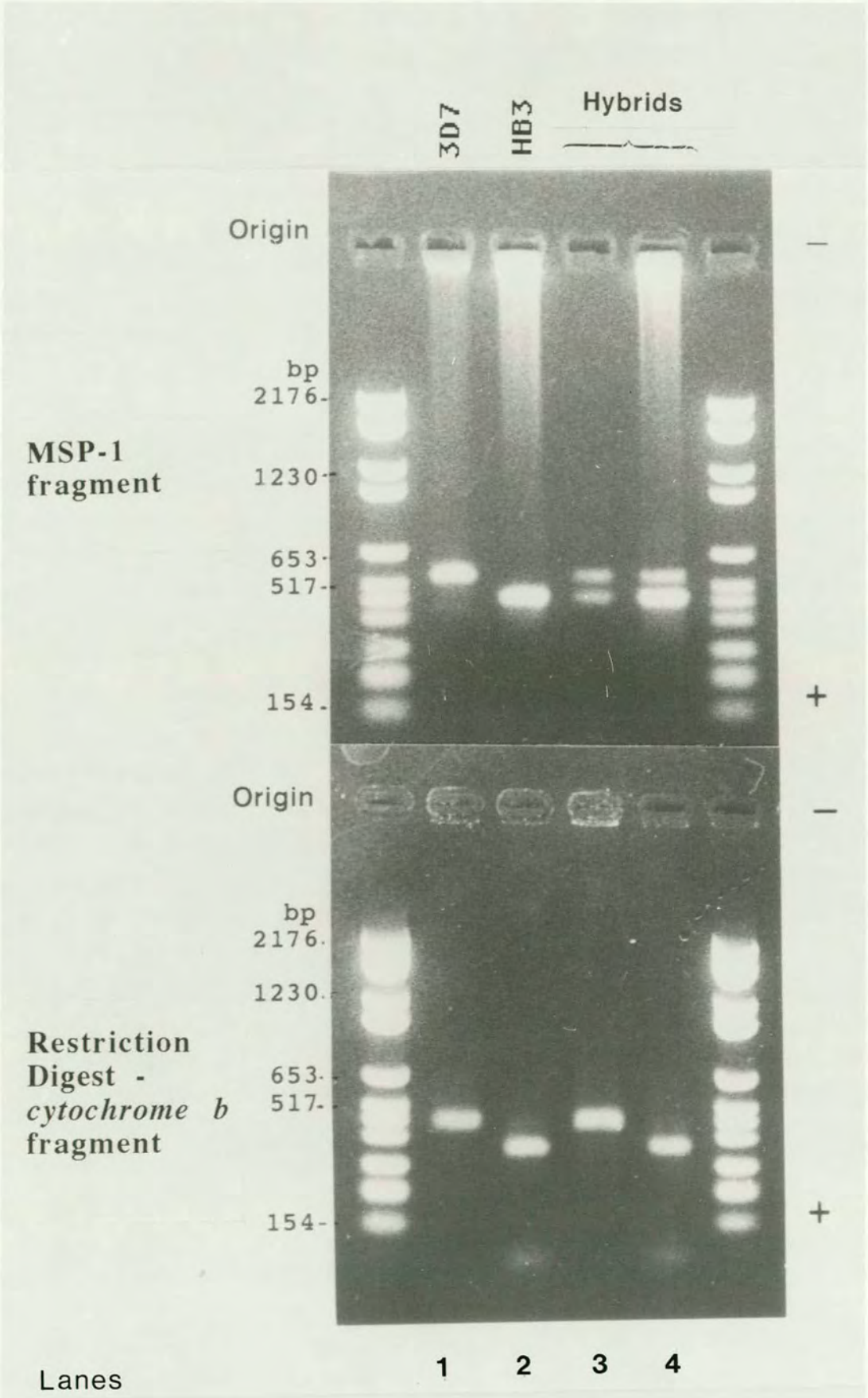
Figure 22.

Gels showing a summary of the results of the analysis of nuclear and extranuclear gene fragments amplified from oocysts of the cross between Plasmodium falciparum clones 3D7 and HB3.

Upper Gel - PCR-amplified fragments of the nulear gene MSP-1

Lower Gel - Restriction digestion products of PCR-amplified fragments from the extranuclear gene cytochrome b

For both gels :-
Lane 1 DNA from asexual culture of clone 3D7
Lane 2 DNA from asexual culture of clone HB3
Lane 3 DNA from hybrid oocyst (oocyst no. 5) from a cross between 3D7 and HB3 showing only the 3D7 form at position 21
Lane 4 DNA from hybrid oocyst (oocyst no.33) from a cross between 3D7 and HB3 showing only the HB3 form at position 21


3D7
HB3
Hybrids
Origin
bp
2176
1230
653
517
154
MSP-1
fragment
Origin
bp
2176
1230
653
517
154
Restriction
Digest -
cytochrome b
fragment
Lanes
1
2
3
4

3.5 Analysis of male and female gamete DNA preparations of P. gallinaceum

The uniparental inheritance of an extranuclear gene marker among hybrid oocysts of a cross prompted the question of whether extranuclear DNA was transmitted through the male or female gamete. One approach to this question was made by probing purified male and purified female gamete DNA using sequences specific for the extranuclear DNA elements.

Since male gametes of P. falciparum are too delicate to purify successfully (R. Carter - personal communication), it was decided to carry out this experiment using gametes of P. gallinaceum, the avian malaria parasite. (Section 2.3.2 and Figure 16). Sequence analysis of rRNA genes suggests that P. gallinaceum is closely related to P. falciparum (Waters et al., 1991).

3.5.1 Results of Southern blot probing of the male and female gamete DNA

DNA from the male and from the female gamete preparations, digested with Hinc II, and blotted onto nitrocellulose, was probed successively for the nuclear gene Pgs25 and the two extranuclear genes, cytochrome b (from the 6kb element) and rpoB (from the 35kb element), using the methods outlined in Section 2.6.3.

a) Nuclear gene probe Pgs 25

This probe had been designed to indicate the total amount of DNA in the tracks of the male preparation and three dilutions of the female preparation.

Visual examination and densitometric readings of the Pgs25 bands of the Southern blots showed that of the three dilutions of female gamete DNA, one track contained approximately three times more DNA than the male, one female track contained slightly more and the third contained approximately five times less than the male gamete preparation. This latter track was chosen for comparison with the male track so that estimation of any hybridisation of probes to the male gamete DNA would be as accurate as possible (Figures 23 A and 24 A).

b) 6kb element cytochrome b probe

The cytochrome b gene probe hybridised very strongly to the female track but was undetectable in the male track after 4 hours exposure to the autoradiograph. (Figure 23 B). Following more prolonged exposure of the film to the blot (8 hours and overnight), a faint band in the male track was detected at the same position as the cytochrome b gene probe on the female track.

Densitometric readings were taken of the strength of hybridisation signal from the cytochrome b gene probe, in relation to that of the nuclear gene probe, in the male and female gamete DNA tracks on the overnight exposure, (Figure 24) and expressed as follows ;

$$\frac{\text{Density female cytochrome b band}}{\text{Density male cytochrome b band}} \times \frac{\text{Density male nuclear band}}{\text{Density female nuclear band}}$$

$$\frac{17{,}710}{1{,}506} \times \frac{3{,}334}{661} = 61.26$$

Thus in relation to the amount of DNA recognised by a probe for the nuclear genome, there appeared to be at least 60 times as much DNA recognised by the probe for the 6kb element in the preparation of female gametes, compared to that in the male gametes.

The same autoradiograph was also analysed using a Molecular Dynamics Phosphorimager (per kind favour of Dr R.J.M. Wilson - Mill Hill London). This demonstrates that the readings for all three exposures of the female band (4 hours, 8 hours and 25 hours) were in linear progression. In the male track where a reading was detectable this was less than 2% of the reading in the female track (Figure 25). Thus in relation to the amount of DNA recognised by the probe for the nuclear genome there was less than 0.4% of the signal for cytochrome b in the preparation of male gametes compared to that in the female gametes.

Figure 23. Southern blot of Plasmodium gallinaceum gamete DNA probed with genes from the nuclear, 6kb and 35kb genomes

Figure 23.

Autoradiograph of a Southern blot of Plasmodium gallinaceum male (M) and female (F) gamete DNA preparations probed successively with three different probes.

A - probed with a fragment of the nuclear gene Pgs25
B - probed with a fragment of the 6kb cytochrome b gene
C - probed with a fragment of the rpoB gene of the 35kb circle

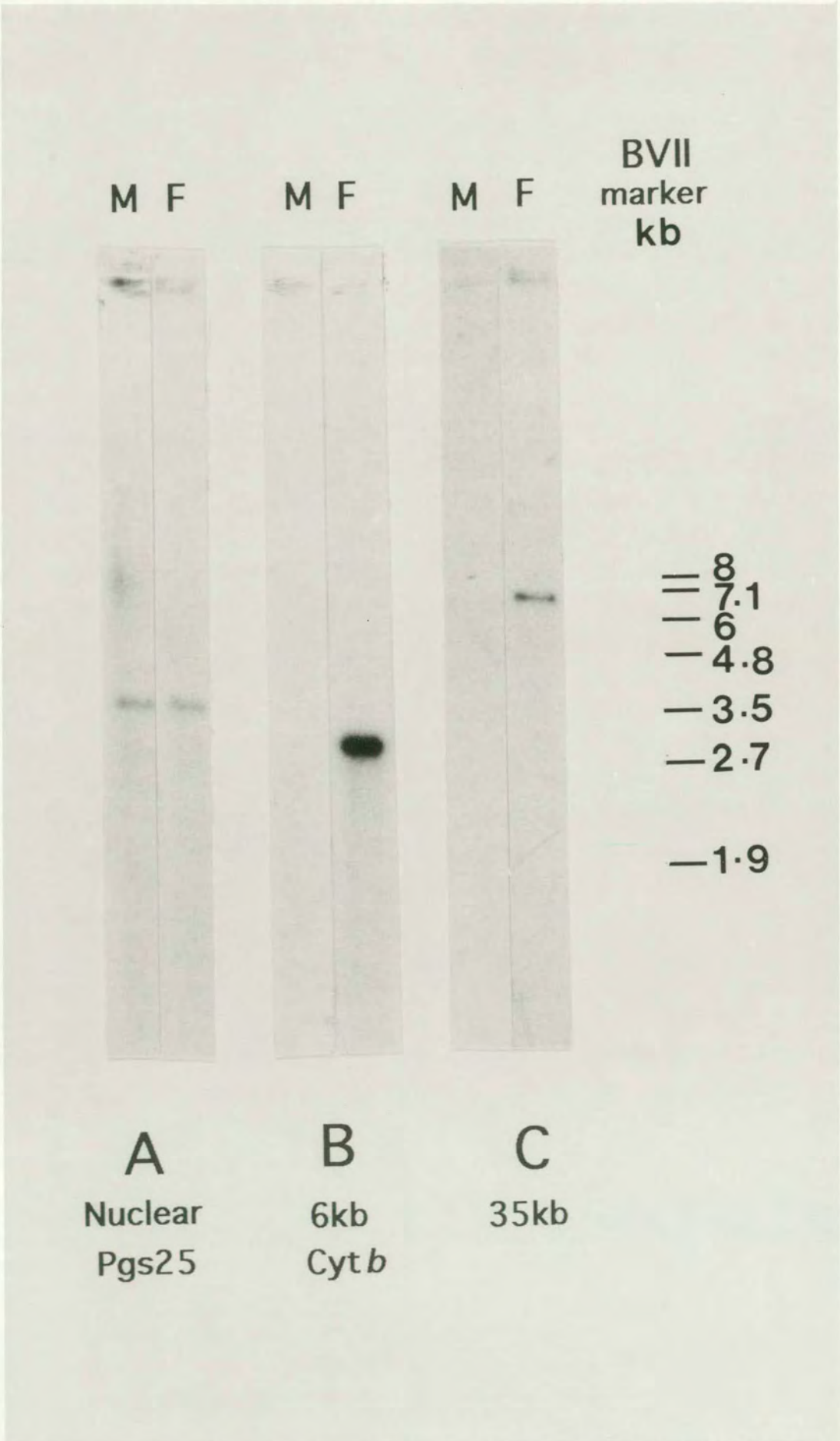

M F
M F
M F
BVII
marker
kb
8
7·1
6
4·8
3·5
2·7
1·9
A
Nuclear
Pgs25
B
6kb
Cyt b
C
35kb

Figure 24. Densitometry of Plasmodium gallinaceum male and female gamete DNA hybridised with probes of nuclear and extranuclear genomes

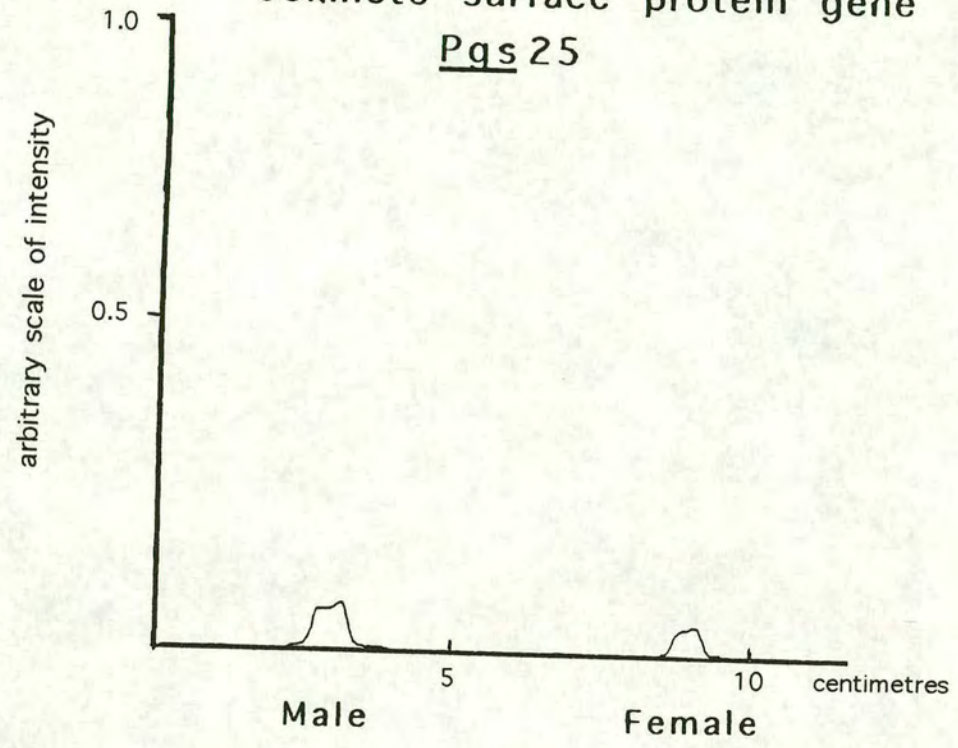
Figure 24.

Densitometric traces of the Southern blot of Plasmodium gallinaceum male and female gamete DNA preparations probed with:-

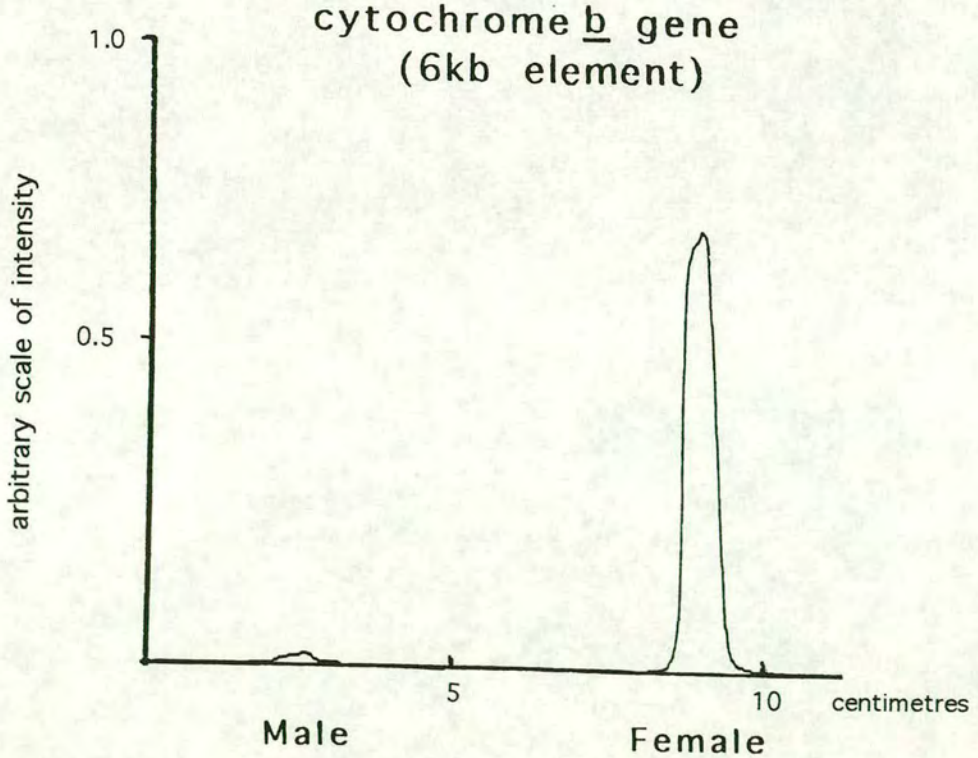
A - a fragment of the P. gallinaceum nuclear gene, Pgs25.
(integral male = 3343, female = 1506)

B - a fragment of the P. gallinaceum extranuclear 6kb element cytochrome b gene
(integral male = 640, female = 17710)

NUCLEAR GENE PROBE
ookinete surface protein gene
Pgs 25



EXTRANUCLEAR GENE PROBE
cytochrome b gene
(6kb element)



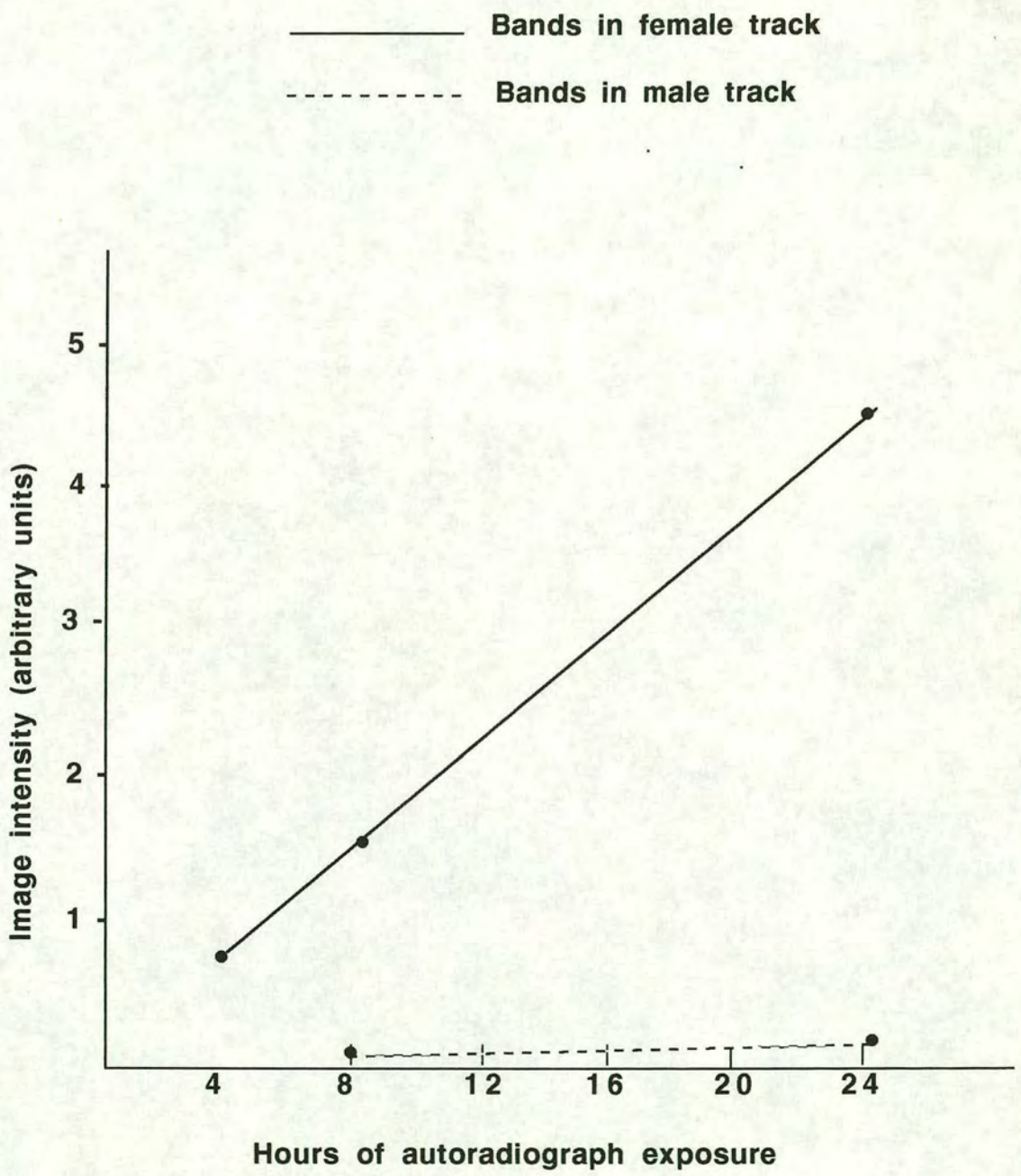


Figure 25. Phosphorimager graph of the fluorometric readings of three different exposures of the Southern blot autoradiograph of Plasmodium gallinaceum male and female gamete DNA preparations probed with the cytochrome b gene sequence. The graph shows that the intensity of the band in the male gamete track is approximately 2% of that in the female track (see text).

c) 35kb element rpoB gene probe

The rpoB gene probe from the 35kb element showed a single clear band in the female track but no visible hybridisation in the male track following 8 hour (Figure 23C) and overnight exposures. Following a six-day exposure of the film to the blot, there was still no band visible in the male track, at the position of the 35kb fragment in the female track.

Hybridisation of the probe for the 35kb element in the female track was at a lower intensity compared to that of the 6kb element. This probably indicates that the P. falciparum probe used contained differences in sequence from that of the homologous sequence in P. gallinaceum. However a lower intensity of hybridisation for this gene probe is also consistent with the lower number of copies of the 35kb (estimated by dot blot analysis as 1-3 copies per cell in Plasmodium spp. (Feagin, 1994)), compared to around twenty copies of the 6kb element per cell in P. gallinaceum (Joseph, et al., 1989).

3.5.2 PCR analysis using male and female gamete DNA

The male and female gamete DNA preparations were used as templates for PCR amplification using primers recognising the P. gallinaceum nuclear gene (Pgs 25) and extranuclear gene (cytochrome b). Fragments of both genes could be amplified from adequate quantities of both the male gamete and the female gamete DNA templates. However, by careful dilution of the templates, it was possible to find a dilution of the male gamete DNA at which the nuclear gene (of which there is one copy per gametocyte) was amplified clearly but at which the extranuclear gene (of which there are about 20 copies per gametocyte) could no longer be amplified. Conversely it was possible to find a dilution of the female gamete DNA at which the nuclear gene could no longer be detected but the extranuclear gene was easily detected. The results of this exercise are presented in Figure 26. Bearing in mind that PCR is not a quantitative technique, these results nevertheless support the view that the quantity of extranuclear DNA in the male gamete preparation was very small and probably a result of contamination with female gamete DNA during preparation.

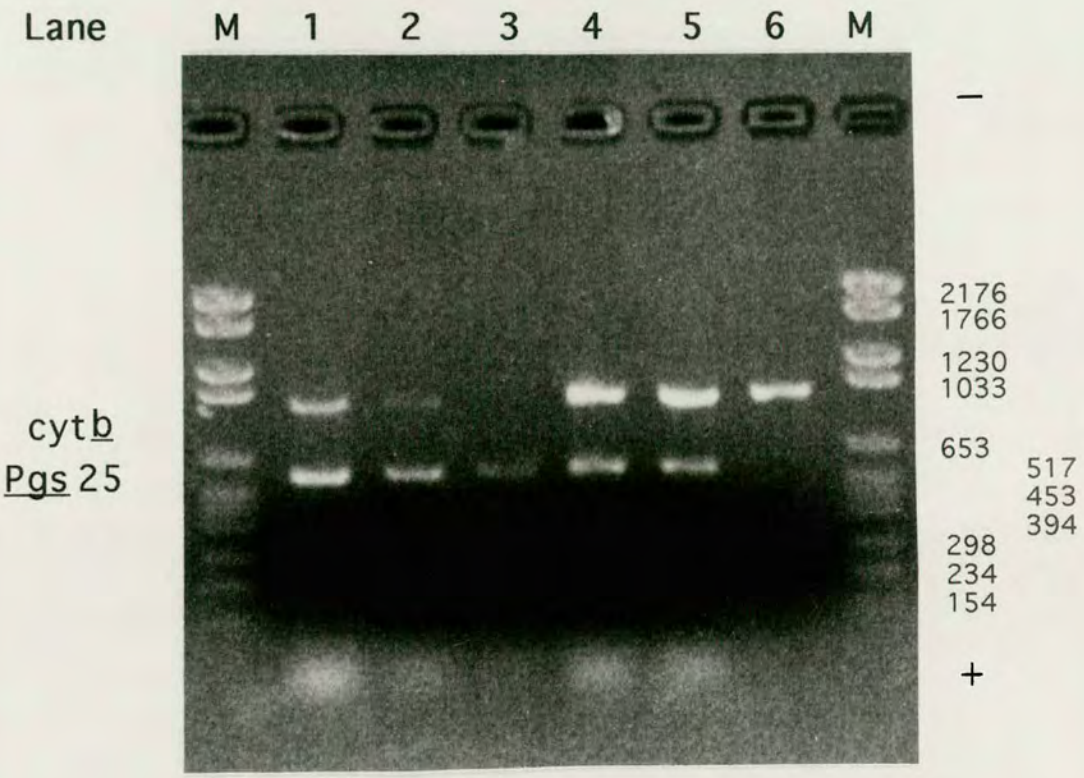
Figure 26. PCR amplification of nuclear and extranuclear gene fragments from Plasmodium gallinaceum male and female gamete DNA

Figure 26.

Gel showing fragments of the Plasmodium gallinaceum nuclear encoded ookinete surface antigen gene Pgs25 and the extranuclear 6kb element cytochrome b gene amplified together by PCR.

The primers used for amplifying the Pgs25 fragment (G251-G252) and the cytochrome b gene fragment (GCB1-GCB3) and the conditions used for the PCR reaction are shown in Figure 16.

Lanes 1-3 - show the products from reactions using decreasing dilutions of male gamete DNA template
Lanes 4-6 - show the products from reactions using decreasing dilutions of female gamete DNA template



Lane 1 2μl male gamete DNA template
Lane 2 1μl male gamete DNA template
Lane 3 0.5μl male gamete DNA template
Lane 4 0.1μl female gamete DNA template
Lane 5 0.01μl female gamete DNA template
Lane 6 0.001μl female gamete DNA template

M= molecular weight markers

3.5.3 Summary

The results of the experiments using the P. gallinaceum male and female gamete preparations show that the extranuclear DNA elements are present almost exclusively in the female gamete (Creasey, et al., 1994). In the context of malaria genetic crossing work, the maternal inheritance pattern of extranuclear gene alleles thus allows the identification of the parental clone contributing the female gamete to a hybrid oocyst. In the cross between 3D7 and HB3 the 58:1 bias in the inheritance of the 3D7 and HB3 alleles of the cytochrome b gene suggests that 58 oocysts were formed from 3D7 female/HB3 male gametes and only one from HB3 female/3D7 male gametes.

3.6 Results of an "artificially controlled" cross

A biological approach to the question of which gamete was transmitting the extranuclear elements was attempted by designing an "artificially controlled" cross between 3D7 and HB3. In this experiment, female HB3 gametes, without male gametes (prepared as described in Section 2.3.2), were mixed with 3D7 gametocytes of both sexes before feeding them to mosquitoes (as in Section 2.3.3). It was expected that this would produce self-fertilised 3D7 progeny, together with hybrid progeny formed exclusively from female HB3 gametes and male 3D7 gametes (Figure 27). If all the hybrid oocysts from this cross showed the HB3 form of the extranuclear markers, then it would demonstrate experimentally that it was the female gamete that was transmitting the extranuclear elements to the hybrids. Furthermore if the ratio of hybrids showing the HB3 form of the extranuclear marker to the self-fertilised 3D7 was roughly 1:1, it could be concluded that there was no intrinsic problem in the production of the HB3 female/3D7 male type of hybrid. The rarity of his type of hybrid in the uncontrolled cross, in this case, must have been the result of some mechanism operating only when both sexes of the two clones were allowed to mix.

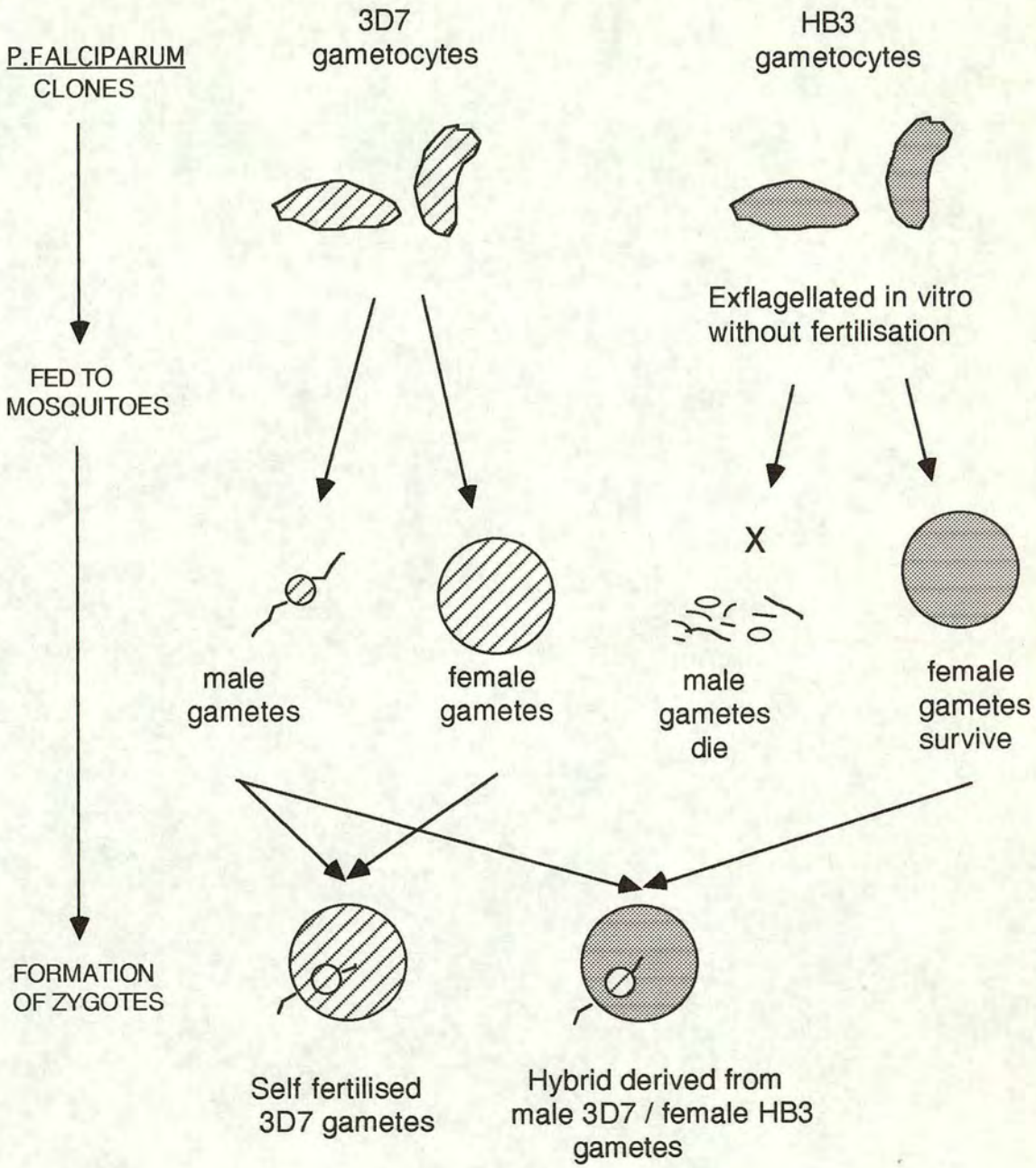


Figure 27. Diagram of the expected outcome of an 'artificially controlled' cross in Plasmodium falciparum parasites between female gametes of clone HB3 and male and female gametes of clone 3D7. The protocol also results in self-fertilisation of clone 3D7.

This "artificially controlled" cross was carried out on eight occasions and a total of 86 single oocysts were harvested. DNA was extracted from the oocysts and analysed by nested PCR amplification for the MSP-1 and MSP-2 polymorphic markers as before (Section 3.4.5). All 86 oocysts obtained from this cross showed only the 3D7 type allele of these nuclear genes indicating that none of them were hybrids. Control experiments in which a sample of the normal HB3 culture prior to exflagellation was fed to A. stephensi produced oocysts on the mosquito guts. However, both the number of mosquitoes which became infected and the number of oocysts per infected gut were lower than those which had been obtained in the HB3 controls for all the 9 feeds in the normal cross experiment analysed earlier. In the 8 "artificially controlled" experiments an average of only 1 in 7 mosquito guts were infected and each of these had only one oocyst per gut.

The failure to find hybrid oocysts in this experiment may be explained in several ways:-

1. It may represent a true mating phenomenon. It could mean that there is some inherent mating preference existing between these two clones which allows male HB3 to mate successfully with female 3D7 but only rarely allows HB3 females to mate with 3D7 males.

2. It may represent an experimental problem. The exflagellation procedure may have destroyed the viability of the HB3 female gametes or the overall infectivity of the HB3 culture may have been low. The HB3 cultures used in this experiment were ones which had been freshly thawed from stocks which themselves had been re-frozen within 3 weeks of thawing from a stabilate which had subsequently produced infective gametocytes for over three months in the initial successful crosses. Cultures from these stocks in the present experiment produced adequate numbers of gametocytes. However, since the mechanism of infectivity is largely unknown it is possible that it was only weakly infective. The lower than expected infection rate of the control HB3 feed would support this explanation. The additional handling of the females may have reduced their viability even further.

3.7 Analysis of the results of the cross between clones 3D7 and HB3.

The analysis of the parental nuclear gene alleles in the oocysts from the 3D7 x HB3 cross suggested that random mating was occurring among the gametes of the two clones since the proportions of homozygotes to heterozygotes did not differ significantly from the Hardy Weinberg Equilibrium (Section 3.4.3) and (Ranford-Cartwright, et al., 1993). If simple random mating[2] were occurring heterozygotes of both the 3D7 female/HB3 male and the HB3 female/3D7 male form will be present and the two forms will appear in a 1:1 ratio (Chapter 1, Figure 8). Any deviation from this ratio should not be significant.

In the analysis of parental maternally-inherited cytochrome b gene alleles it was found that, of the 59 heterozygotes identified, 58 had been formed from 3D7 female/HB3 male and only one had been formed from HB3 female /3D7 male gametes. The results including the extranuclear gene analysis are :-

3D7 x 3D7 homozygotes	37
HB3 x HB3 homozygotes	14
3D7 female/HB3 male	58
HB3 female/3D7 male	1
Total	110

From these observed oocyst results the actual numbers of male and female gametes of each clone participating in the cross were :-

3D7 females	=	(37 + 58)	=	95
3D7 males	=	(37 + 1)	=	38
HB3 females	=	(14 + 1)	=	15
HB3 males	=	(14 + 58)	=	72
Total				220

[2] The term mating is used here for convenience although in fact the oocyst data represents the mid-gut selected products of the mating process which took place in the gut. The zygotes formed by mating develop into ookinetes which pass through the gut wall to develop into oocysts on the outer wall of the gut. There is a great reduction in total numbers of parasites between the ookinete and oocyst stages but it is not known at present whether this selection process involves a change in the proportions of homozygote and heterozygote progeny.

and the relative proportions of successful male and female gametes in the mixture of the two clones :-

Of male gametes 3D7 = (38/38+72) = 0.34545

HB3 = (72/38+72) = 0.64545

Of female gametes 3D7 = (95/95+15) = 0.86364

HB3 = (15/95+15) = 0.13636

If random mating was occurring between these gametes, then the estimated frequencies of the four possible matings become:-

3D7 female x 3D7 male = 0.86364 x 0.34545 = 0.298

HB3 female x HB3 male = 0.13636 x 0.64545 = 0.088

3D7 female x HB3 male = 0.86364 x 0.64545 = 0.557

HB3 female x 3D7 male = 0.13636 x 0.34545 = 0.047

and the expected numbers of homozygote oocysts and the two forms of heterozygote oocysts will be :-

3D7 homozygous = 0.298 x 110 = 32,78

HB3 homozygous = 0.088 x 110 = 9.68

3D7 female/HB3 male = 0.557 x 110 = 63.47

HB3 female/3D7 male = 0.047 x 110 = 5.17

These expected numbers differ significantly (at the 0.05 level) from the actual oocyst numbers observed in the cross as is shown in the calculation below:-

	Observed	Expected	Deviation	$\frac{D^2}{E}$
3D7 x 3D7	37	32.78	+4.22	0.5432
HB3 x HB3	14	9.68	+432	1.9279
3D7 f./HB3 m.	58	63.47	-5.47	0.4717
HB3 f./HB3 m.	1	5.17	-3.57	2.4652

$x^2 = 5.4077$

$p < 0.05$ $(1df)^3 = 3.84.$

[3] Degrees of Freedom. Since these are the actual oocyst proportions in the cross there is no extrinsic data used in these calculations and there is only one degree of freedom i.e. knowing the frequency of one class it is possible to calculate the frequency of the other three classes.

These results conflict with the earlier analyses using nuclear gene markers which showed that the proportion of homozygous to heterozygous oocysts in this cross was consistent with the Hardy-Weinberg equilibrium for random mating. This conflict arises because of the extreme bias in the two forms of heterozygote.

3.7.1 Analysis of sex ratios

One of the important factors relevant to the bias in the two forms of heterozygote is, of course, the sex ratio bias of the two parental clones. The 3D7 and HB3 gametocytes which were fed to the mosquitoes at a ratio of 1:1, had significantly different sex ratios in culture (Table III).

These were :- 3D7 - females 0.92 : males 0.08

HB3 - females 0.82 : males 0.18

During exflagellation in P. falciparum each male gametocyte may produce up to 8 microgametes

Assuming that each clone produces the same number of microgametes per microgametocyte at exflagellation then this might have given gamete sex ratios of the order of :-

3 m.gametes/m.g'cyte	5 m.gametes/m. g'cyte	8 m.gametes/m.g'cyte
3D7 f 0.79 : m 0.21	3D7 f 0.70 : m 0.30	3D7 f 0.59 : m 0.41
HB3 f 0.60 : m 0.40	HB3 f 0.48 : m 0.52	HB3 f 0.36 : m 0.64

From the 110 oocysts obtained the actual sex ratio of the gametes of the two clones participating in the cross was:-

3D7 - females 0.71 : males 0.29
(95/133) : (38/133)

HB3 - females 0.17 : males 0.83
(15/87) : (72/87)

The sex ratio of the 3D7 clone in the actual results could, therefore, theoretically be achieved simply by assuming that with the ratio fed to the mosquitoes female gametocytes produced one female gamete and male gametocytes produced approximately 5 male gametes at exflagellation. But for the HB3 clone exflagellation

rates alone cannot theoretically produce the actual sex ratios of gametes observed in the cross from the sex ratios of the gametocytes which were fed to the mosquitoes.

In the mating process obviously the viability of both the female and male gametocytes may vary in addition to exflagellation rates. A matrix giving theoretical examples of this variation may be constructed for each clone (Tables IVa and IVb). These Tables illustrate that the sex ratios of the gametocyte cultures fed to mosquitoes could have produced the sex ratios of the gametes observed in the cross progeny by varying the exflagellation rate and/or fitness of the male gametes with the fitness of the female gametes in several ways.

The sex ratio data for this cross may therefore be summarised as follows.

1. There is a difference between the estimated sex ratios of the culture fed and the sex ratio of the oocysts resulting from the cross.

2. There is a significant difference in the sex ratios of the two clones in culture.

3. There is a difference between the sex ratios of two clones in the oocyst progeny ; the 3D7 clone is contributing far more females than males and the HB3 is contributing far more males than females. Of female gametes the 3D7 is contributing 86% and HB3 14%. Of the male gametes the 3D7 clone is contributing 35% and the HB3 65%.

5. The most dramatic difference between the estimated and actual sex ratios occurs in the HB3 clone.

The possible mechanisms by which the differences between the sex ratios of the gametocytes going into the cross, and the sex ratios of the oocysts produced by the cross may have arisen and the wider implications of an apparently non-random style of mating in malaria are discussed further in Chapter 5.

TABLE IV

MATRICES SHOWING THEORETICAL MALE GAMETOCYTE EXFLAGELLATION RATES AND FEMALE GAMETE FITNESS REQUIRED TO ACHIEVE THE OBSERVED RESULTS IN THE 3D7 X HB3 CROSS

Observed gamete sex ratios in the 1:1 gametocyte feed	3D7	.92 : .08
	HB3	.82 : .18

Gamete sex ratios in oocyst progeny	3D7	.71 : .29
	HB3	.17 : .83

TABLE IVa

No. of male gametes per male gametocyte

Proportion of successful female gametes per female gametocyte: 3D7	1	3	5	8
1	.92:.08	.79:.21	**.70:.30**	.59:.41
0.8	.90:.10	.75:.25	.65:.35	.53:.67
0.6	.87:.13	**.70:.30**	.58:.42	.40:.60.
0.4	.82:.18	.61:.39	.48:.52	.27:.73.
0.2	**.70:.30**	.35:.65	.32:.68	.13:.87

TABLE IVb

No. of male gametes per male gametocyte

Proportion of successful female gametes per female gametocyte: HB3	1	3	5	8
1	.82:.18	.60:40	.48:.52	.36:.64
0.8	.78:.22	.55:.45	.42:.58	.31:.69
0.6	.73:.27	.48:.52	.35:.65	.25:.75
0.4	.65:.35	.38:.62	.27:.75	**.18:.82**
0.2	.48:.52	.23:.77	**.15:.85**	.10:.90

Chapter 4

SOME INVESTIGATIONS ARISING FROM THE STUDY OF THE INHERITANCE OF EXTRANUCLEAR DNA OF MALARIA PARASITES

Section I

Investigation of the melanisation of certain strains of P. falciparum in the mosquito stage of the life-cycle

4.1 Introduction

From the early days of malariology it was observed that under certain conditions the malaria parasite is prevented from completing its life cycle through the mosquito because a process of melanisation traps it within the mosquito midgut wall (Garnham, 1966). Gametocytes which are taken up by the mosquito apparently form normal zygotes but as they turn into early ookinetes and enter the gut wall a coat of melanin is deposited over the parasite. This melanin entombs the ookinete within the gut tissue and prevents it reaching the outside of the gut wall and progressing to the oocyst stage. Onward transmission of these parasites to a vertebrate host is thus impossible (see Life cycle Chapter 1 Figure 1). Similar mechanisms of melanin deposition have been well described as an immunological reaction in other Diptera (Soderhall, 1982; Christensen and Forton, 1986; Charalambidis et al., 1994; Boman and Hultmark, 1987: Lackie, 1988).

A number of studies have demonstrated that factors such as flight distance, probing time and longevity of mosquitoes may be affected by malaria infection, although these studies have generally been carried out under laboratory conditions using far greater infection levels than would be expected in the field (Rossignol, et al., 1984 ; Chege and Beier, 1990; Klein, et al., 1986). It seems likely that melanisation may be one of a number of general non-specific refractory defense mechanisms mounted by the mosquito against infection with the malaria parasite (Warburg and Miller, 1991;Vernick, 1995). Melanisation occurs very typically, however, when parasites

are taken up by a species of mosquito which is not its natural host (Garnham, 1966). Through long association with their 'natural' mosquito host parasites can apparently adapt to evade this mechanism.

4.1.1 Recent Studies

A number of the recent studies of the melanisation phenomenon have been precipitated by work of Collins and his collaborators using Anopheles gambiae (Collins et al., 1986). A. gambiae, is the natural and most widespread vector of P. falciparum in Africa but it also supports infection (and produces infective sporozoites), with a range of different simian, avian and rodent malarias for which it is not the natural host (Garnham, 1966; Collins et al., 1986).

When a line of A. gambiae from West Africa, denoted G3, was infected with P. cynomolgi, the simian malaria, many of the mosquitoes displayed normal oocysts in the gut wall. A small proportion however, showed large numbers of melanised structures with haem pigment granules and subcellular features typical of Plasmodium ookinetes. These were sometimes found together with a few normal oocysts and sometimes in the absence of any normal oocysts. A selection protocol against P. cynomolgi resulted in the production of two mosquito lines, one of which was fully refractory and the other fully susceptible to infection with this parasite. These two lines were then tested for infectivity to different species of human, simian, avian and rodent malaria parasites. In some cases various isolates or clones of the same species were tested. The results of these experiments are summarised in Table V.

4.1.2 Present Investigations

Of particular interest in the context of the present thesis were the results of the infections with P. falciparum. Different clones of P. falciparum showed different proportions of melanised to non-melanised oocysts in the refractory line. The lines of African origin (NF54, LE5 and Tan 1), whose natural mosquito host is A gambiae, are far more resistant to melanisation than the two South American lines (7G8 and SL) and the one Asian line (Indo3), all of whose natural mosquito hosts are other Anopheles

Species	Strain	Natural Host	Natural* Vector	Geographical Origin	Susceptible Line 100% normal	mixed	100% mel.	Refractory line 100% normal	mixed	!00% mel.
P. berghei	NK	Rodent	A.dureni	C. Africa	100	0	0	0	2	98
P. gallinaceum	8A	Chicken	Ae.aegypti	Sri Lanka	100	0	0	0	0	100
P. cynomolgi	Ceylon	Primate	A.hackeri/	Indochina	100	0	0	0	1	99
	B		A.balabacensis		92	8	0	0	0	100
P. gonderi		Primate	Anopheles sp.?	W. Africa	100	0	0	0	3	97
P. inui	N34	Primate	A.hack./leucosp.	Asia	100	0	0	0	0	100
P. knowlesi	H	Primate	A.hackeri	Asia	100	0	0	0	0	100
P. brazilianum	PI	Primate	A.kompi?	S&C. America	100	0	0	75	2	3
P. vivax	NK	Human	Anopheles sp.	Asia /	100	0	0	0	7	93
	Chesson		(many)	S. Pacific/	95	5	0	0	6	94
	PNG				100	0	0	0	5	95
P. ovale	G	Human	A.gambiae	E. Africa	92	8	0	0	34	66
P. malariae	Uganda II	Humnan	A.gambiae		100	0	0	95	5	0
P. falciparum	SL	Human	A.albimanus	El Salvador	100	0	0	4	4	92
	7G8		A.darlingi	Brazil	100	0	0	5	5	90
	Indo3		A.dirus	Indochina	100	0	0	6	6	88
	TanI		A.gambiae	Tanzania	95	3	2	26	61	13
	LE5		A.gambiae	Liberia	100	0	0	85	5	10
	NF54		A.gambiae	Netherlands (W Africa?)	100	0	0	64	36	0

Table V. The melanisation response of different species of Plasmodium and different strains of some of the species to transmission through the susceptible and the melanisation-selected refractory lines of Anopheles gambiae mosquitoes.
(Adapted from Collins et al. 1986)

* This list obviously does not include all possible vectors. In some cases the main vector is not known with complete certainty

species. This would seem to indicate that the African P. falciparum parasites have adapted to avoid the melanisation response while in general the non-African parasites show varying degrees of sensitivity to melanisation in the A. gambiae mosquito.

The process of melanisation of malaria parasites in the mosquito host must involve complex interaction between the adaptive mechanisms of both organisms. A number of workers are studying the mechanisms and genes involved in melanisation (Paskewitz et al., 1989; Vernick and Collins, 1989; Vernick et al., 1989; Romans et al., 1991 Brey et al., 1995). Relatively little work has been done so far to investigate the differences between the melanisation-sensitive and melanisation-resistant lines of the parasite. Although extranuclear genes themselves are unlikely to be involved in the response to melanisation, they will be a useful tool in such studies, since they provide a meams of identifying the sex of parental contributions to a hybrid oocyst in crosses between parents with differing phenotypes.

4.1.3 Proposed study

It is proposed to carry out a parasite cross between a melanisation-sensitive and a melanisation-resistant line of P. falciparum in the A. gambiae line which has been selected for melanisation . Individual oocysts will be harvested from this cross and identified as homozygous or heterozygous by their possession of alleles of one or more polymorphic nuclear genes as described earlier in this thesis (see Section 3.4.3 and Figure 28). It is expected that self-fertilised progeny will display the parental melanisation phenotype. The cross-fertilised heterozygotes could follow one of three paths :-

1. All heterozygotes may be melanised.
2. None of the heterozygotes may be melanised
3. Some, but not all heterozygotes may be melanised

If heterozygotes survive the melanisation process it will then be important to know whether only one, or both, possible forms of heterozygote are surviving. This information would indicate whether the sex of the gamete transmitting the melanisation-resistant phenotype was relevant to the expression of that phenotype.

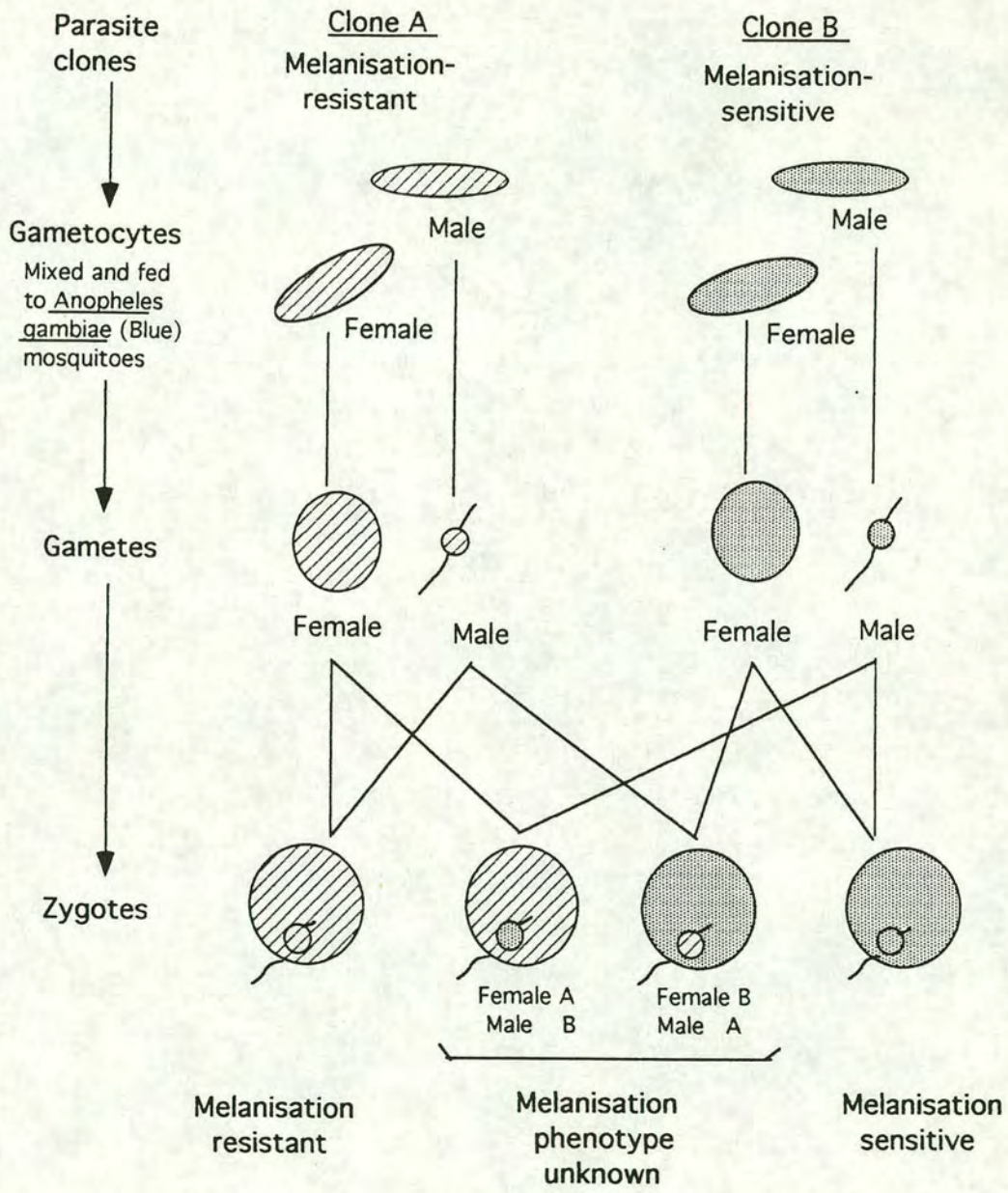


Figure 28. Diagram of a cross between two Plasmodium falciparum clones showing different melanisation phenotypes in the selected melanising line of Anopheles gambiae mosquitoes. The two types of heterozygote expected from this cross may be differentiated using maternally inherited extranuclear markers.

Since melanisation is reported to occur very soon after fertilisation the particular proteins expressed on the female gamete surface may be important in the control of the mechanism. The extranuclear genes which are uniparentally and maternally inherited should enable the identification of the maternal parental contribution to a hybrid oocyst..

4.2 Results so far

4.2.1 The A. gambiae melanisation-selected line

The refractory A. gambiae line which was selected for melanisation by Collins (Collins et al., 1986) has been acquired and established in Edinburgh. It is hereafter referred to as the A. gambiae (Blue) line.

4.2.2 The melanisation-resistant x melanisation-sensitive cross in P. falciparum

In seeking clones which would be suitable parents for the melanisation cross, three criteria needed to be fulfilled.

1. The clones should produce gametocytes infective to mosquitoes.
2. They should differ in allelic markers for both nuclear and extranuclear genes.
3. One parental clone would need to be sensitive and the other resistant to melanisation in the selected A. gambiae (Blue) line.

In addition to these criteria, it would need to be shown that the parasites chosen produced both types of heterozygotes (ideally in roughly equal numbers), in a cross through a non-melanising mosquito. Since successful crossing work had already been carried out using the clones 3D7 and HB3 it was decided to investigate these clones as parents for a melanisation-resistant x melanisation-sensitive cross.

a) 3D7 x HB3 cross

3D7 gametocytes fed to A. gambiae (Blue) produced normal looking oocysts, confirming work by Collins who reported that isolate NF54, (the parental isolate from which 3D7 was cloned), was completely resistant to melanisation (Collins et al., 1986). This incidentally also strengthens the evidence that NF54 is of African origin.

It was anticipated that HB3, from the South American continent (Honduras), might be suitable as the parent sensitive to melanisation in A. gambiae (Blue). This was based

partly on the fact that other South American clones 7G8 and SL were shown to undergo melanisation in the melanising A. gambiae (Blue) line (Table V) and partly on the belief that non-African isolates may not have adapted to avoid melanisation in African mosquitoes.

The known polymorphisms in the nuclear MSP-1 and MSP-2 genes and the in the extranuclear cytochrome b gene between 3D7 and HB3 would allow the male and female contributions in the hybrid oocysts from such a cross to be determined.

While preparatory work on this cross was being carried out, the analysis of the 3D7xHB3 cross in the non-melanising A. stephensi revealed a very strong bias towards the 3D7 female/HB3 male form of heterozygote; the HB3 female/3D7 male form occurring only once in 59 hybrid oocysts examined (Section 3.3.4). Since the melanisation cross experiment depended on being able to determine the fate of both types of heterozygote during the melanising process, 3D7 and HB3 were obviously unsuitable as the parents of this cross.

b) The search for P. falciparum clones suitable for a melanisation cross study

Four non-African isolates, which were close to their date of isolation, have been cultured and assessed for gametocyte production. DNA was extracted from the asexual parasites of each culture and examined by PCR amplification, restriction digest and/or sequencing for the known polymorphism in the cytochrome b gene. So far no suitable clone has been found. Selection for a mutation in the cytochrome b gene using Atovaquone treatment may provide a useful technique for selecting for a suitable polymorphism for this work (Wilson, et al., 1996).

4.2.3 Demonstration of the melanisation phenomenon using P. chabaudi

To examine the phenomenon of melanisation, a clone of Plasmodium chabaudi, called ER, which transmits normally through A. stephensi (which also transmits P. falciparum well), was found to melanise in the A. gambiae (Blue) selected line. Mosquitoes infected with P. chabaudi ER were dissected 7 days after infection and 7 out of 10 guts examined were found to be heavily infected with between 50-150 dark brown oval or pear -shaped bodies each with a lighter, narrower rounded point at one

end as shown in Figure 29. When viewed under polarised light the narrower lighter 'points' were seen to contain small amounts of haem pigment. No normal oocysts were seen.

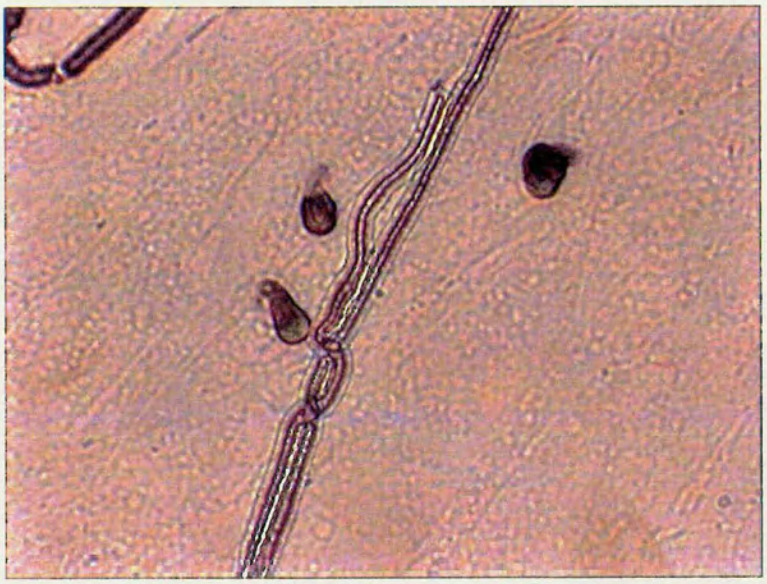
Unfortunately there are no reports of any attempts to transmit P. chabaudi through normal unselected A. gambiae. However in this experiment P. chabaudi ER produced at least three times (and often up to ten times) the number of melanised ookinetes in the melanising A. gambiae (Blue) line than it does normal oocysts in the non-melanising A. stephensi. This may reflect the species difference. Alternatively it may be that when ookinetes develop normally in a mosquito gut wall the first few (and possibly the fittest?), trigger mechanisms which inhibit the entry or development of further ookinetes. If ookinetes are melanised as they enter the wall such inhibition may not develop thus allowing a large number to enter or the melanisation may make visible what normally occurs. Similar phenomena have been investigated before particularly in the context of the role of the peritrophic membrane as a barrier to parasite infection in the mosquito (see Section 1.2.1 c) and Warburg and Miller, 1991;Sieber et al., 1991; :Vaughan, et al., 1994: Shahabuddin, et al., 1995: Billingsley et al., 1995).

4.3 Discussion

If the ability to melanise exists in all mosquito vectors of malaria then all successful parasites must possess a gene or genes for avoiding melanisation in their "natural" hosts. The proposed cross in P. falciparum between a melanisation-sensitive x melanisaton resistant clone in A. gambiae (Blue) should indicate whether the sex of the gamete carrying these genes is important. It should also provide information as to the dominance or recessiveness of these genes and the speed at which they may be expressed after fertilisation.

If melanised oocysts are present but no heterozygotes are found in the progeny, the assumption is that they have been melanised and that the gene or genes for avoiding melanisation are recessive. The sex of the gamete carrying them would be irrelevant.

If heterozygotes of both types survive melanisation, it could be assumed that the genes for avoiding melanisation are dominant, (whichever gamete is carrying them), and



x100

Figure 29. Melanised oocysts viewed x100 on the gut of an Anopheles gambiae (Blue) mosquito from the line seleced for the melanisation trait. The mosquito was infected with Plasmodium chabaudi (clone ER).

probably expressed on the zygote membrane soon after fertilisation, well before the melanisation response is triggered. The earliest that deposition of melanin on ookinetes has been observed, so far, is 16 hours post infected blood-meal (Paskewitz et al., 1988).

If the heterozygotes surviving melanisation are all of one type, the experiment will show that the sex of the gamete determines the heterozygote's response to melanisation. In this case, the heterozygotes surviving melanisation might be those which had received the female gamete from the melanisation-resistant parent . At fertilisation the male gamete contributes prinicipally its nucleus to the zygote: its surface membrane fuses with the female gamete membrane but forms only a tiny proportion of the total surface area of the zygote (Carter and Graves, 1988).

4.4 Conclusions

With continued screening it should be possible to identify clones which would be suitable for a melanisation -sensitive x melanisation-resistant cross. However if the dramatic inheritance bias towards one form of heterozygote observed in the 3D7 x HB3 cross is common in all P. falciparum crosses, (and possibly in animal Plasmodium models as well), then the analysis of such experiments will be difficult.

Further investigation of parasite response to melanisation and to other refractory mechanisms is of biological interest in itself. On a practical level those involved in investigating these phenomena in the mosquito envisage eventual practical applications in control strategies (Vernick, 1989; Vernick, et al., 1989; Curtis, 1992a; Curtis, 1992b). It is important that variation in the parasite's response to melanisation is included in these considerations.

Section B

Investigation of the rpoB polymerase gene of the 35kb circle

4.4 Introduction

At the time of writing, the function and cellular location of the 35kb element are unknown. The retention of this element in Plasmodium species and in other Apicomplexa suggests that it is functionally important to the survival of the whole organism. Its non-eukaryotic-type sequences make it potentially important as a drug target.

4.4.1 Antisense oligodeoxynucleotide inhibition

Synthetic antisense oligodeoxynucleotides (antisense oligos.) have been shown to inhibit viral and cellular gene expression. This is thought to be achieved by sequence-specific hybridisation to target mRNAs, where it would interfere with translation as well as cause the activation of RNase H which leads to accelerated degradation of the target mRNA (Crooke, 1991; Bayever et al., 1992; Politz et al., 1995).

Much research is being carried out into therapeutic use of antisense oligo-deoxynucleotides in cancer therapy (Yuan, 1995; Cerutti et al., 1996), in the treatment of viruses (Blum et al., 1995), including the human immunodeficiency virus (HIV) (Wickstrom, 1991) and in many cellular proteins in in vitro and in vivo screening systems (Burch and Mahan, 1991).

4.4.2 Antisense-oligonucleotide studies in malaria

Studies of the inhibitory effect of antisense oligos. have not been extensive in malaria. The studies published to date have been considering the effects of antisense oligos. with a view to their human therapeutic efficacy (Sartorius and Franklin, 1991; Rapaport et al., 1992; Clark et al., 1993; Dawson et al., 1993; Barker et al., 1996) and so far all have involved nuclear genes. A summary of some aspects of these studies is presented in Table VI.

From a practical point of view the cost involved in producing oligodeoxynucleotides seems to preclude their widespread use for mass treatment. However, the studies

TABLE VI - ANTISENSE OLIGONUCLEOTIDE INHIBITION IN MALARIA

AUTHOR	Gene Targetted	Oligo length/ conc etc..	Detection system	Results (inhibition)
Sartorius et al 1991	DHFR/TS	Cell-free 20-40mer 6-285μM	Transcription assay	40-90% (49mer 45μM)
Rappaport et al. 1992	DHFR/TS p195	sync. cultures 18-21mer 1-5μM/24hr mod. oligos	^{3}H uptake	20-88% but some non-specific w. controls
Clark et al. 1993	DHRF/T	sync. cultures 10-40mer 1-10μM/24hr mod. oligos	FACS + slides	77-89% at 10μM but non-specific
Dawson et al. 1993	HPRT	sync cultures 24mer 20-40μM/24hr 20-40μM	^{3}H uptake	25-60%
Barker et al. 1996	DHFR/TS EBA 175 TPI, RHO RR, polα	sync. cultures 21-33me .1-1μM mod. oligos	^{3}H uptak + slides	Differs acc to target best 0.2-0.5mM

A brief summary of the current literature on the use of antisense oligodeoxynucleotide inhibition of malaria parasite genes.

using antisense oligos. in malaria frequently show definite and specific effects relative to untreated controls and to controls treated with random (non-sense) or sense oligos. It was proposed to use antisense oligos. to target P. falciparum genes, not necessarily to kill the parasite but to observe the effect of inhibiting specific genes in living cultures of parasites in a similar manner to that of a transfection system. Preliminary investigations using antisense oligos. to various cell cycle control genes including crk-1, map-1 in parasite cultures showed encouraging effects that were both dose-dependent and specific to each gene relative to controls (Carter, Doerig and Creasey - unpublished observations). As part of this larger exercise, it was proposed to add antisense oligos. against the rpoB polymerase gene of the 35kb circle, to cultures of P.falciparum, in order to observe their possible effects on the living parasite.

If, as has been suggested, the 35kb circle is located within an organelle and the rpoB gene polymerase processes only those genes located on the circle, antisense oligos. may not be able to penetrate that organelle to hybridise with the mRNA of this gene. In this case the addition of the oligos, would have no effect on the living parasite, and this would add evidence towards it having an organellar location. If a specific effect was observed then it might show either that the 35kb circle was located outside an organelle or that the wall of the organelle allowed access to oligo-nucleotides. Either way, some additional information about this molecule might be obtained.

4.4.3 Design of the protocol

In designing the protocol for this work a review of current antisense studies was carried out on the following considerations; size and position on the gene of the oligos. used, the use of single oligos. or multiple oligos. to the same gene in combination, concentrations of the oligos., modifications to the oligos to protect against nuclease degradation, timing and duration of treatment, synchronicity of the parasites and design of controls.

4.5 Method

4.5.1 Design of oligos

Two antisense oligos were used for this work. The first consisted of a 28nt primer (G39 - Figure 13) at the 5' end of the rpoB gene. It was anticipated that this oligo. might interfere with the initiation of the message. The second oligo. was a 13nt primer, G27 (Figure 12) situated roughly half-way down the gene. A control oligo. consisted of a random sequence 17nt long with the same codon usage as the experimental oligos, and was designed by Dr Thomas Brown of the Oswel DNA Service, Southampton.

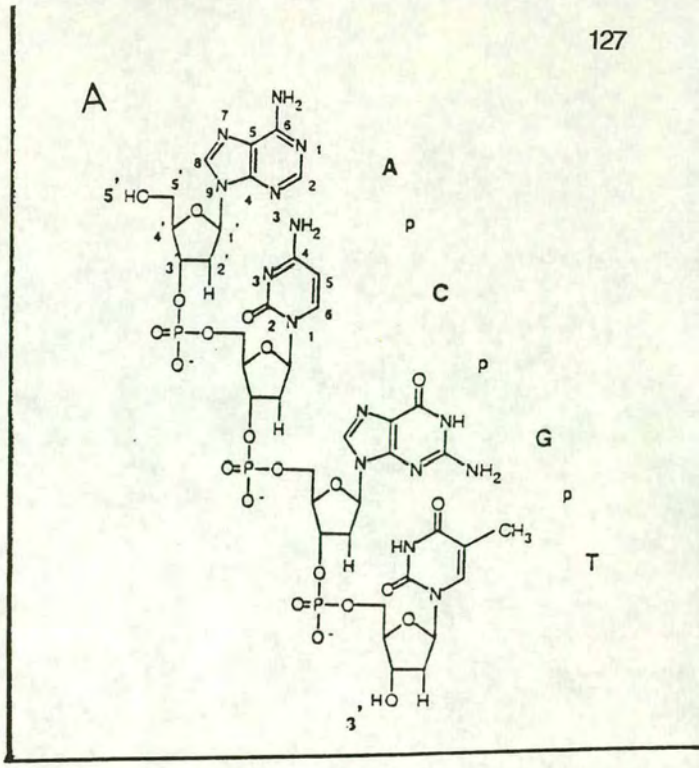
4.5.2 Protection against nucleases

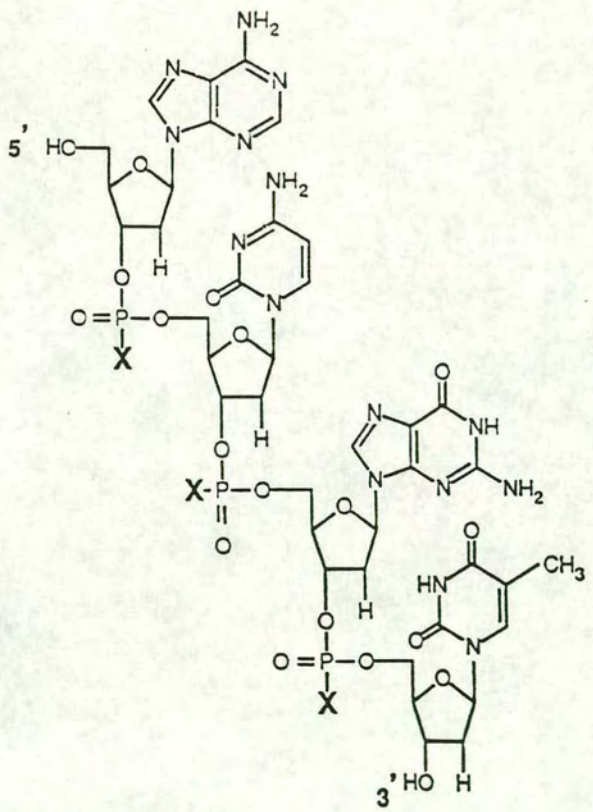
Initial experiments were carried out using oligos. designed for PCR work and thus were not specially protected against nuclease attack except by the normal routine of keeping the stocks freeze-dried or frozen. These oligos were used at concentrations of 2μM ,10μM and 20μM, this range having been determined from dose-dependent curves using the crk-1 and map-1 oligos mentioned above.

Following advice given by other workers and the Oswel DNA Services, another set of the same oligos. were modified by the addition of two phosphorothioated bases at the 5' end and one phosphorothioated base at the 3' end to protect against nuclease attack (Figure 30). The freeze-dried oligos, were re-constituted in complete culture medium immediately prior to use.

4.5.3 Parasites and protocol

Unsynchronised 3D7 and HB3 cultures were diluted to a parasitaemia between 0.5-1.0% and dispensed in 100μl volumes into the wells of 96-well flat-bottomed microtitre plates (Linbro). The plates were gassed, incubated at 37ºC and allowed to settle for two hours. The normal medium was removed and replaced with the same volume of medium containing 2μM, 10μM or 20μM concentrations of the antisense oligos., or medium containing the control oligo. at the same concentrations, or with normal medium without oligos. The cultures were maintained as normal asexual cultures (Section 2.2) and the respective media in the cultures was changed at the





X=S' phosphorothioate

Figure 30. Chemical structure of the backbone of phosphorothioate-modified antisense oligonucleotides. The structure of unmodified oligonucleotides is shown in inset A for comparison.

same time daily. After 72 hours thin smears of each culture were examined, parasite counts recorded and photographs taken. The experiment was repeated once more with the un-phosphorothioated oligos, and twice more using the phosphorothioated oligos.

4.6 Results

There was a clear and consistent difference between the parasites which had been treated with antisense oligos. and both the untreated controls and the random oligodeoxynucleotide controls using un-phosphorotioated oligos. Morphologically, the cultures treated with antisense oligos. showed deformities in 60% of the schizonts and trophozoites and 20-30% of the rings while those treated with random control oligos. looked normal (Figure 31). The inhibitory effect of the antisense oligos. was most marked at the 20mM concentrations but still clear at 10mM and 2mM concentrations. It was noted that the morphology of the treated cultures closely resembled cultures which have been grown at temperatures of 38-39ºC.

The parasite counts of trophozoites and schizonts showed a slight reduction in the treated cultures but the most striking feature was the almost complete absence of ring stages at 72 hours at the highest dosage (Figure 32). The random oligo. control showed a slight inhibition relative to the untreated control.

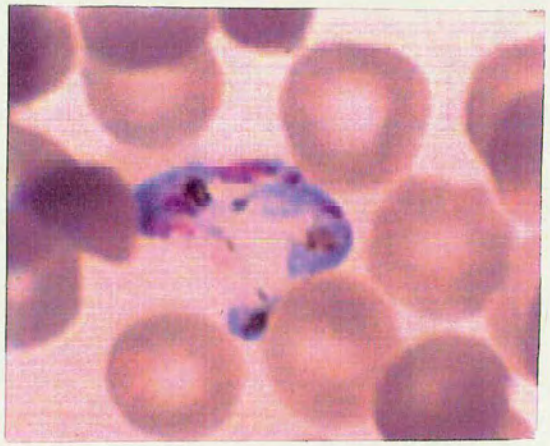
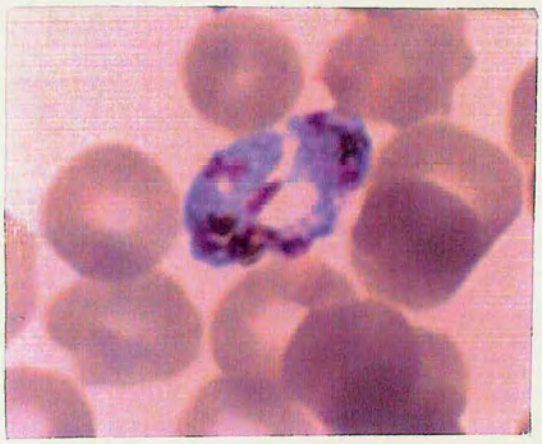
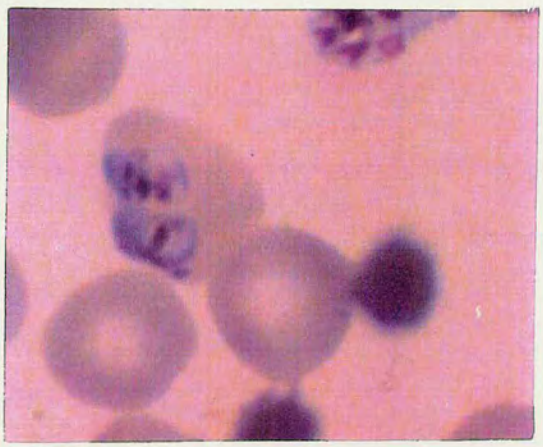
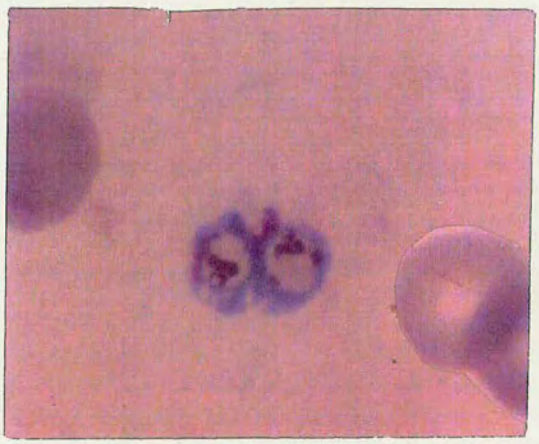
In the experiments using phosphorothioated oligos. the cultures treated with antisense oligos. again showed clear inhibition relative to the untreated control cultures and the cultures treated with random oligos. However the cultures treated with the random oligo. also showed some degree of inhibition relative to the untreated control.

4.7 Discussion

The experiments described here were carried out simply to observe the possible effects of addition of rpoB gene antisense sequences to living parasites. It is not certain that the apparent specific effects observed represent anstisense oligonucleotide hybridisation with the target mRNA. In view of the inconsistencies experienced in the whole field of anti-sense inhibition studies both in malaria and in other organisms, (Clark, et al., 1993; Ramasamy, et al., 1996; Lehr, 1996; Tolou, et al., 1996) the results of this experiment, while very interesting, should be treated with caution. The

Figure 31. Photograph of Plasmodium falciparum cultures treated with antisense oligodeoxynucleotides of the rpoB gene

20μM treatment (72 hours)

Control (72 hours)

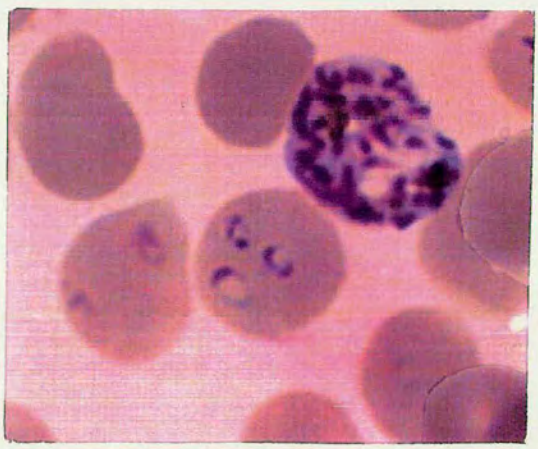
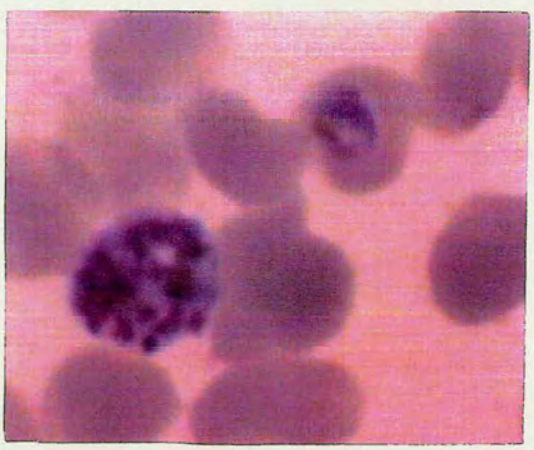



Figure 32. Results of antisense oligodeoxynucleotide inhibition experiments on the rpoB gene

Figure 32

Graphs showing parasitaemia of cultures treated for 72 hours with antisense oligodeoxynucleotides of the rpoB gene and samples of the same cultures untreated or treated with random oligodeoxinucleotide controls.

R = Rings
T = Trophozoites
S = Schizonts

A and B represent the results of experiments using unmodified oligonucleotides
C and D represent the results of experiments using phosphorothioated oligonucleotides.

All the parasitaemias represent the means of duplicate sets of results.

UNMODIFIED OLIGODEOXYNUCLEOTUDES

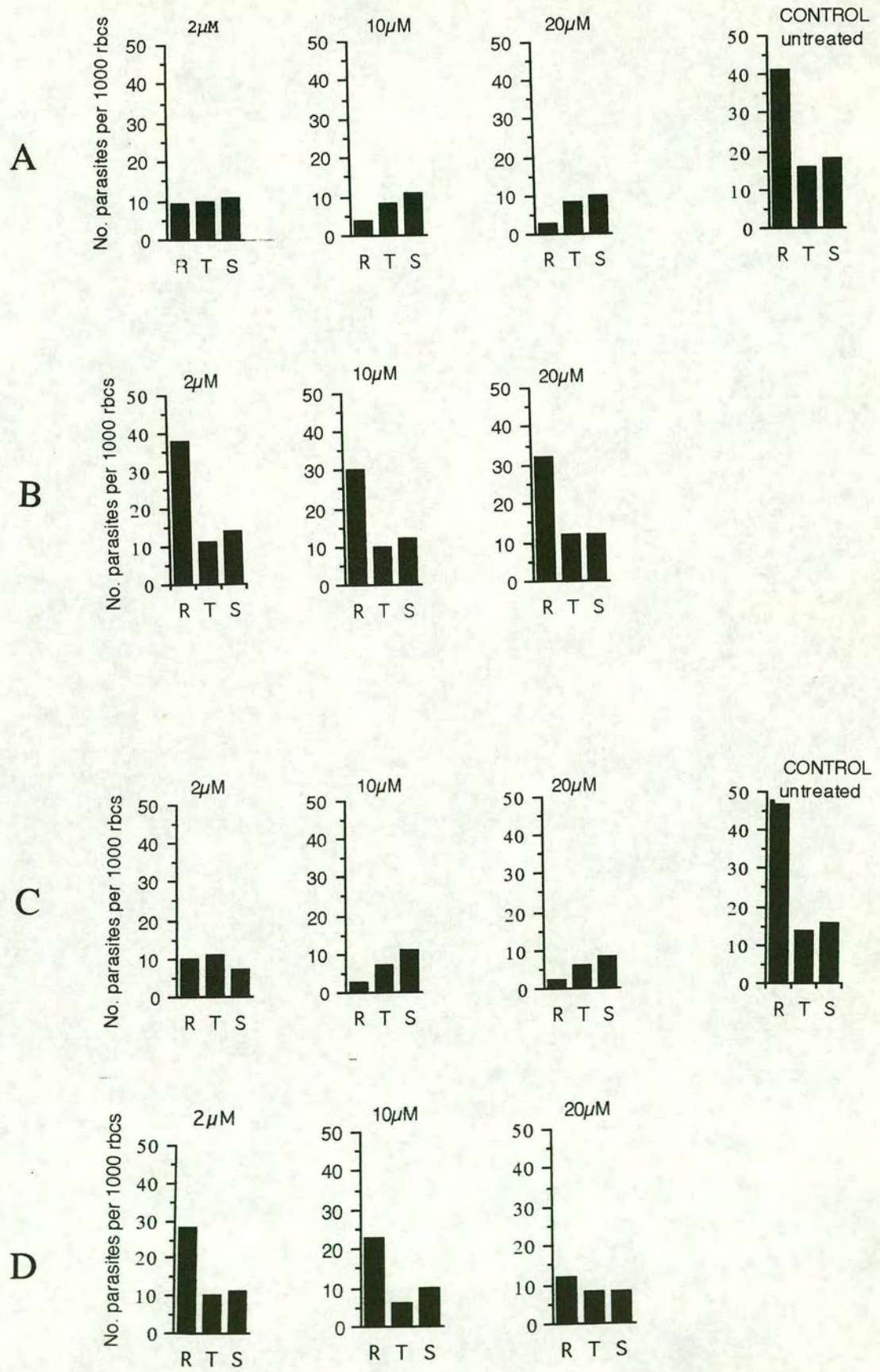
A - Parasitaemias of culture treated with unmodified antisense oligodeoxynucleotides (antisense oligos.) at 2μM, 10μM and 20μM concentrations compared to the untreated control cultures set up from the same stock at the same time.

B - Parasitaemias of cultures from the same stock as in A, treated with unmodified non-specific random oligos with the same codon usage as the antisense oligos.

PHOSPHOROTHIOATED OLIGODEOXYNUCLEOTIDES

C - Parasitaemias of cultures treated with non-specific olionuleotides at 2μM, 10μM and 20μM concentrations compared to untreated culture set up at the same time from the same stock.

D - Parasitaemias of the same culture as in C but trated with non-specific oligonucleotides with the same codon usage as the antisense oligos,


A
B
C
D
No. parasites per 1000 rbcs
2μM
10μM
20μM
CONTROL
untreated
R T S
0
10
20
30
40
50

differences in the binding capacity of different oligodeoxynucleotides, (even when they have the same codon usage), make it possible for differences similar to those observed between experimental and control oligos to be due to the sequences rather than inhibition of the target gene : concentrations of oligos. causing efficient inhibition also varies according to the particular gene targetted (Barker et al., 1996). Similarly differences between untreated controls and those treated with oligodeoxynucleotides may simply be reflecting a generalised toxic effect of adding any substance to parasites in culture.

The protection against nuclease attack provided by phosphorotioation was expected to prevent degradation of the oligos. and increase their sensitivity without affecting their specificity (Helene and Toulme, 1990). When used at the same concentrations as the un-modified oligos. non-specific inhibition by the random control was observed while the specific effect of the antisense oligos was no greater than with the un-modofoed oligos. Similar findings to those observed in our experiment have been reported by other workers. They suggest several mechanisms by which the process of oligo. modification may in itself cause non-specific inhibition of parasites. They also report that it is possible to restore specificity of phosphorotioated oligos. by greatly educing their concentrations in the cultures to below 0.5μM (Barker, et al., 1996). In the present experiments, although the addition of the un-modified oligos. gave a specific effect at 20μM concentrations, the actual concentration may be considerably lower as a result of degradation of the DNA.

If it could be confirmed (possibly by some form of in situ labelling), that the inhibitory effect of adding antisense oligodeoxynucleotides against the rpoB gene observed in our experiments is a specific effect, application of this mechanism to therapy becomes a possibility. The extreme A+T richness and prokaryote features of the rpoB gene sequences (Gardner, et al., 1991) make it unlikely that they would cross-hybridise with human DNA sequences. Furthermore if the phosphorothioate modification of the antisense oligos. allows them to be used at much lower concentrations than had previously been thought, their cost. for treatment would be greatly reduced.

As far as the investigation of the function of the rpoB gene is concerned, the advent of techniques for the stable transfection of malaria genes potentially provides a more reliable way of gaining information than does antisense oligonucleotide inhibition (Yimin Wu, et al., 1995; review Waters, 1996). The application and development of these techniques for this particular gene, however, may have to wait until more information about the location of the 35kb DNA and its replicative mechanisms is available.

Section III

Preliminary investigations into the level of homology of the cytochrome b gene in five Plasmodium species and comparison with the phylogenetic trees constructed using rRNA sequences from the nuclear genome.

4.7 Introduction

Primate, rodent and avian malarias are frequently used as models to study the pathology and immune response of the human malarias. The phylogenetic relatedness of these Plasmodium species is one of a number of important aspects both in the choice of model and the interpretation of the results of such studies (Mons and Sinden, 1990).

A number of phylogenetic studies using the SSU rRNA genes of the nuclear genome have been carried out (Waters, et al., 1991; Waters and al., 1993; Waters et al., 1993). Phylogenetic studies to determine the evolutionary origin of the plastid-like 35kb circle have also been carried out for the purposes (Palmer, 1992; Williamson, et al., 1994; Egea and Lang-Unnasch, 1995).

The mitochondrial cytochrome b gene is widely used for phylogenetic studies (Avise, 1994). It is considered to be conserved enough for looking at distantly-related organisms such as mammals and birds (Irwin, 1991; Edwards, et al ., 1991) but variable enough for examining questions about closely-related species and even within populations (Wenlink, et al., 1990). In the present study, primers designed to amplify the P. falciparum cytochrome b gene (Chapter 2, Figure 11) easily amplified homologous fragments from P. gallinaceum, P. simiovale, P. gonderi, P. yoelii, P. berghei, P. chabaudi and P. vivax DNA. A preliminary study was instigated to sequence the cytochrome b gene from some of these species in preparation for constructing a phylogenetic tree based on gene to see whether it would confirm the relatedness suggested by the nuclear SSU rRNA trees.

4.8 Method

Approximately 500 bases in three separate blocks of the cytochrome b gene from P. vivax and P. chabaudi were sequenced and aligned to each other and to the same

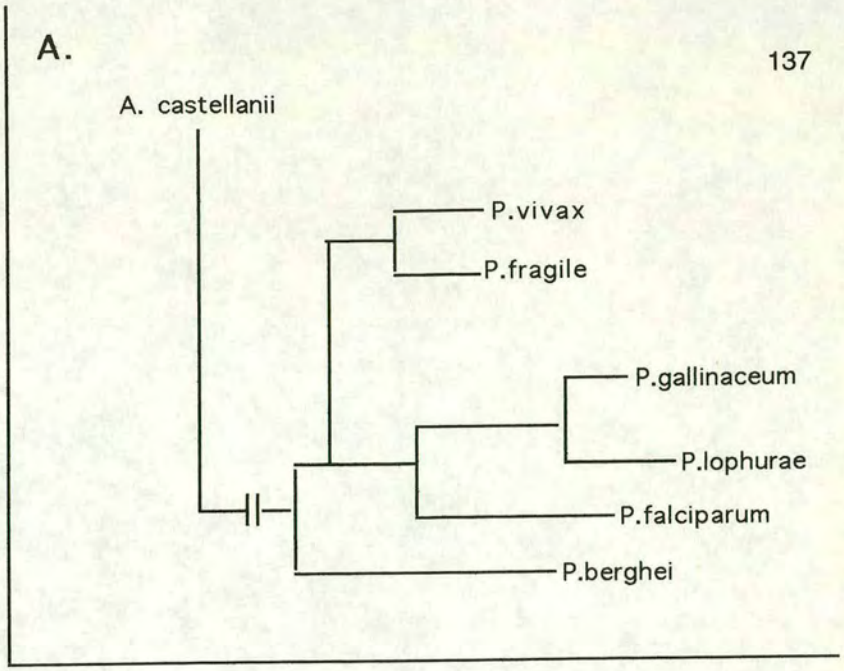
fragments from P. falciparum, P. yoelii and P. gallinaceum (Appendix III). The cytochrome b gene of Theileria annulata was used as an outgroup since being an apicomplexan it is closely related to Plasmodia, but is not of the same Subclass (Megson, et al., 1991; Thorne and Kishino, 1992). These sequences were analysed using the data matrix method of Fitch-Margoliash (Saitou and Imanishi, 1989). In simple terms this calculates phylogenetic distances by counting the number of differences between sequences and dividing it by the number of sites considered. Support for the trees was calculated by the conventional "bootstrapping" method (Felsenstein, 1985). This takes random samples of sites from the alignment to generate new trees. Probability scores for each branch of the tree are expressed as percent probabilities of the number of times a branch appears in the same place on the tree by re-sampling. In the present study the data were re-sampled 100 times.

4.9 Results

The tree produced by the Fitch-Margoliash analysis is illustrated in Figure 33. This very preliminary examination of the phylogeny of the cytochrome b in these parasites does not show the close grouping of P. gallinaceum with P. falciparum shown in the analysis of the nuclear rRNA genes (Waters, et al., 1991) although it is thought that there may be some inaccuracies in the published P. gallinaceum sequence in the gene data base (personal observations). It does, however, suggest early separation of P. vivax and P. falciparum shown by other studies (Waters et al., 1993) and suggests that the use of rodent models can be justified in studies relating to P. falciparum. This tree, (as do all trees based on analysis of a single gene), reflects how closely these particular genes are related to each other. This is not necessarily the same as the relatedness of the particular species to each other.

4.10 Discussion

With such a limited study no firm conclusions should be inferred from these results. Although cytochrome b has been used so widely for this type of work, recent study has pointed out some of the disadvantages of its use for phylogenetic purposes (Meyer, 1994). These include base compositional biases, rate variation between



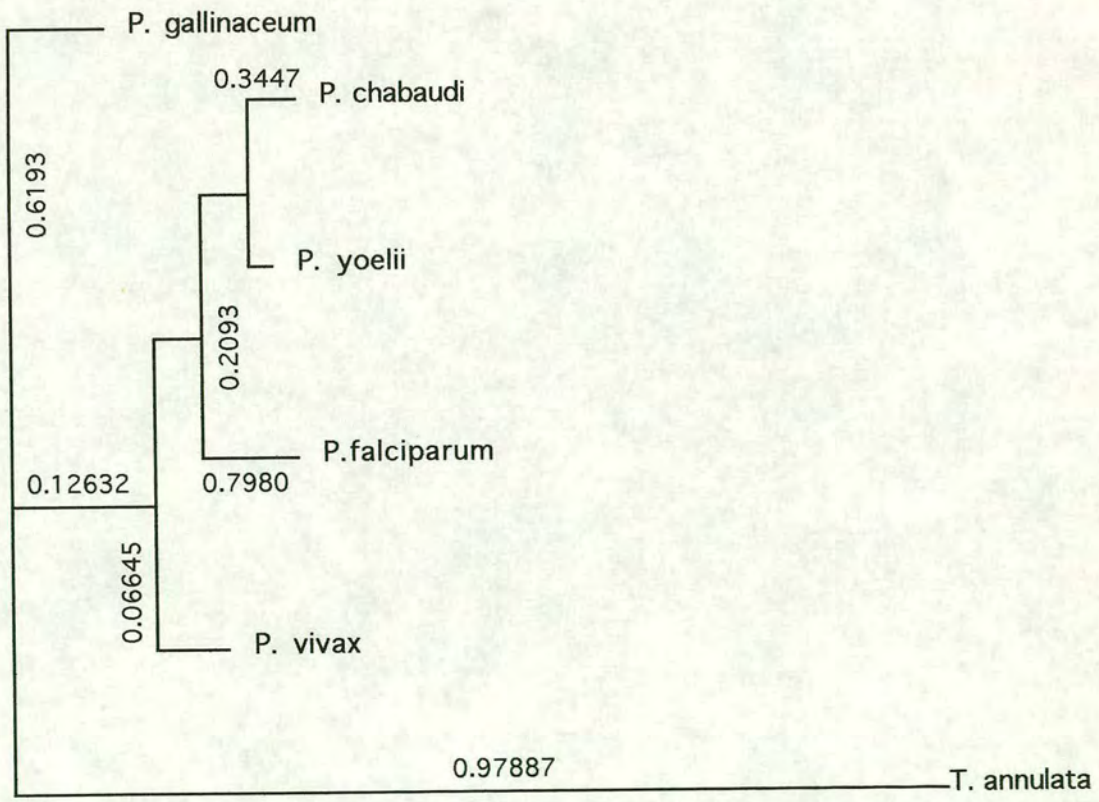


Figure 33. Phylogenetic tree of five Plasmodium species constructed from the mitochondrial cytochrome b gene sequences using the Fitch-Margoliash method. The inset A shows for comparision, a tree constructed by standard parsimony methods using asexually expressed SSU rRNA sequences. (from Waters et al. 1991).

lineages, early saturation of third codon positions and limited variation of first and second codon positions. Some nuclear copies of this gene have also been identified in certain groups of animals (Kornegay, et al., 1993; Smith, et al., 1992; Quinn, 1992). Among Plasmodia, the use of phylogenetic trees to infer evolutionary relatedness and especially lateral transference has been controversial (Ayala and Fitch, 1992). Earlier workers believed that the species infecting humans may have become adapted independently from previous zoonotic associations (Bruce-Chwatt, 1988). However, the results of this and other phylogenetic studies will be useful contributions to this field: the more genes that are analysed in this way the more accurate will be the prediction of the actual evolutionary relatedness of malaria parasite species.

Chapter 5 DISCUSSION

This project has shown that a mitochondrial gene marker was inherited uniparentally in 59 hybrid oocysts examined from a cross between Plasmodium falciparum clones 3D7 and HB3, and that this inheritance was almost certainly through the female gamete. A maternal inheritance pattern allows us to identify which parent contributed the female gamete, and which the male gamete, to a hybrid oocyst. In this particular cross there was a dramatic bias in the two forms of heterozygote oocyst. This suggested that oocyst progeny were not being produced (or at least not surviving) according to simple random mating principles as had previously been deduced from analysis of nuclear gene markers in the same cross (Ranford-Cartwright, et al., 1993). A number of questions regarding the biology and the genetics of the parasite are stimulated by these investigations.

5.1 Conservation of the extranuclear elements of malaria parasites

The difficulty of finding allelic markers to follow extranuclear DNA through a cross confirmed previously reported conservation of extranuclear gene sequences between P. falciparum clones (Vaidya, et al., 1989). More than 75% of the cytochrome b gene from the 6kb element and 70% of the rpoB gene from the 35kb element were sequenced for the two clones 3D7 and HB3. Only one synonymous substitution was found in the cytochrome b gene.

This conservation in extranuclear gene sequences contrasts markedly with the degree of sequence diversity which seems to be well tolerated among nuclear genes (Walliker, et al., 1988; Kemp, et al., 1990; Anders et al., 1993; and Introduction Section 1.4.4). A high level of homology of the cytochrome b gene was also demonstrated among 5 Plasmodium species. Five hundred bases of the cytochrome b gene of P. falciparum, P. gallinaceum, P. yoelii, P. vivax and P. chabaudi showed close alignment between 75-90% at the nucleotide level and between 92% and 97% at the amino acid level in a preliminary phylogenetic exercise (Chapter 4 Section 3). A high level of conservation within species and homology between species in the cytochrome b gene is not unexpected since it is well conserved across most of the animal kingdom (Kotcher,

1989). High levels of conservation of genes in the 35kb DNA may be of interest since the functions of these genes are still largely unknown (Gardner, et al., 1991; Wilson, et al., 1991).

5.1.1 Implications of conservation and homology in extranuclear genes.

a) Functional efficiency

In theory, genes which are essential for housekeeping functions (such as the cytochrome b gene), and for which adaptation to external selection pressure holds little advantage may be more efficient when consigned to a non-recombining genome where they are not subject to re-arrangement at meiosis and where any deleterious mutations should be quickly eliminated from the population. Genes for which more rapid adaptation to external conditions would be important (such as antigen genes for example) would conversely have a more advantageous position in the rapidly recombining nuclear genome. The conservation of the extranuclear DNA and the plasticity of the nuclear DNA may perhaps give the malaria parasite an advantageous combination of stability and adaptability. However, the conservation of the extranuclear DNA combined with its uniparental inheritance, which does not allow rapid adaptation through recombination, theoretically make extranuclear genes more vulnerable than nuclear genes to disruption and they may therefore be suitable targets for drugs or other parasite-based control measures.

b) Mutation rates

The contrast in the degree of sequence polymorphism in nuclear and extranuclear genes suggests very different mutation rates between these genomes. Lack of polymorphism in the extranuclear genes suggests that mutations may occur rarely or, if they occur more frequently, they must be lethal. The fact that the 6kb element is almost all coding implies that it has already been reduced to a functional minimum where even a small substitution could be deleterious. It may be that the number of copies of the 6kb element per cell (± 20 in P. falciparum but ±150 in P. yoelii) allows for such losses. If deleterious mutations were being lost, however, survival of synonymous substitutions might still be expected. The difficulty of finding even a

single synonymous substitution between the cytochrome b gene sequences of 3D7 and HB3 suggests that mutation rates are low in this gene. Recent reports on the development of clinical P. falciparum resistance to the hydroxynapthoquinone, Atovaquone, however, have shown that under pressure from this drug a mutation in the cytochrome b gene relatively rapidly and tolerated successfully (Wilson et al., 1996). It will be of interest to investigate this observation further in vitro.

If the Plasmodium extranuclear genes have a slower mutation rate than their nuclear genes then malaria differs from the typical animal pattern and certainly differs greatly from the human pattern (Gray, 1989). Human mitochondrial DNA undergoes at least a 5-fold higher rate of mutation than human nuclear DNA in its coding regions and up to a 10-fold higher rate in intergenic regions (Brown et al., 1979). In contrast to animal DNA, plant mitochondrial DNA, in general, diverges at a much lower rate than plant nuclear DNA. It has been estimated that plant mitochondrial DNA evolves at least 5x more slowly than its nuclear DNA (Wolfe et al., 1987), and up to 100x more slowly than the average animal mitochondrial DNA (Atlan and Couvert, 1993).

5.2 Geographical variation in extranuclear genes

The single base pair difference used in this study was found between the cytochrome b genes of the Honduran clone HB3 and the putative African clone 3D7. However the HB3 form of this base difference was not found among 18 clones examined from other parts of the world including 8 others from South America and the one other isolate (M23) which was available from Honduras. There is evidence that among nuclear-encoded genes there may be some natural variation in frequencies of certain alleles among P. falciparum parasites from different geographical areas (Creasey et al., 1990). It is impossible to know at present whether any variation between geographical regions may occur in the extranuclear DNA until more markers are identified.

5.3 Evolution of the three genomes

In general terms the eukaryotic nuclear genome is assumed to have evolved in the very distant past from a 'protoeukaryote genome' that itself may have evolved from prokaryote-like DNA (Fincham, 1994). The mitochondrial genetic system is thought

to have arisen by endosymbiosis of primitive archebacteria within the evolving eukaryotic cell (Anderson, et al., 1981; Iwabe, et al., 1991). Other cytoplasmic organelles, including plastid-type molecules like the 35kb circle, are also thought to be endosymbiotic in origin but acquired more recently in evolution than the mitochondria (Grun, 1976).

The malaria parasite thus possesses three genomes of very different origin and it may be that these differences are important in enabling it to undergo its complex life-cycle successfully. In trypanosomes it has been shown that species such as T. equiperdum and T. evansi which do not have an insect host, produce some strains that have mitochondria (or kinetoplasts) entirely lacking the large quantity DNA of other trypanosomes. They do not have cytochromes, their respiration is cyanide-resistant and they are classified as dyskinetoplastic. In contrast, species that spend part of their life-cycle in an insect gut namely, T. brucei, T. gambiense and T. rhodesiense, have mitochondria which posses large quantities of DNA, produce cytochromes and function normally with cyanide-sensitive respiration (Hoare, 1954; Trager, 1965). It is possible that the different origins and characteristics of the three genomes of malaria parasites are important in providing the flexibility necessary for survival in the very different environments of arthropod and non-arthropod hosts.

5.4 Uniparental Inheritance

Fifty-nine out of the 110 single oocysts from the 3D7 x HB3 cross possessed both parental alleles of a nuclear gene marker (one allele from the parent contributing the male gamete and the other from the parent contributing the female gamete) but each possessed only one parental allele of the extranuclear gene marker. The conclusion that extranuclear DNA uniparentally inherited in this cross was clear and unambiguous.

5.4.1 Uniparental extranuclear inheritance in other P. falciparum crosses

Subsequent to the present work another investigation of the inheritance of extranuclear DNA was carried out in cloned recombinant progeny from two crosses transmitted from mosquitoes through a chimpanzee host (see Introduction Section 1.10.3)

Sixteen recombinant clones from a cross between clones HB3 and Dd2 (Walker-Jonah, et al., 1992) and 9 from the cross between 3D7 and HB3 (Walliker, et al., 1987) were examined for their possession of an extranuclear gene marker (Vaidya, et al., 1993). (This marker was the same as that used in the present project since clone Dd2 possesses the same allele of the cytochrome b gene as clone 3D7). All 25 clones possessed one form only of the extranuclear gene marker and all were the 3D7/Dd2 form.

Although highly indicative of uniparental inheritance, this analysis does not specifically prove it. Unlike hybrid oocysts, which each contain the products of a diploid zygote, the recombinant clones are haploid and in theory could have been a result of high selective pressure on one allelic type among the progeny of a zygote in which alleles from both parents were represented. These results nevertheless are consistent with the oocyst data in showing uniparental inheritance of the extranuclear marker.

5.4.2 The evolution of uniparental inheritance

"The variety of molecular and cellular mechanisms [of uniparental inheritance] found in different organisms is matched only by the variety of hypotheses devised to explain the evolution of the phenomenon." This statement (Birky, 1995) well summarises the wealth of theoretical work in this field. A few of the main themes of the theories of evolution of uniparental inheritance are discussed below.

a) Limitation of 'selfish' mutations

Traditionally one explanation given for the development of uniparental extranuclear inheritance has been that it prevents the deleterious effects of 'selfish' DNA (Grun, 1976; Jacobs and Lonsdale, 1987; Hastings, 1992). This argument states that the ability of extranuclear DNA to regulate its own replication can at times be disadvantageous to the organism as a whole. If by mutation or recombination a new form arises which is deleterious to the 'host' cell but has its own replication advantage, it may spread to become the dominant form. The replication advantage of the

extranuclear DNA in this case could be greater than the overall selective pressure of the nuclear DNA against the deleterious trait.

In both yeast and various filamentous fungi, which do not display uniparental extranuclear inheritance, mitochondrial DNA mutants that have lost certain essential functions by deletion or mutation, have turned out to be more efficient at replication than the wild type which they tend to replace as growth proceeds. Examples of such genomes are the suppressive 'petite' mitochondrial mutants in yeast and the senescence mutants in Neurospora (Beale and Jurand, 1966; Yang and Griffiths, 1992; Piskur, 1994).

Uniparental transmission of extranuclear DNA allow deleterious mutations to be transmitted vertically only. This might lead to the extinction of a line but would not spread through the whole population. This is a group argument however and it is difficult to see how individual organisms could gain selective advantage by this mechanism alone.

b) The development of anisogamy

More recent theories have proposed that extranuclear DNA inheritance is closely involved in the development of anisogamy and sexual differentiation. In these arguments the disadvantages of bi-parental extranuclear inheritance as described above select for nuclear gene mutations which prevent the entry or survival of other (possibly deleterious) cytoplasm from other individuals. In this situation, such new mutants as well as the original 'wild type' without the mutation will in turn favour linkage with mutations that promote self-incompatibility because of selection for hybrid fitness. Thus, two populations of individuals arise within the same species one of which can transmit its cytoplasm readily, the other of which cannot, and both of which can in time only mate successfully with the other - in other words two sexes only one of which transmits the extranuclear elements (Coleman, 1982; Hutson and Law, 1995). Anisogamy thereafter brings its own benefits in the form of more efficient allocation of resources between the two mating types.

A number of variants of this basic idea have been proposed some of which demonstrate how the degree of inbreeding or outbreeding in an organism's life-style may explain the diversity of patterns that exists between uniparental and bi-parental inheritance as well as the optimum sex ratios for each system (Hurst, 1994).

c) The costs of sex

Another group of theorists consider the reasons for the development of uniparental patterns of extranuclear DNA to be entirely related to the relative costs and benefits of sex for different types of genes or genomes, (see review, Birky, 1995). For the transmission of genetic material, uniparental inheritance precludes the acquisition of a mixed population and/or formation of heterozygotes of extranuclear genes and is thus similar in effect to asexual multiplication.

Some of the benefits of sex include features such as repair of damaged DNA, rapid selection and efficient adaptation through recombination. These theorists claim that the benefits of sex are principally an advantage to genes with polymorphic loci on a large nuclear genome, but are not so important to the many fewer extranuclear genes which may also not be very polymorphic. In most organisms there are on average 100 to 1,000 times as many genes on the nuclear genome as on the extranuclear genome. Specifically in malaria parasites there are around 7,500 genes in the nucleus (Reddy, 1995), around 60 on the 35kb genome (Preiser et al., 1995) and counting the fragmentary rRNA genes around 18-20 on the 6kb genome (Feagin, 1994). With bi-parental inheritance the contribution of extranuclear genes to recombinational frequency and linkage disequilibrium would therefore be very small. The costs of sex on the other hand would involve the two-fold reduction in reproductive capacity. For the extranuclear genomes, the costs of sex may outweigh its benefits, and the asexual-type or uniparental pattern of transmission may thus be the more efficient system. It has also been proposed that in times of nutritional deprivation the two fold advantage of extranuclear asexual reproduction over sexual reproduction may allow the cell to sacrifice some of its extranuclear DNA to utilise its essential nucleotides - a theory

known as the Salvage/Turnover/ Repair (STOR) model (Sears and VanWinkle-Swift, 1994).

One of the main disadvantages of an asexual-type multiplication and a uniparental pattern of inheritance, however, is that advantageous forms of extranuclear mutations may occasionally be lost by random drift. In the absence of bi-parental inheritance and recombination this loss is irreversible. Non-lethal deleterious mutations will be allowed to accumulate and the population fitness will gradually decline. This phenomenon, known as Muller's ratchet, may lead to eventual extinction. In practice, however, there may be a number of mechanisms that may stop this process or slow it sufficiently for environmental change, low level bi-parental inheritance or compensating mutations in either the nuclear or extranuclear genomes to restore population fitness.

5.5 Maternal Inheritance

5.5.1 Analysis of the P. gallinaceum gamete preparations

In the experiment using preparations of P. gallinaceum male and female gametes, extranuclear gene probes hybridised strongly to the female gamete DNA preparation but did not hybridise or hybridised very weakly to the male gamete DNA preparation.

It is not possible to ensure that the preparations were 100% pure for each type of gamete using present techniques. 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 containing extranuclear DNA from the female gametocytes. Small amounts of extranuclear DNA detected in the male preparation could thus be due to contamination. Since the cross in P. falciparum indicated that inheritance of the cytochrome b gene in malaria was uniparental it can be concluded, with a reasonable degree of confidence, that the 6kb extranuclear element is transmitted through the female gamete only. Likewise, since the 35kb circular DNA probe hybridised to the female but not to the male DNA preparation it is most probable that this element too is maternally transmitted.

5.5.2 Mechanisms of maternal inheritance

Where maternal inheritance of extranuclear elements occurs, there are many different physical mechanisms by which it may be achieved (Reich and Luck, 1966; Lee and Taylor, 1993) (Table VI). It is not always possible to determine whether the male gamete did not carry any extranuclear elements at all (Group A in Table VI), whether it carried the elements but during zygote formation inserted only the nucleus into the female gamete leaving the extranuclear contents behind (Group B in Table VI), or whether extranuclear elements from both parents were carried to the zygote and one set outgrew the other or actively inhibited it (Group C in Table VI).

5.5.3 Mechanisms of maternal inheritance in P. falciparum

The male gametocyte in P. falciparum has a large mitochondrion visible both by light and electron microscopy. Following exflagellation this mitochondrion appears to be left behind as part of the residual body. This has been shown microscopically by staining exflagellating gametocytes with Rhodamine 123 (Vaidya, et al., 1993). Electron microscopic examination of the male and female gametes in malaria species also indicates that each of the 6-8 male gametes, derived from a single male gametocyte, contains little more than a nucleus, surface membrane and an axoneme, whereas the female gamete, derived from a single female gametocyte, contains a full complement of extranuclear organelles (Aikawa et al., 1984; Sinden, 1982; Aikawa, 1988). The failure to find a 6kb element DNA marker or a 35kb element marker in the purified male gametes suggests that at exflagellation the extranuclear DNA is not replicated and presumably is left behind together with the mitochondrion and with whatever organelle is associated with the 35kb element.

5.5.4 Uniparental and bi-parental extranuclear DNA inheritance in other organisms

Among eukaryotes a uniparental maternal pattern is the commonest form of extranuclear DNA inheritance in both plant and animal kingdoms. Almost exclusive maternal inheritance in the higher eukaryotes, however, may have been overestimated. Mice, for example, were thought to display typically maternal transmission of mitochondrial gene markers (Gyllensten et al., 1985) but with the advent of sensitive

TABLE VI

MECHANISMS OF MATERNAL INHERITANCE

MECHANISM	EXAMPLE
A Pre-zygote	
i) Differential growth of large egg and small sperm	Mouse
ii) Exclusion of organelles from male gametes	Crayfish **Plasmodium?**
iii) Disintegration of extranuclear DNA in male gamete	Green alga Bryopsis
B At fertilisation	
i) Exclusion of male organelles from zygote	tunicate Ascidia
ii) No organelles exchanged	ciliate Paramecium
C Zygote stage	
i) Selective disintegration of male extranuclear DNA	green alga Chlamydomonas
ii) Exclusion of male extranuclear DNA from subsequent developmental stages	angiosperm Pelargonium
iii) Random replication only	yeast Saccharomyces

With the exception of that for Plasmodium, (Creasey et al., 1994) Bryopsis (Kuroiwa et al., 1991) and crayfish (Moses, 1961) the informtion and references to the mechasnism in each organism are given in Beale and Knowles 1978 and Beale et al., 1972).

PCR-based detection techniques transmission through spermatozoa was shown to occur at a low level (Gyllensten, et al., 1991). Some eukaryotic animals display more unusual patterns of inheritance. The marine blue mussel Mytilus edulis displays a type of bi-parental transmission which is determined by the sex of the progeny. No female carries mitochondrial DNA from the father but all sons carry mitochondrial DNA from both the father and the mother (Zouros et al., 1994).

Other simpler organisms like the unicellular green alga Chlamydomonas reinhardtii, and the myxomycete Physarum polycephalum (Meland et al., 1991) transmit their cytoplasmic DNA bi-parentally but one parental form later degenerates. In Paramecium spp., conjugating cells do not exchange mitochondria (Beale, 1954; Beale et al., 1972). Among plants, some like the banana Musa acuminata, for example transmit mitochondrial DNA through the female gamete and chloroplast DNA through the male (Faure et al., 1994).

Even in organisms showing bi-parental inheritance of extranuclear genes, parental ratios are often skewed so that, when heterozygotes are formed, they may be quickly dispersed by somatic segregation (see reviews (Brooks, 1988; Kondrashov, 1993)). Furthermore studies of organisms with bi-parental inheritance, such as the blue mussel cited above, suggest that even where both male and female extranuclear genes are inherited by the progeny, actual recombination events between them may be rare.

It seems that in evolution there must be good reasons for maintaining uniparental transmission and minimising the possibility of extranuclear genes being derived from both parents. If plants and animals whose patterns of extranuclear inheritance are known at present are placed in a phylogenetic tree (constructed from rRNA sequence homology), most branches exhibit organisms with uniparental maternal inheritance of extranuclear DNA (Figure 34). It seems likely therefore that evolution of uniparental maternal inheritance may have occurred, not once, but many times probably for a variety of reasons and by a variety of mechanisms.

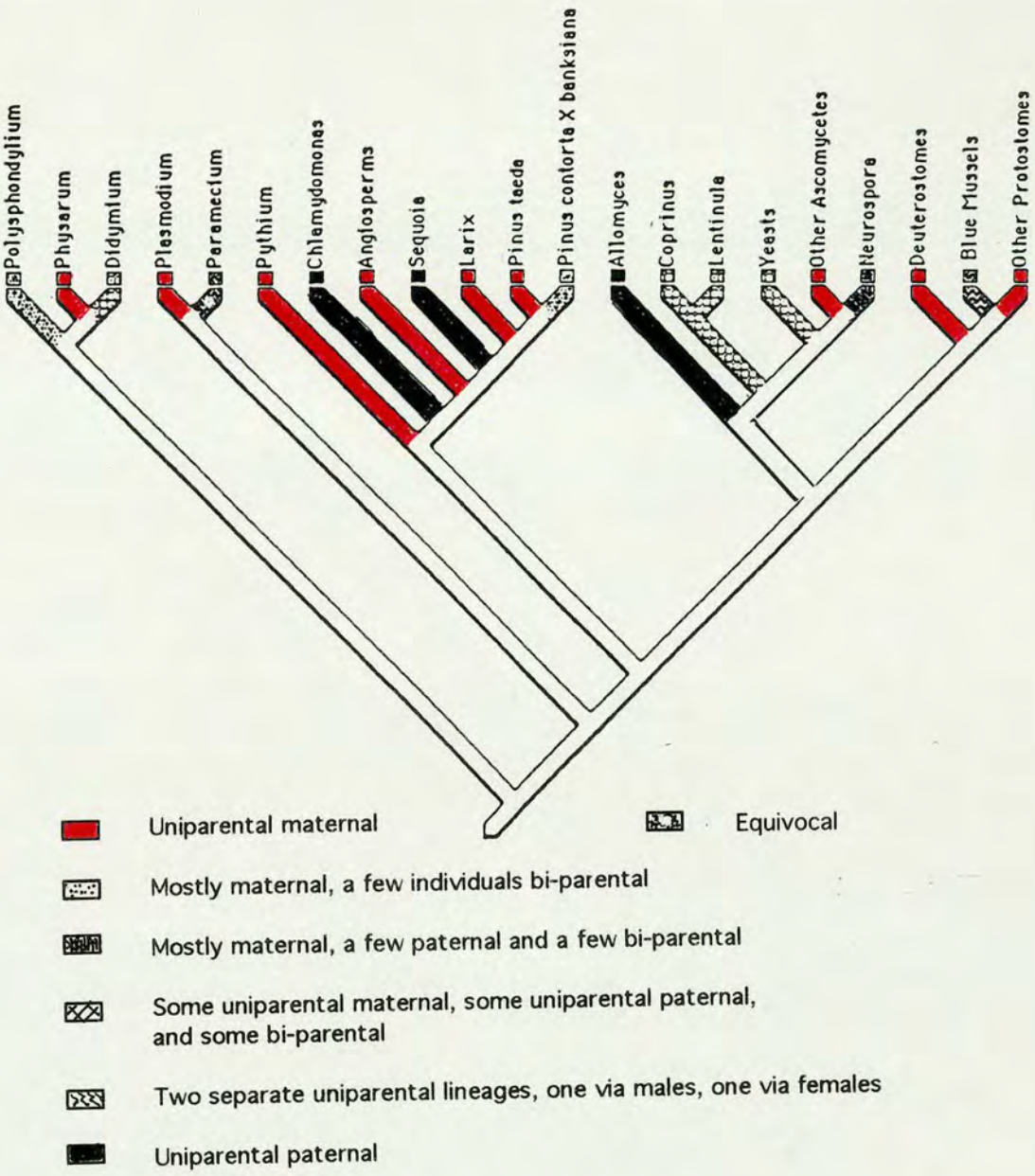


Figure 34. Phylogenetic tree of selected organisms whose mitochondrial DNA inheritance pattern is known. The branching pattern of the tree is based mainly on 18S rRNA gene sequences and indicates that the maternal inheritance pattern appears to have arisen independently several times during evolution.
Organisms are classified according to the major form of inheritance observed. Ancestral forms are coloured white since their inheritance patterns are unknown.

References
Mycetozoa : Slliker & Collins 1988: Meland et al., 1991: Mirfakhrai et al., 1990 #414: Kawano et al., 1987: and 1993. Plasmodium: Creasey et al., 1994. Parameceum : Beale et al.,1972. Pythium Martin, 1989. Chlamydomonas : Sears & VanWinkle-Swift, 1994. Plants : Neale et al., 1989: Wagner et al., 1991. Fungi : Birky, 1994: Yang & Griffiths, 1992: Borkhardt & Olson, 1993: Fukuda et al., 1995. Higher animals Skibinski et al, 1994 Zouros et al, 1994: Magoulas & Zouros, 1993: Meusel & Moritz, 1993.

The exclusively uniparental pattern of extranuclear DNA inheritance in malaria is based, so far, on the small sample of 59 oocysts. The possibility that other types of extranuclear inheritance might occasionally occur cannot be excluded without the examination of a larger sample of hybrid oocysts and/or use of more sensitive techniques.

5.6 Inheritance bias

The most unexpected result of following an extranuclear gene marker through the 3D7 x HB3 cross was the dramatic bias found in the two forms of heterozygote. In summary:

1. One form of heterozygote (HB3 female/3D7 male) was extremely rare.
2. The other form of heterozygote (3D7 female/HB3 male), shows a dramatic preferential advantage over the other heterozygote and to a lesser extent over the homozygotes.
3 At the same time the frequencies of the two homozygotes are not significantly different from those expected if all the gametes were mating randomly in the cross (Ranford-Cartwright, et al., 1993).

A number of factors are relevant to consideration of these results

5.6.1 Factors relating to the parental clones

a) 3D7 and HB3 differ in 'vigour' in the experimental systems used.

In culture the 3D7 clone tends to grow faster and to reach a higher parasitaemia without morphological deterioration than the HB3 clone. A higher production of gametocytes, rate of exflagellation and infectivity to mosquitoes was a consistent finding in 3D7 compared to HB3 in the present work and in almost every successful cross reported between these clones (Walliker, et al., 1987; Ranford-Cartwright, et al., 1993). This situation is reflected in the lower number of self-fertilised HB3 oocysts found in the cross although this difference is not statistically significant (Ranford-Cartwright, 1992). Such variations in growth, gametocyte production and transmission through mosquitoes between different clones of P. falciparum are well

documented although the factors influencing it are largely unknown (Ponnudurai, et al., 1982b; Carter and Graves, 1988).

b) 3D7 and HB3 self-fertilise competently.

3D7 and HB3 are successfully transmitted through mosquitoes when fed separately (as shown by the control feeds) and also when mixed together as shown by the appearance of self-fertilised progeny of both parents in the cross.

By comparison, in the cross between HB3 and Dd2 (Vaidya, et al., 1993) cloning of the isolate from the chimpanzee did not produce any self-fertilised progeny of the Dd2 parent even though a low number of oocysts had been observed in the mosquitoes infected with this clone alone. This cross yielded one form of heterozygote only (Dd2 female/HB3 male) (Walker-Jonah, et al., 1992). The possibility of defectiveness of the Dd2 male gametocyte has already been proposed (Vaidya, et al., 1995). The lack of homozygote progeny in the 3D7 x HB3 cross through the chimpanzee (Walliker, 1987) may be due to some selective process after the oocyst stage, perhaps in the chimpanzee or during the cloning procedures.

5.6.2. Factors influencing the proportions of gametes in a cross

a) Gametocyte/ gamete sex ratios

In the experimental cross 3D7 and HB3 gametocytes were mixed and fed to the mosquitoes in a 1:1 ratio (Tables II and III) (Ranford-Cartwright, et al., 1993). However there was significant difference in male to female sex ratio of gametocytes between the two cultures, the 3D7 clone showing a greater female bias than the HB3 clone (3D7 - 0.92 female ; 0.08 male, HB3 - 0.82 female ; 0.18 male). The chance of an HB3 male fertilising a 3D7 female is therefore greater than that of a 3D7 male fertilising an HB3 female. The difference in gametocyte sex ratios therefore shifts the fertilisation probabilities in the direction of the observed bias although on its own it is not great enough to account for it. The actual bias observed between the gamete sex ratios required to produce the oocysts was much greater (3D7 - 0.71 females ; 0.29 male, HB3 - 0.17 female ; 0.83 male (Results Section 3.1.1).

b) Gametocyte maturation rates

This factor is not known for the 3D7 or HB3 clones. The gametocyte cultures of the two clones were set up on the same day and mixtures of 14 and 17 day cultures fed to the mosquitoes on each occasion. However it is difficult to determine precisely the day on which both cutures are at a similar (and/or maximum) level of infectivity, using morphological means (Jeffrey and Eyles, 1955). If the gametocytes in the two clones mature at slightly different rates this will affect their relative efficiency in the cross.

c) Microgametocyte exflagellation / gamete survival rates.

The actual numbers of male gametes produced per microgametocyte at exflagellation between the two clones 3D7 and HB3 is again unknown. The process of exflagellation was observed by looking microscopically at a drop of the infected blood fed to the mosquitoes. 3D7 displayed a higher exflagellation rate than HB3 as assessed by both the number of occasions on which exflagellation was observed and by the numbers of exflagellating microgametocytes seen per slide examined. However, exflagellation on a microscope slide represents a very artificial environment. compared to that of the mosquito gut so that the relevance of these counts is uncertain. It seems biologically unlikely that each clone produced the same average of male gametes per microgametocyte at exflagellation. In P. falciparum, exflagellation rates range from 3-8 microgametes per microgametocyte (Carter and Graves, 1988).

If exflagellation rates alone were accounting for the bias in the cross then theoretically (using the ratios seen in culture) the 3D7 microgametocytes could have been producing 5 microgametes per microgametocyte if all the macrogametocytes were producing one successful macrogamete each. However even if HB3 were producing the maximum 8 microgametes per microgametocyte the observed proportions of oocysts could not have been produced with random mating with all the macrogametocytes producing one successful macrogamete. The fitness of the macrogametes would also need to vary in order to produce the sex ratios observed in the cross. Several theoretical combinations of exflagellation rate and female gamete fitness for both 3D7 and HB3 will produce these ratios as is shown in Table IV (Results section 3 6 3).

Another important aspect of the fitness of the gametes will also involve the length of time which each gamete remains viable for fertilisation in the mosquito gut. If one sex of gamete remains viable slightly longer than the other sex in either or both of the clones, then the observed bias may be easily achieved.

i) The Dd2 x HB3 cross

In the Dd2 x HB3 cross it has been reported that there was a lower exflagellation rate as well as a lower number of microgametes per microgametocyte in the Dd2 clone than in the HB3 clone. This leads the authors to conclude that the lack of the HB3 female/Dd2 male form of heterozygote in their progeny clones could be accounted for by defective Dd2 male gametocytes (Vaidya et al., 1995). They showed further that this trait was apparently linked to a marker on chromosome 12 (Vaidya, et al., 1995). Defective Dd2 males may be the major factor in this particular cross. However there was no corresponding evidence in the cross between 3D7 and HB3 that the 3D7 male was defective to cause the rarity of 3D7 male/HB3 female hybrid. The observed exflagellation rate of 3D7 on a microscope slide was better than that of HB3 and self-fertilisation of 3D7 was successful. Different mechanisms may be at work in these two crosses. It may also be significant that both crosses involving the HB3 clone produced a similar bias.

A summary of the results of the two P. falciparum crosses 3D7xHB3 and HB3xDd2 is presented in Figure 35. Only further crosses will resolve the question of whether a bias in the inheritance of the extranuclear elements is common in crosses between all P. falciparum clones or only those involving the HB3 clone and/or a Dd2 clone with defective males. It will be interesting to determine whether the bias in the parental gamete contributions to heterozygotes exists in other clones and in other malaria species.

d) Laboratory generated clones and conditions

Both 3D7 and HB3 used in this cross have been maintained in the laboratory and have been artificially cloned. This may have critically altered the genetic content of one or

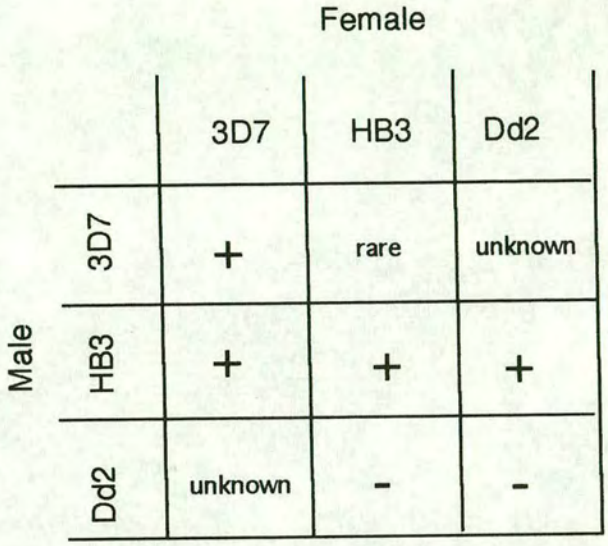


Figure 35. Summary of the mating success* of the male and female gametes in the two Plasmodium falciparum crosses in which the two forms of heterozygote have been distinguished using extranuclear markers.

* in the strict sense for the HB3 x Dd2 cross this represents mating success plus any selection pressures exerted during subsequent growth in the chimpanzee, in vitro and in the cloning process

both of these clones so that the observed bias in this cross could be artifactual and very different from the normal mating behaviour of malaria parasites.

e) 'Unnatural' mosquito hosts and laboratory conditions

The A. stephensi line which originated in the Indian sub-continent is not the 'natural host' of either the Honduran HB3 or the (putative African) 3D7 clone. While both clones are transmitted successfully through A. stephensi previous laboratory studies suggest that infectivity is affected by geographically determined genetic features of both mosquito and parasite (Ponnudurai, et al., 1982b; Collins, et al., 1986).

The effect on infectivity of unnatural parasite/host relationships as well as mosquito maintenance and/or the membrane feeding systems in the laboratory is unknown.

5.6.3 Factors related to the analysis of the cross

In addition to the factors affecting the biological fitness of the gametes two other factors which might have influenced the results of the cross.

a) PCR of the nuclear gene fragments

From the DNA of certain of the oocysts the nuclear gene fragment of MSP-1 could be amplified successfully but not the MSP-2 gene fragment. From others, the MSP-2 gene fragment could be amplified but not the MSP-1 (see Section 3.4.3). In theory, this could have changed the proportions of oocysts recorded. If the allele from one clone was present but could not be amplified it would cause an under-estimate of the heterozygotes although under normal conditions it is possible to detect a 3D7 or an HB3 allele even if it is present at a dilution of 1000x less than the other (Ranford-Cartwright, 1992). Much less likely would be an overestimation of heterozygotes caused by a non-specific band of similar size to that of one of the clones.

b) Sample size

It has been argued that the sample size of 59 might have allowed the unusual bias in the oocysts to have occurred by chance. Since only one oocyst of the HB3 female/3D7 male was recorded no reasonable prediction can be made regarding when the next one might have been found.

5.6.4 Gamete fitnesses and random mating

It is clear that any or all of the above factors which affecting gamete fitness may have contributed to the eventual proportions of the four types of oocyst produced in this cross. ("Fitness" here being defined in the classical population genetics sense as "the proportion of gametes represented and able to reproduce in the next generation" [Falconer, 1960]).

As has been seen in Table IV (Chapter 3 Section 3.7.1), there are a number of different ways in which the ratios of the two clones might have varied to produce the proportions observed in the oocyst results. A more statistical approach was then taken to determine which of these theoretically possible proportions gave the best comparison with those expected if mating were occurring randomly between the male and female gametes in the parasite mixture.

5.6.5 A theoretical model testing for random mating while manipulating gamete fitness.

A mathematical model has been presented in which the viability of the four types of gamete involved in the 3D7xHB3 cross was theoretically manipulated (Ranford-Cartwright, 1994). This analysis was first carried out on the 60 oocysts for which both the MSP-1 and the MSP-2 gene fragments had been successfully amplified and typed.

a) A sample size of 60

In this model the relative fitness of the 3D7 female gamete was given a value of 1 and the relative fitnesses of the other three gametes allowed to vary freely. The numbers of homozygous and heterozygous progeny which would result during random mating from each set of fitnesses of the four gametes was calculated and compared for goodness of fit (G-test) with the actual numbers of homozygotes and heterozygotes observed in the cross. The closest comparison was achieved when the proportions of the gametes relative to the 3D7 female (1) were between 4.5-4.6 for the 3D7 male, between 3.8-6.4 for the HB3 male and between 0.15-0.25 for the HB3 female. At these values of relative fitness the observed and predicted proportions of the four types

of gametes were not significantly different as assessed by the goodness of fit test (G-test) = 1.7). The lack of significance of this G-test showed that it was possible to obtain the observed results during random mating if the viability of the male gametes of the two clones is approximately equal but the viability of the female HB3 gametes is five times lower than the viability of the female 3D7 gametes. This result coincides with the rough estimates presented in Table IV of the Results Section 3.7.1 page 109.

b) A sample size of 110

In the current study the sample size was increased to 110. This set of samples included all the hybrid oocysts typed for either one or two nuclear genes.

The computer model was re-run as before (using a fitness of 1 for the 3D7 female gamete and allowing the 'fitness' of the other three to vary freely from 0.05-10). All the theoretically possible manipulations of fitness between the gametes from the 110 oocyst sample were significantly different at the 5% level (2 degrees of freedom) from those which would be expected during random mating. The closest comparison was achieved when the proportions of the gametes relative to the 3D7 female (1) were between 4-5 for the 3D7 male, between 2-7 for the HB3 male and between 0.1-0.3 for the HB3 female. The best goodness of fit G-scores, however, comparing the observed to the possible manipulated gamete proportions were all around 7.4 - 7.5 while the probability of obtaining these results at the 5% level is 5.99 ($p > 0.05 = 5.99$). Therefore, using the 110 oocyst sample, simple random mating principles cannot not account for the proportions of the progeny in this cross. The 3D7 female gamete appears to be mating more frequently than expected with HB3 males than with 3D7 males while the HB3 females appear to be mating more frequently than expected with their own HB3 males than with 3D7 males.

5.6.6 Summary - the question of random mating in the 3D7 x HB3 cross

Random mating between malaria parasites has been assumed for most genetic analysis of the parasite to date (Wellems and Howard, 1986; Walliker, et al., 1987; Wellems, Walliker et al., 1987; Walliker, et al., 1988; Wellems, et al., 1990) and analysis of nuclear gene inheritance in this cross at first confirmed this assumption. However by

distinguishing between the two forms of heterozygote it was possible to find out the actual numbers of male and female gametes of both parents producing the four types of oocysts in the cross. These numbers were used to calculate the expected proportions of the four types of oocysts if mating was random. These predicted numbers differed significantly from the observed numbers and so random mating could no longer be invoked.

5.6.7 Theoretical explanations for non-random mating

The inability to account for the observed proportions of the four types of oocyst progeny in the 3D7 x HB3 cross using random mating principles can lead only to the conclusion that some form of non-random mating is occurring. Mating bias due to gamete fitness for fertilisation has been discussed at length above and this may be the most likely cause of apparent non-random mating in malaria. The following are some explanations for non-random mating which have been observed in other organisms:-

a) Extranuclear incompatibility

Extranuclear incompatibility is usually defined as the situation in which a cytoplasmically inherited factor prevents the successful development of zygotes from matings between individuals carrying the factor and those without it (Barr, 1980). Frequently these incompatibilities are caused by the rickettsia-like bacteria particularly the Wolbachia spp. (O'Neil and Patterson, 1992; Rousset et al., 1992; Werren and Jaenke, 1995). Typically the incompatibility is asymmetric; males from the infected parent being able to affect uninfected females although females of the infected line are not themselves affected (Spencer, 1995).

There are a number of well-studied examples of this phenomenon particularly among invertebrate species including Culex mosquitoes (Fine, 1978), Drosophila spp. (Turelli, 1992) and Tribolium flour beetles (Stevens and Wade, 1990). Early experiments in Culex mosquitoes for example showed that when different strains of wild caught Culex pipiens mosquitoes were crossed in the laboratory different results were obtained depending on the strains used as parents and on which parent was used as the male parent and which the female parent (Laven, 1959). In a cross between

strains Ha (Hamburg) and Og (Oggelshausen), female Ha crossed with male Og produced mostly viable eggs (87%). The reciprocal cross female Og with male Ha was either sterile or produced very few (less than 0.2%) viable eggs. The presence of a rickettsia-like bacterium was later shown to be involved in this phenomenon.

It is difficult to determine whether such a system could be operating in malaria. No reports have been found indicating the presence of endosymbionts in malaria parasites. Experimentally it might be possible to test whether an endosymbiont was conveying the bias by treating the clones with antibiotics before repeating the cross, although sensitivity of P. falciparum cultures to many common antibiotics might make this approach impractical. However it would be relatively simple to probe for the rickettsias using the panel of probes available (J. Werren - personal communication).

b) Mating hierarchy

In microorganisms the mating type phenomenon is usually defined as the sub-division of a species into groups based on their mating behaviour. In this system only individuals of different mating types will mate. This is obviously not the case in the 3D7 x HB3 P. falciparum cross. However the results may indicate male and female mating preferences which, if examined in many clones, might reveal a hierarchy of preferences. If the mutation in the cytochrome b gene which is reported to have arisen under Atovaquone pressure proves to be stable and is different from that found between 3D7 and HB3, then a clone produced in this way might be crossed with both 3D7 and HB3 to help unravel such a system.

c) Early speciation

It has been suggested that, since the clones used in the crosses were from different parts of the world, the bias observed in the progeny may represent an early phase of reproductive isolation of P. falciparum clones from different geographical regions (Vaidya, et al., 1993).

d) An aberration of the particular clones in the laboratory cross

Apparent non-random mating observed in P. falciparum crosses may simply reflect defectiveness (perhaps induced by in vitro culture) of one or more of the particular

clones used. Crosses between other different clones might help to answer this question. The more important question is whether non-random mating between malaria parasites is observed only in 'artificial' crosses in the laboratory or whether it actually occurs in the natural population of parasites in the field. A recent study has begun to address this question by studying the parasite population structure in malaria patients from 6 villages in Papua New Guinea (Paul et al., 1995).

5.6.8 Final summary of the results of the cross.

The proportions of the homozygotes and the two forms of heterozygotes in the progeny of the cross were significantly different those which were expected from the material which was fed to the mosquitoes. We know, however, that there are a large number of variable parameters in the whole process the cultures being fed to the survival of the oocysts, and so this result is not entirely unexpected.

More intriguingly, if we ignore all the factors biasing the cross up to the oocyst stage and look only at the distribution of the four types of gametes among the four types of oocyst progeny what survived, it is obvious that something other than normal chance is causing the observed proportions of homozygotes and heterozygotes.

Briefly the 3D7 females, (for whatever reason), apparently are more likely to mate with HB3 males than their own males. There are around double the number of heterozygotes involving 3D7 females than would be expected. Conversely the HB3 females apparently prefer to mate with their own males rather than 3D7 males. The HB3 female x 3D7 male is very rare but the HB3 self-fertilised oocysts survive at an expected level given the low number of total HB3 female gametes present.

Speculation into the various biochemical mechanisms to explain different levels of attraction and/or survival of gamete combinations as well as into the evolutionary strategies which may be involved are also intriguing. Outbreeding (as apparently preferred by 3D7 females) carries greater chance of improving DNA but also greater risk of encountering less healthy or incompatible DNA. Inbreeding (as apparently preferred by HB3 females) carries the security of mixing DNA that is always compatible but has no possibility of improvement by recombination.

The forced sub-division of parasite populations in both human and mosquito hosts imposes a degree of inbreeding by virtue of their life-cycle (Read and Day, 1992). Recent studies of field populations of P. falciparum parasites in Papua New Guinean people have inferred that populations are inbreeding even where mixed infections have been detected (Paul, 1996).

5.7 Practical applications of the features of extranuclear elements in malaria

5.7.1 Identification of female gamete contributions in a cross

The ability to follow a uniparentally inherited marker in the hybrid progeny of a cross may be useful in the analysis of crosses set up to examine the inheritance of almost any heritable trait in the parasite since it can identify the relative survival rates of the two forms of heteroztgote.

Identification of the female gamete might be of particular relevance where genes of interest are thought to be located in the extranuclear elements themselves. Furthermore, it might be important to know which parent had contributed the female gamete to a hybrid if the surface of one or other of the gametes is playing a role in the development of the phenotype, as in the response to melanisation of the melanisation of the parasite in the mosquito (Section 1 Chapter 4).

The ability to identify male and female contributions in hybrid progeny of a cross may also for the first time allow some investigations into the influence of gametocyte sex ratios on the population genetics of Plasmodium parasites.

5.7.2 Malaria control using inhibitors of extranuclear DNA and/ or extranuclear element function

The high degree of conservation of both the 6kb and 35kb elements points to their functional importance in the parasite. The unusual nature of some of the genes on the extranuclear elements in malaria parasites highlights one of their potentially most important practical features, - namely that their genes may differ significantly from the homologous genes in their hosts. The highly conserved protein-coding genes, the much-reduced and scrambled rRNA genes of the 6kb element and the very ' un-

eukaryotic' structure of the 35kb genes are obvious targets for drugs which may disrupt the metabolic processes of the parasite but leave the host unaffected.

5.7.3 Studies of drug resistance

It is envisaged that techniques developed in this project may have a part to play in the on-going studies to investigate the mechanisms of drug resistance in malaria.

Recent studies in P. falciparum and P. chabaudi using the progeny of crosses between a chloroquine-resistant clone and the chloroquine-sensitive clone from which it was derived are contributing to the search for genes involved in chloroquine resistance (Wellems, 1990; Walliker et al., 1987; Carlton, 1995). In these studies a large number of markers were mapped to the nuclear genomes of the progeny to look for linkage with the drug sensitivity phenotype. In a similar manner it would be possible to investigate whether resistance to various drugs is linked to extranuclear genes. Several drugs in current use are known to act on the mitochondria (Chapter 1, Section 1.9.1). Recent identification of mutations in the cytochrome b gene of P. falciparum linked to high-level resistance to Atovaquone have already been mentioned (Wilson et al., 1996) and(Chapter 1, Section 1.9.1 a).

5.7.4 Useful practical procedures

The study has provided some useful practical procedures for the analysis of experimental crosses. In particular it has developed methods for finding rare mutations in the highly conserved extranuclear DNA, especially in the A+T rich areas of sequence.

5.8 Wider implications of the study

The results of the inheritance of the extranuclear elements in the cross between clones 3D7 and HB3 show clearly that progeny from a P. falciparum do not occur in the proportions expected from simple uncomplicated random mating. This observation may have far-reaching implications. Much of the theoretical work on the spread of drug resistance, for example, relies heavily on the assumption of random mating of the parasite in the mosquito. Similarly much of the work on the genetics of the parasite and its diversity in the field assumes random mating for its analysis.

The critical issue with regard to random mating is whether the results observed in this laboratory cross are true for all P. falciparum matings and whether the same phenomenon is occurring in the field. It should be possible to answer the first part of this question if crosses using different clones are analysed in a similar way to the 3D7 x HB3 cross. The second part of this question of whether this is what happens in the real world of malaria parasites in nature will be much more difficult to assess, but perhaps knowledge of this aspect of the parasite's biology will provide better understanding of the dynamics of malaria infection and ultimately better control of the disease.

However the oocyst stage represents the fertilisation products which have already passed through selection through the zygote and ookinete stages mosquito mid-gut. They therefore represent the mid-gut selected products of mating rather than mating in the strictest sense. The zygotes which are the actual progeny resulting from the mating between two differring parasites have not yet been analysed in this way although this would be possible using in situ techniques. However the oocysts provide a good indication of the products of mating which are going to be transmitted via the sporozoites as the next generation to the infecting the human host.

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APPENDIX I

Plasmodium species mentioned in this thesis together with their natural hosts

Species	Natural host
P. berghei	Rodent
P. brazilianum	Primate (+human - experimental)
P. cynomolgi	Primate
P. fallax	Guinea fowl (+other wild birds?)
P. falciparum	Human
P. gallinaceum	Chicken (originally Asian jungle fowl?)
P. gonderi	Primate
P. innui	Primate
P. knowelsi	Primate
P. lophurae	Duck
P. malariae	Human
P. yoelii	Rodent
P. vivax	Human

APPENDIX II

THE MITOCHONDRIAL bc_1 COMPLEX

The functional unit of the bc_1 complex normally consists of at least 11 proteins, only 3 of which carry redox centres[JaGouw, 1986 #436]. These 3 are cytochrome b, with 2 redox centres (haem) cytochrome c_1 with 1 redox centre (haem), and the ferredoxin-type iron-sulphur (or Rieske) protein (Fe_2S_2).with one redox centre. There are two catalytic sites within the complex, the Q_0 site on the cytoplasmic side (c-side) of the membrane where ubiquinol (QH_2) oxidation occurs, and the Q_1 site on the matrix side (m-side) of the membrane where ubiquinone (Q) reduction occurs. (Figure A.1 A)

The reaction sequence appears to be as follows :

At the Q_0 site, ubiquinol (also known as coenzyme Q or CoQ) is oxidized to ubiquinone in two steps, one electron being transferred to the iron sulphur centre and then via cytochrome c_1 to cytochrome c, while the second electron is transferred to the low potential heam b_{566} and from there to the high potential haem b_{562} of the cytochrome b (Figure A.1 B). The oxidation of ubiquinol is accompanied by the release of two protons to the c-side.

At the Q_1 site ubiquinone is re-reduced in a two-step mechanism by the haem b_{562}, so that half the electrons set free during ubiquinol oxidation return to their point of origin, the quinone pool (Q/QH_2 in Figure A.1 A). The dashed arrows indicate where the oxidized ubiquinone formed at the c-side of the mitochondrial membrane is re-reduced at the m-side by various dehydrogenases.

During the flow of two electrons from ubiquinol to cytochrome c_1, four protons are released to the outer space while two protons are taken up at the inside.

Numerous inhibitors of this reaction are available and these fall into at least four groups:

Group 1: Various ß-methoxyacrylates binding at the Q_0 site and blocking two reactions at the same time i.e. electron transfer from the QH_2 to the iron sulphur centre and electron transfer onto the haem b_{566}.

Group 2: Hydroxyquinone analogues, binding at the Q_0 site and blocking electron transfer from the iron-sulphur centre to cytochrome c_1 as well electron transfer onto the haem b_{562}.

Group 3: Antimycin. funiculosin and certain quinone analogues binding at the Q_1 site and blocking electron transfer from the haem b_{562} centre to ubiquinone.

Group 4. The chromone inhibitors (stigmatellins) which completely block the Q_0 centre but show binding properties completely different to both Groups 1 and 2.

Because many of these inhibitors are structurally similar to ubiquinol they are believed to act through competative binding to either Q_0 or Q_1.

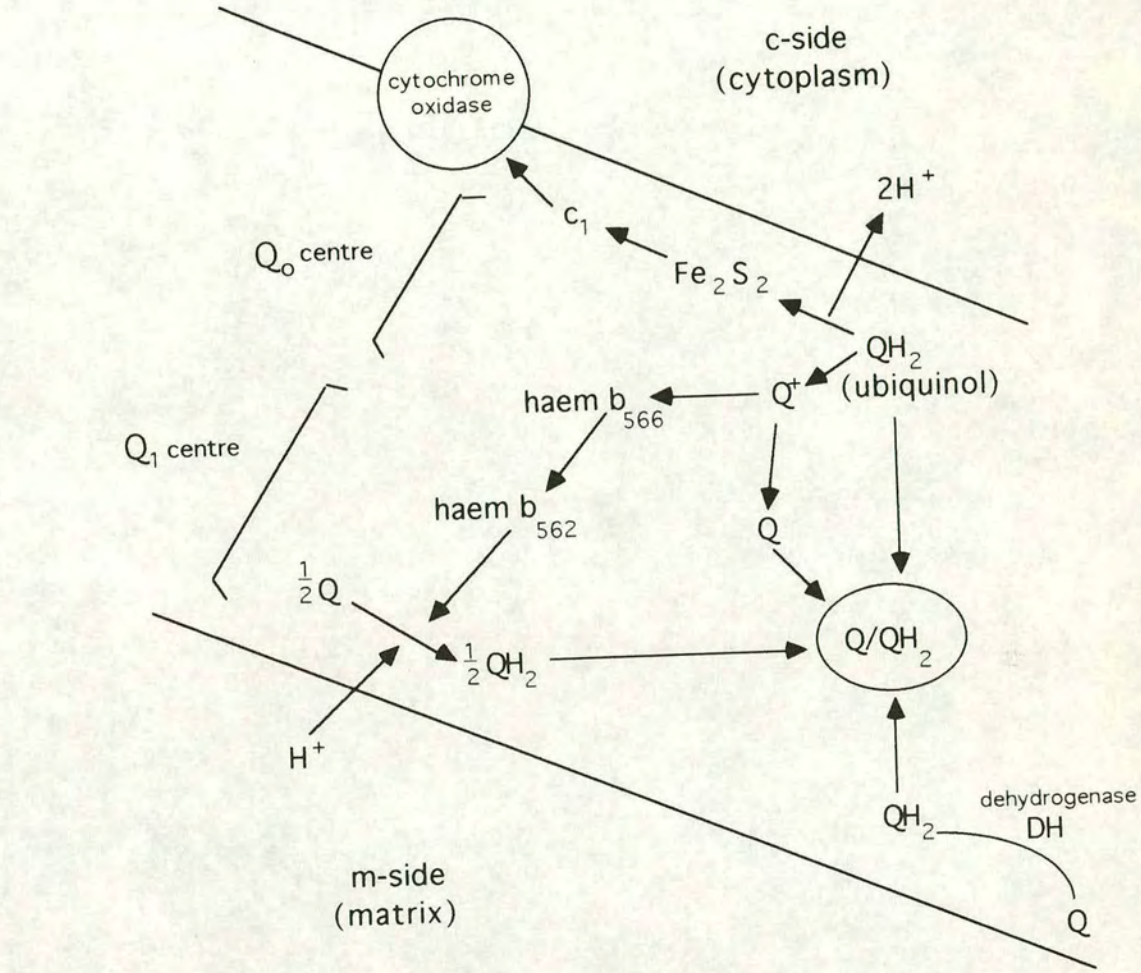
Figure A1. The mitochondrial bc_1 complex

Figure A.1

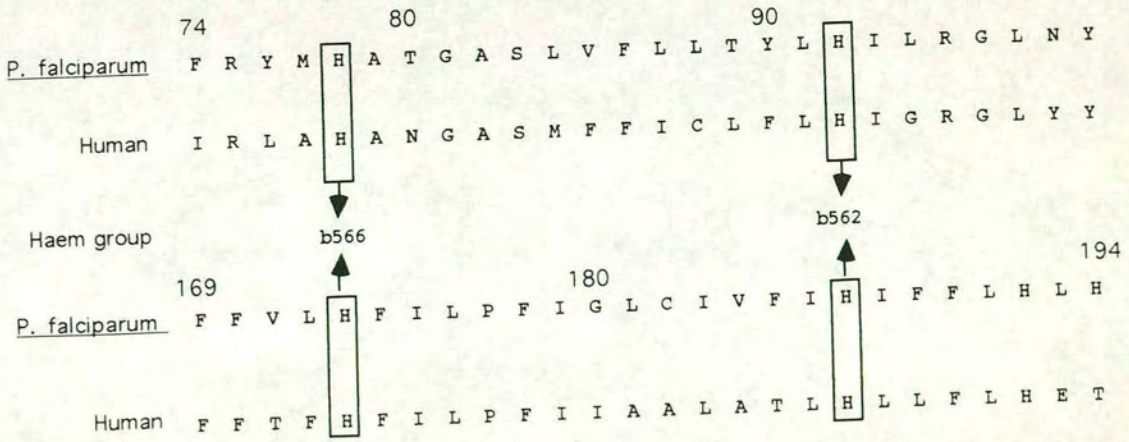
A - Diagramatic representation of the bc_1 complex

B - Diagramatic representation of the haem binding sites in the Plasmodium falciparum cytochrome b gene compared to those of the human cytochrome b gene.

A


cytochrome oxidase
c-side (cytoplasm)
2H+
c1
Qo centre
Fe2S2
QH2 (ubiquinol)
Q+
haem b566
Q1 centre
haem b562
Q
½Q
½QH2
Q/QH2
H+
QH2
dehydrogenase DH
m-side (matrix)
Q

B


74
80
90
P. falciparum F R Y M H A T G A S L V F L L T Y L H I L R G L N Y
Human I R L A H A N G A S M F F I C L F L H I G R G L Y Y
Haem group
b566
b562
194
169
180
P. falciparum F F V L H F I L P F I G L C I V F I H I F F L H L H
Human F F T F H F I L P F I I A A L A T L H L L F L H E T

APPENDIX III

PARTIAL SEQUENCES OF THE CYTOCHROME B GENE FROM FIVE PLASMODIUM SPECIES AND FROM THEILERIA ANNULATA USED TO CONSTRUCT A PHYLOGENETIC TREE

(Numbers refer to the P. falciparum gene bases).

```
                10        20        30        40        50        60
Pf ATGAACTTTTACTCTATTAATTTAGTTAAAGCACACTTAATAAATTACCCATGTCCATTG
Pg ATGAATTATTACTCTATTAATTTAGCTAAAGCACACTTACTTCATTATCCATGTCCACTA
Py ATGAACTATAACTCAATTAATTTAGTAAAAACACATTTAATTAATTACCCATTCCATTAA
Pv ATGAATTATNNNICTATTAATTTAGCTTTTGCACATTTATTAAATTACCCATTCCCATTA
Pc ATGAACTATAATTCTATTAATTTAGTAAAAACACATTTAATGAATTATCCATGTCCATTA
Ta                                        CATTTGCTTTCGTATATGGTACCTAAA
                70        80        90       100       110       120
Pf AACATAAACTTTTTATGGAATTACGGATTCCTTTTAGGAATAATATTTTTTATTCAAATT
Pg AATATTAATTTCTTATGCAATTATGGATTTCTTTTAGGAATAGTAAGAGCAATTATAGGA
Py AATATTAATTTCTTATGGAATTATGGTTTCCTTTTAGGAATAATATTTTTTATTCAAATA
Pv AATATTAATTTCTTATGGAATTATGGATTTCTTTTAGGTATTATATTCTTTATACAAATA
Pc AACATTAATTTCTTATGGAATTATGGATTCCTATTAGGAATTATATTTTTTATTCAAATC
Ta AATTTGAATTTGAATTGGAATTTTGGGTTTATACTTGGAATATTGTTAGTACTCCAAATT
              130       140
Pf ATAACAGGTGTATTTTTAGCAAGTCGA
Pg ATGGATGGTGTTTTAGATATATATGCAT
Py TTAACAGGTGTATTTTTAGCAAGTCGTT
Pv TTAACAGGTGTATTTTTAGTCAGTGCTA
Pc TTAACAGGTGTATTCTTAGCTAGTCG
Ta ATATCCGGGTTGATGCTTTCTTTTTT
  453     460       470       480       490       500       510
Pf AGTTATTGGGGTGCAACTGTAATTACTAACTTGTTATCCTCTATTCCAGTAGCAGTAATT
Pg TGTTTCATGGGGTCAAATGAGTTTCTGAAATACTACTGTTATAACTAATTTACTTTATTTAT
Py AGTTATTGGGGTGCAACTGTTATTACTAATTTATTATCTGGTATACCAGCATTAGTAATT
Pv AGTTATTGGGGTGCAACTGTTATAACTAATTTATTATCTTCTATTCCAGTATTAGTAATT
Pc AGTTATTGGGGTGCAACTGTTATTACTAATTTATTATCTGGTATTCCAGCATTAGTAATT
Ta AGCTTCTGGGGAGCTACAGTCATAGGTGGTTTATTGAAATTTTTCGGAAAAGCTAATGTT
            520       530       540       550
Pf TGGATATGTGGAGGATATACTGTGAGTGATCCTACAATAAAA
Pg TCCGGACTTGTTTCATGGATTGTGGTGGATATCTTGTAACTG
Py TGGTTATGTGGAGGATACACTGTTAGTGATCCAACAATAAAA
Pv TGGTTATGTGGAGGATATACTGTAAGTGATCCTACAATTAAA
Pc TGGTTATGTGGAGGATACACAGTTAGTGATCCAACAATTAAA
Ta CTAATTTTTGGAGGCCAAACAGTTGGTCCAGAGACATTAGAG
  1102    1110      1120      1130      1140      1150      1160
Pf ATTCTTATTAGCAGAACAAAGAAGTTTAACAACTATAATTCAATTTAAAATGATTTTTGGT
Pg ATTCTATTAGCAGAACAAAGAAATTTAACAACTCTTATCCATTTTAAATTTGCTTTTTGGT
Py ATTCTTATTAGCAGAACAAAGAAATTTAACAACTATTATACAATTTAAAATGGTTTTTAGT
Pv ATTTTTGTTAGCTGAACAANNNNNNTTAACTACTATAATAGAATTTAAAATGACATTTGGT
Pc ATTTTTATTAGCAGAACAAAGAACTTAAACAACTATTATACAATTTAAAATGGGTTTTAGT
Ta AGTATTATTAGTTGAATCTAGAACTTTAAACAGACTGTTTCAGCTATGAATTATCATAGAG
           1170      1180      1190      1200      1210
Pf GCTAGAGATTAT  TCTGTTCCTATTATATGGTTTATGTGTGCATTCTATGCTTTATTAT
Pg GCTAGAGAATAT  TCAGTTCCTACAATTTGTTATAGTTGTGCATTTCTATGCTTATATT
Py GCTAGAGAATAT  TTCAGTTCCTATTATATGGTTTATGTGTTCATTCTATGCTTTATTT
Pv GCTAGAGAATAT  TCAGTACGAANNNTATGGTTCATGTGTTCATTCTATTCTTTATTAT
Pc GCTAGAGATTAT  TCTGTTCCTATTATATGGTTTATGTGTGCATTCTATGCTTTACTAT
Ta TATGGACTACATCAAGTGTACCAATTT
 1220      1230      1240      1250
Pf TATGGATTGGATGTCAA    TTACCACAAGATAT
Pg TATGGATTGGATGTCAA    TTACCACAAGATATT
Py ATAGGATAGGATGTCAA    TTACCACACAAGATA
Pv TATGGATTGGATGTCAG    TTACCACAAGATANN
Pc TATGGATTGGATAGGAC    TTACCACAAGATA
Ta AAAGATGGTTGTTCATGTTGATCTCTCATAGCTATT
```

Source of sequences

P.falciparum	Gardner et al 1993
P. yoelii	Vaidya et al GCG Acc No M 23009
P. gallinaceum	Aldritt et al GCG Acc No. j80040
P. vivax	Partial sequence Creasey unpublished
P. chabaudi	Partial sequence Creasey and Murray unpublished
T. annulata	Megson et al GCG Acc No. V50118

PUBLICATIONS ARISING FROM THESE STUDIES

Creasey, A.M., Ranford-Cartwright, L.C., Moore, D.J., Williamson, D.H., Wilson, R.J.M., Walliker, D. and Carter, R., (1993). Uniparental inheritance of the mitochondrial gene cytochrome b in Plasmodium falciparum. *Curr. Genet.* 23:360-364.

Creasey, A., Mendis, K., Carlton, J., Williamson, D., Wilson, I. and Carter, R., (1994). Maternal inheritance of the extrachromosomal DNA in malaria parasites. *Mol. Biochem. Parasitol.* 65:95-98.

Curr Genet (1993) 23:360–364

Current Genetics

Uniparental inheritance of the *mitochondrial* gene cytochrome b in *Plasmodium falciparum*

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Abstract. The inheritance of an extrachromosomal 6-kb element has been examined in the human malaria parasite *Plasmodium falciparum*. A single base pair difference in the cytochrome b gene from the 6-kb element of two different cloned lines of the parasite was identified, and used as a marker in a cross in the mosquito stage of the life cycle. Analysis of 59 individual hybrid oocysts resulting from this cross clearly demonstrated that inheritance of the cytochrome b gene was uniparental. This observation makes it possible to investigate the inheritance and evolution of cytoplasmic traits, including certain forms of drug resistance, in natural populations of this parasite.

Key words: Plasmodium falciparum – Cytochrome b – Uniparental inheritance – Mitochondrial

Introduction

Malaria parasites have two dissimilar extrachromosomal DNAs (Wilson et al. 1991). One is a 35-kb covalently closed circular molecule of uncertain cellular location (Gardner et al. 1991). The other comprises multiple tandem repeats of a sequence known as the 6-kb element. This element is believed to be mitochondrial since it carries characteristic mitochondrial genes, including subunits of cytochrome oxidase, cytochrome b (*cytb*), and fragmentary rRNA genes (Vaidya et al 1989; Feagin et al. 1992, Feagin 1992), and is enriched in a mitochondrial subcellular fraction (Wilson et al 1992). While genetic analysis of malaria parasites by classical crossing methods has shown that nuclear genes are inherited in a Mendelian fashion (Walliker et al. 1987), nothing is known about the inheritance of the extrachromosomal elements.

The malaria parasite *Plasmodium falciparum* is haploid for most of its life cycle. It produces both male and female gametocytes in the human host which are taken up by the second host, the mosquito, when it takes a blood meal. Within the mosquito, the gametocytes mature into gametes which undergo fertilisation to form diploid zygotes. Meiosis occurs shortly after zygote formation, each zygote giving rise to an oocyst, on the outside of the mosquito midgut, which contains several thousand haploid progeny (Sinden and Hartley 1985). These mature as sporozoites, which are the forms infective to humans. Genetic crosses between malaria parasites are made by feeding mosquitoes on a mixture of gametocytes of two clones (Walliker et al. 1987). Since each parental clone produces both male and female gametes, random fertilization events will produce homozygous parental-type zygotes by selfing and heterozygous types by crossing.

Until recently it was not possible to analyze the genotypes of individual zygotes directly. However advances in PCR technology permit the examination of the DNA of single oocysts (Ranford-Cartwright et al. 1991 b), and by their possession of both parental alleles of certain genes it is possible to identify those which are derived from heterozygotes. In this paper we use this technique to study the inheritance of the *cytb* gene of the 6-kb element in the human malaria parasite *P. falciparum*. We first located a single base pair difference in the *cytb* gene for use as a marker to distinguish two cloned lines, 3D7 and HB3. The two clones were then crossed in mosquitoes, and hybrid oocysts identified by their possession of both parental alleles of two nuclear genes *MSP-1* and *MSP-2* (Ranford-Cartwright et al. 1991 a, Fenton et al. 1991). These oocysts were then analysed with respect to the *cytb* gene marker. Our results indicate that the *cytb* gene is inherited uniparentally in all hybrid oocysts.

Materials and methods

P. falciparum clones. The clones used were 3D7, derived from a *P. falciparum* isolate originating from a patient in The Netherlands, and HB3 from an isolate from Honduras. They were cultured in the laboratory by methods described previously (Walliker et al. 1987).

Genetic cross between clones 3D7 and HB3 in mosquitoes and identification of hybrid oocysts. Gametocytes of 3D7 and HB3 were cul-

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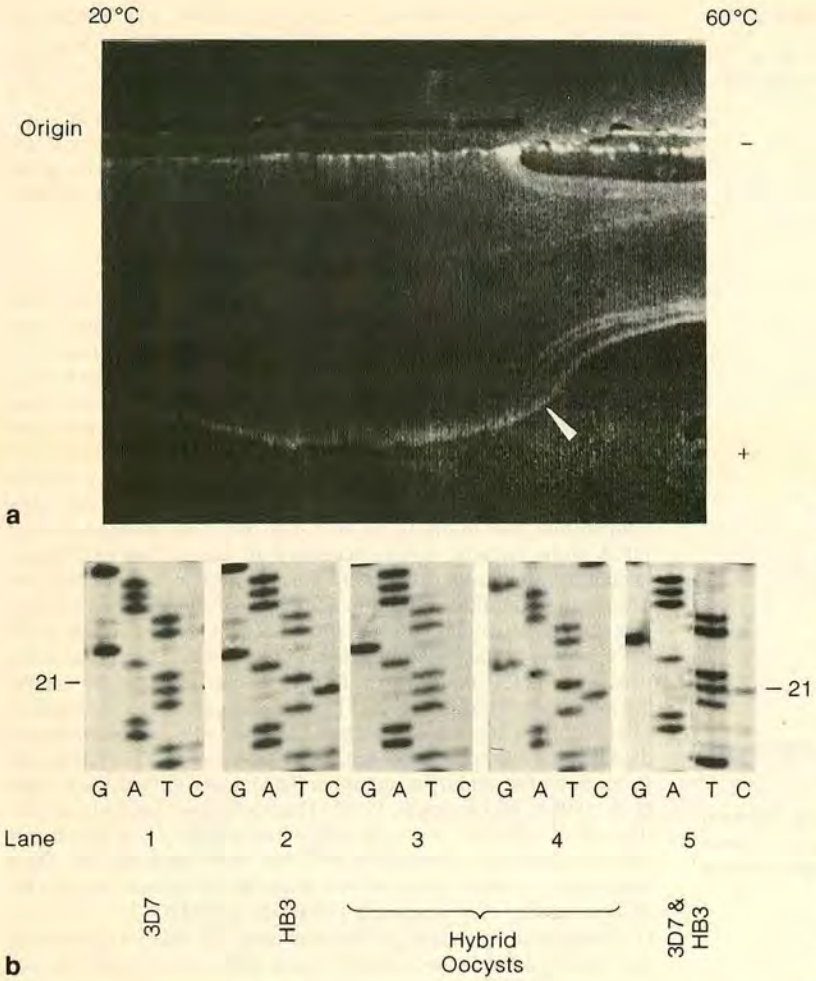

Fig. 3. **a** TGGE analysis of a PCR fragment of the *cytb* gene from two *P. falciparum* clones, 3D7 and HB3. The homoduplex molecules of 3D7 and HB3 appear together as the fastest running band (*arrowed*). The two heteroduplexes of 3D7 and HB3, being less stable, denature at a lower temperature and appear as the two slower running bands. **b** Sequencing gels of DNA fragments covering the mutant site of the *cytb* gene in the clones 3D7 and HB3. *Lane 1*, DNA from blood-stage parasites of clone 3D7; *lane 2*, DNA from blood-stage parasites of clone HB3; *lane 3*, DNA from hybrid oocyst from a cross between 3D7 and HB3 showing only the 3D7 form at position 21; *lane 4*, DNA from hybrid oocyst from a cross between 3D7 and HB3 showing only the HB3 form at position 21; *lane 5*, DNA from oocysts of each of the parental clones 3D7 and HB3, mixed together before amplification and sequencing showing both forms at position 21

no sequence differences between the two strands, all the renatured fragments in the sample will denature at the same temperature and appear as a single sigmoid-shaped band on the gel (Fig. 3a). If the two strands differ in base sequence, the resulting mismatched heteroduplex molecules will denature at lower temperatures than the homoduplex molecules and appear as two slower-running bands on the gel, corresponding to the two possible versions of the heteroduplex fragments (Fig. 3a). In the present study, amplified fragments of the *cytb* gene from 3D7 and HB3 were mixed and subjected to TGGE. One pair of fragments, amplified with primers 1 and 3, produced a three-band pattern on the gel, indicating a sequence difference between the two genes (Fig. 3a).

Sequencing of the fragments revealed a single base pair transition at position 21 of the predicted *cytb* open reading frame, T in 3D7 being replaced by C in HB3 (Fig. 3b, lanes 1 and 2). This produced no change in the expected amino acid leucine (TTA to CTA) at this position. No other differences were found between 3D7 and HB3 in the 70% of the gene that was sequenced (data not shown).

The HB3 form of the gene, but not the 3D7 form, includes the sequence ATCTAG recognised by the restriction enzyme *Bfa*-1. Digestion of each PCR product confirmed the results obtained by direct sequencing; the 3D7 product remained uncut and the HB3 product was cut into two fragments of the predicted sizes 80 bp and 359 bp (Fig. 4b, lanes 1 and 2).

Uniparental inheritance of the cytb gene

DNA from 59 individual oocysts, each of which was identified as hybrid for the nuclear gene markers *MSP-1* and *-2*, was analysed for the *cytb* transition at position 21, using the 'nested' PCR technique.

Direct sequencing of DNA fragments from each oocyst was carried out at least twice. All 59 oocysts showed only one of the two parental *cytb* types (Fig. 3b, lanes 3 and 4). 58 oocysts possessed the 3D7 form, and only one oocyst possessed the HB3 form. As controls, DNA from oocysts of both parental types were mixed together (1:1) amplified and sequenced. Both forms of the gene were detectable in this mixture (Fig. 3b, lane 5).

The results obtained by direct sequencing were further confirmed by digestion of the oocyst PCR products with *Bfa*1. A second restriction digest was carried out using PCR products in which [α^{32}P]dATP (Amersham) was incorporated in the second nested PCR reaction for the final five cycles. The products of the digests were run on 1.5% agarose gels stained with ethidium bromide. The radioactive gels were dried and exposed to X-ray film for

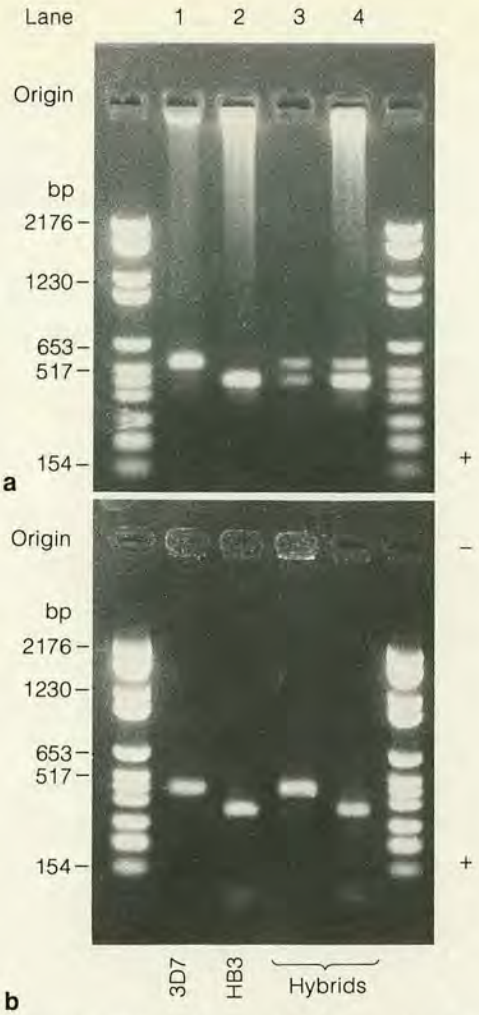

Fig. 4a, b. PCR products of nuclear and cytoplasmic gene fragments from hybrid and parental type oocysts of a cross between *P. falciparum* clones 3D7 and HB3. **a** The upper gel shows DNA fragments amplified using primers for the *MSP-1* gene showing the size polymorphism in the two parental types and the presence of both parental alleles in the hybrid oocysts. *Lane 1*, products of parental type 3D7 oocysts; *lane 2*, products of parental type HB3 oocysts; *lanes 3 and 4*, products of hybrid oocysts resulting from the cross between 3D7 and HB3. **b** The lower gel shows DNA fragments from the *cytb* gene digested with the enzyme *Bfa-1* for 1 h at 37 °C. *Lane 1*, product of parental type 3D7, uncut; *lane 2*, product of parental type HB3, cut at the mutation; *lane 3*, product of the same hybrid oocyst as that in gel **a** lane 3, showing only the 3D7 form of the *cytb* fragment; *lane 4*, product of the same hybrid oocyst as that in gel **a** lane 4 showing only the HB3 form of the *cytb* fragment

4 h. The results of the restriction digest on both cold and radio-labelled products demonstrated that each hybrid oocyst contained only one type of *cytb* and that in each case it was the same type as that indicated by direct sequencing.

We conclude from this study that, like the inheritance of the mitochondrial genome in invertebrates and most plants (Neale et al. 1989), the inheritance of the 6-kb element in *P. falciparum* appears to be uniparental. Our experiment does not allow us to determine the sex of the gamete which transmits the mitochondrial genome, nor does it eliminate the possibility that both gametes carry the mitochondrial genome to the zygote and only one is subsequently replicated, as occurs in *Chlamydomonas* chloroplasts (Sager and Grabowy 1983). However, equal parental cytoplasmic contributions to the zygote of *Plasmodium* are unlikely because electron microscopic studies indicate that each of the 6–8 male microgametes produced from one male gametocyte contains little more than its nucleus, surface membrane and axoneme, whereas the female macrogamete produced from a single female gametocyte appears to carry a normal complement of cytoplasmic organelles (Aikawa et al. 1984). Thus, as in many other organisms in which mitochondrial inheritance is uniparental, the female gamete is likely to be the carrier.

The strong bias for the 3D7 form of the *cytb* gene in hybrid oocysts might suggest that most are produced by fertilisation of 3D7 females with HB3 males. The reason for an apparent inequality in viability of the two types of female gametes is not understood.

Studies on the inheritance of mitochondrial genes in *P. falciparum* are of practical importance, since it has been demonstrated that resistance to certain drugs is encoded by these elements and that mitochondria are the target for some groups of antimalarials (Peters 1987; Fry and Pudney 1992). Furthermore, the finding that cytoplasmic genes are inherited in a uniparental manner opens new possibilities for the analysis of the genetic structure of parasite populations malaria. The distribution of cytoplasmic elements, such as the 6-kb element and the 35-kb circle, in populations of *P. falciparum* may give clues to the evolution of this parasite that cannot be obtained by studying the rapidly recombining nuclear genome.

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Maternal inheritance of extrachromosomal DNA in malaria parasites

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ELSEVIER

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

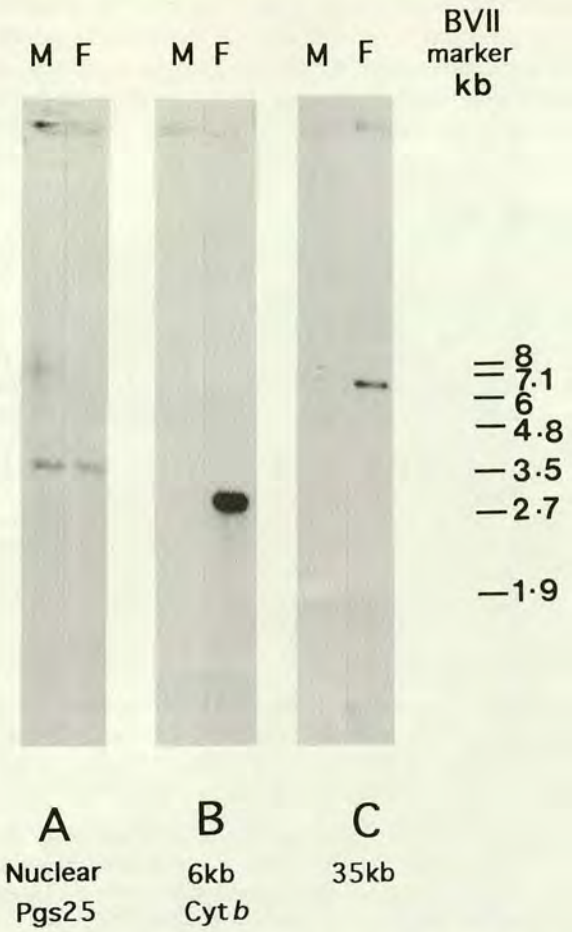

Fig. 1. The same Southern blot of male (M) and female (F) *P. gallinaceum* DNA gamete preparations probed with three different probes: (A) probed for a fragment of the nuclear gene *Pgs25*; (B) probed for a fragment of the 6-kb cytochrome *b* gene; (C) probed for a fragment from the 35-kb circle.

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.

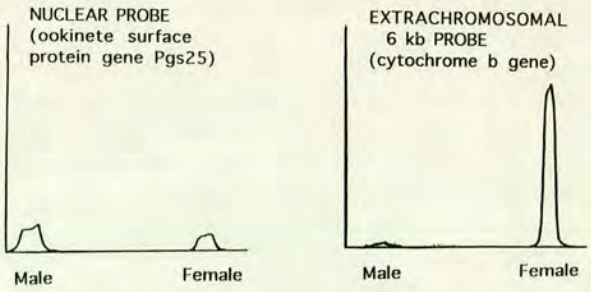

Fig. 2. Densitometric traces of Southern blot of *P. gallinaceum* male and female gamete preparation DNA probed with: (A) a nuclear gene fragment *Pgs25* (integral: male = 3334, female = 1506), and (B) a fragment from the cytochrome *b* gene on the extrachromosomal 6-kb element (integral male = 640, 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

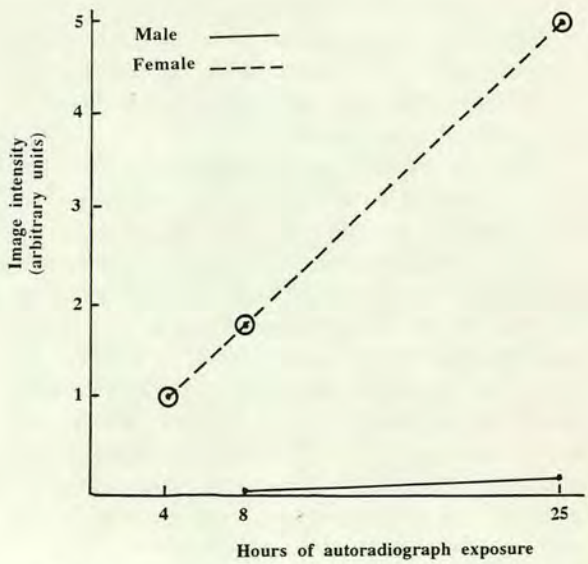

Fig. 3. Readings taken on the Molecular Dynamics Phosphorimager of three different exposures of the male and female *P. gallinaceum* gamete preparations probed with the cytochrome *b* gene probe.

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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