ASPECTS OF PYRIMETHAMINE RESISTANCE IN THE HUMAN MALARIA PARASITE *PLASMODIUM FALCIPARUM*

SHIU-WAN CHAN

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Institute of Cell and Molecular Biology
University of Edinburgh

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DECLARATION

I hereby declare that I alone have composed this thesis, and that, except where stated otherwise, the work presented within is my own.

SHIU-WAN CHAN

DEDICATION

DAD *in memoriam*

MUM

LUAN JING-SHENG

Professor JOHN G. SCAIFE *in memoriam*

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ABSTRACT

Pyrimethamine is a commonly used drug in prophylactic and therapeutic treatment of human malaria caused by the parasite *Plasmodium falciparum*. It has been postulated that the mode of action of pyrimethamine is by inhibition of the enzyme dihydrofolate reductase (DHFR). This was tested genetically in the first part of the study using a cross between a pyrimethamine sensitive and a pyrimethamine resistant cloned line 3D7 and HB3. The results of restriction-fragment-length-polymorphism (RFLP) analysis on the pattern of pyrimethamine resistance inheritance in the progeny confirm that the mutation causing resistance to pyrimethamine in HB3 is closely linked to the dihydrofolate reductase-thymidylate synthetase (DHFR-TS) gene and may be located within it, as previously inferred from sequencing and biochemical results.

Structural alteration to DHFR resulting from point mutation in the DHFR gene has been suggested as one way in which resistance to pyrimethamine might occur. However, it is possible that other mechanisms e.g. over-production of the enzyme DHFR may also be important. The second part of the study investigated this possibility. Quantification of the enzyme level revealed no differences between the parents of the cross, 3D7 and HB3. However, pyrimethamine resistant mutant T9/94 (M1-1) selected *in vitro* was found to produce twice as much protein as the sensitive parent clone T9/94, suggesting that over-production of the enzyme can increase resistance to pyrimethamine. Over-production of the protein was also observed in a naturally occurring pyrimethamine resistant clone PR70/CB3.

Since a mutant over-producing the enzyme was identified, it was decided to initiate studies that would ultimately be able to assess the role of

transcriptional control in pyrimethamine resistance. As a first step, the 5' flanking regions of the DHFR genes from 3D7 and HB3 were sequenced and their transcriptional start sites mapped. Minor variations in the two sequences were found. The relationship of the sequence variations to the RFLP used in the genetic analysis is discussed.

ABBREVIATIONS

A or T	adenosine or thymidine
amp	ampicillin
ATP	adenosine-5'-triphosphate
bp	base pair(s)
BSA	bovine serum albumin
°C	degrees centigrade
C or G	cytosine or guanine
cDNA	complementary deoxyribonucleic acid
Ci	Curie(s)
cm	centimetre(s)
cpm	counts per minute
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
(d)dATP	2'(3'-di)deoxyadenosine-5'-triphosphate
(d)dCTP	2'(3'-di)deoxycytidine-5'-triphosphate
(d)dGTP	2'(3'-di)deoxyguanosine-5'-triphosphate
(d)dTTP	2'(3'-di)deoxythymidine-5'-triphosphate
dGTP	deoxyguanosine-5'-trophosphate
DHFR	dihydrofolate reductase
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide-5'-triphosphate
dTMP	deoxythymidylate
DTT	dithiothreitol

dTTP	deoxythymidine-5'-triphosphate
dUMP	deoxyuridylate
F-dUMP	fluorodeoxyuridylate monophosphate
EDTA	ethylenediaminetetraacetic acid
g	gram(s)
GTP	guanosine-5'-triphosphate
>	greater than
^3H	β emitting isotope of hydrogen (tritium)
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
HEPES	N-2-hydroxyethylpiperasine-N'-2-ethanesulphonic acid
hr(s)	hour(s)
k	kilo (x1000)
kb	kilobase
kDa	kilodalton
Klenow	large fragment of DNA polymerase
l	litre(s)
min.	minute
M	molar
mg	milligram(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
MOPS	3-[N-Morpholino]propane-sulfonic acid
mRNA	messenger ribonucleic acid
MW	molecular weight
$NADP^+$	β-nicotinamide-adenine dinucleotide phosphate, oxidized form

NADPH	β-nicotinamide-adenine dinucleotide phosphate, reduced form
ng	nanogram(s)
nm	nanometre(s)
nM	nanomolar
NMM	N-methylmorpholine
NP-40	nonidet P-40
OD	optical density
^{32}P	β emitting isotope of phosphorous
PABA	*p*-aminobenzoate
PABG	*p*-aminobenzoylglutamate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PPO	2,5-diphenyloxazole
%	percentage
pH	$-\log_{10}$ (hydrogen ion concentration)
pmol	picomole(s)
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
S	Svedberg unit
^{35}S	β emitting isotope of sulphur
SDS	sodium dodecyl sulphate
SSC	standard citrate saline
TEMED	NNN'N-tetra-methyl-1,2-diamino-ethane
Tris	tris(hydroxymethyl)-amino-methane

Triton X-100	octylphenoxypolyethoxyethanol
TS	thymidylate synthetase
Tween 20	polyoxyethylenesorbitan monolaurate
U	unit(s)
UV	ultraviolet
μCi	microcurie(s)
μg	microgram(s)
μl	microlitre(s)
μm	micrometre(s)
μM	micromolar
V	volt(s)
W	watt(s)
w/v	weight per volume

STANDARD AMINO ACID ABBREVIATIONS

Ala	A	alanine
Cys	C	cysteine
Asp	D	aspartic acid
Glu	E	glutamic acid
Phe	F	phenylalanine
Gly	G	glycine
His	H	histidine
Ile	I	isoleucine
Lys	K	lysine
Leu	L	leucine
Met	M	methionine
Asn	N	asparagine
Pro	P	proline
Gln	Q	glutamine
Arg	R	arginine
Ser	S	serine
Thr	T	threonine
Val	V	valine
Trp	W	tryptophan
Tyr	T	tyrosine

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1.1 BIOLOGY AND GENETICS OF PLASMODIUM FALCIPARUM

1.1.1 Life Cycle of Plasmodium falciparum

Malaria is a cosmopolitan disease causing a high degree of mortality and morbidity in large parts of the world every year. The disease is caused by a parasitic protozoan, *Plasmodium* of which four species are responsible for human malaria: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale. P. falciparum* is the most virulent form of these species.

The parasite undergoes a complex life cycle with asexual reproduction occurring in mammalian hosts and sexual reproduction in the anopheline mosquito vectors (Fig. 1.1). The infection in man begins when the sporozoites are inoculated into the bloodstream from the salivary glands of a mosquito. The sporozoites then invade the liver cells where they undergo the exo-erythrocytic phase of development. During this phase the sporozoites mature to become trophozoites which then undergo schizogony to form thousands of merozoites. These are released from the liver and enter red blood cells in which they start a second phase of erythrocytic development. During this phase the parasite again develops into ring and trophozoite and then undergoes schizogony to form merozoites which will invade un-infected red blood cells and cause the cycle to be repeated. Some of the merozoites upon invasion develop into gametocytes which are infective to mosquitoes. In the stomach of the mosquitoes, the gametocytes mature into microgametes and macrogametes. Fertilization results in the formation of zygotes which become motile (ookinetes) and move through the stomach or midgut wall to form a cyst (oocyst) in which hundreds of sporozoites develop. The sporozoites then migrate to the salivary glands of the mosquitoes where they become mature and infective to man.

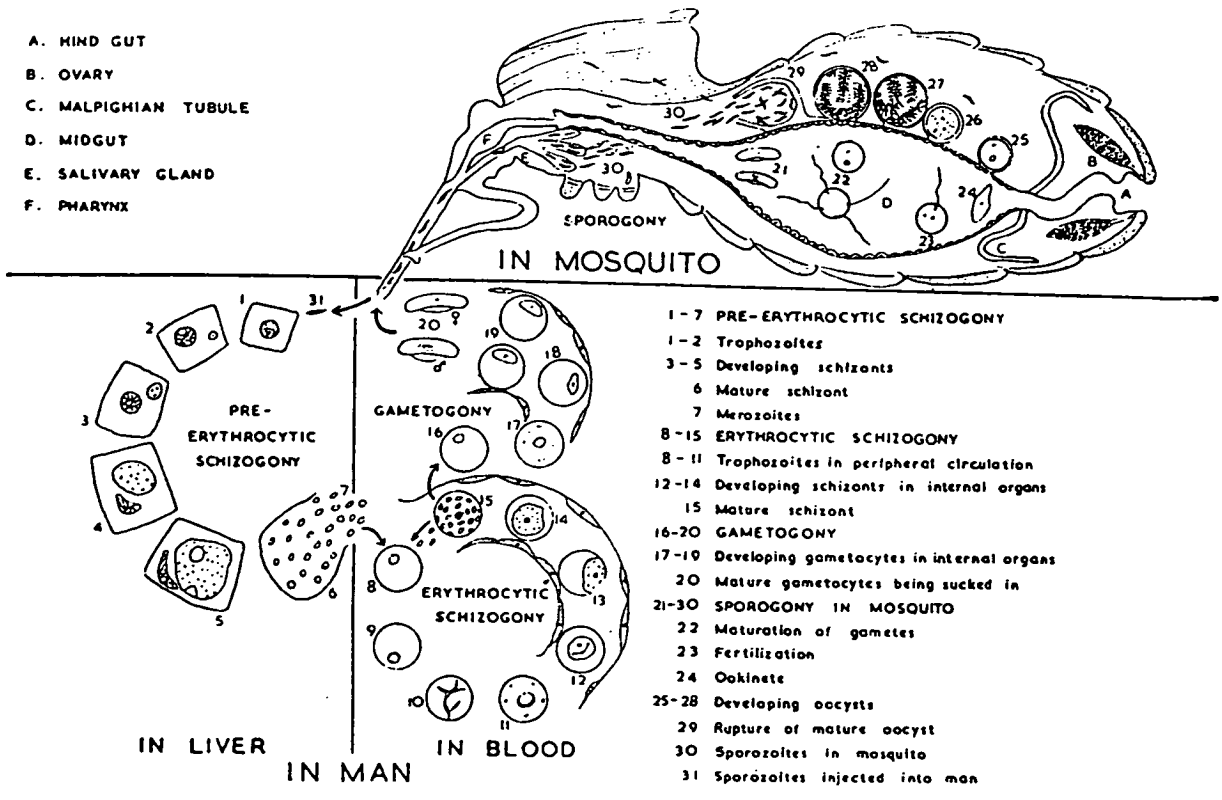


Fig. 1.1 Life cycle of *Plasmodium falciparum* (adapted from Sandosham & Thomas, 1983).

1.1.2 Speciation

Plasmodium is classified as follows:

Phylum: Protozoa

Sub-phylum: Sporozoa

Class: Telosporea

Sub-class: Coccidia

Order: Eucoccida

Sub-order: Haemosporidiidea

Family: Plasmodiidae

Historically, *Plasmodium* species were grouped according to the host range based on the belief that the parasites have evolved with their hosts and that there is greater relatedness among parasites in related hosts than those in hosts greatly separated in evolution. Thus, the species are classified into primate, rodent, avian and lizard malarias (Wernsdorfer, 1980). Further subdivision is based on morphological (e.g. shapes of gametocytes) and biological characters (e.g. cycle periodicity).

However, with the growing discipline of molecular parasitology, the *Plasmodium* species have been re-grouped according to the base composition and organization of their DNA. By this approach, species differing widely in host range, morphology and biological characters are grouped together (McCutchan *et al.*, 1984a).

The diversity of different characters in field isolates or clones of human or rodent malaria (Walliker, 1983) makes it necessary to subdivide the species into subspecies or strains. The heterogeneity in isozyme types, protein variants, drug sensitivity and antigenic types has been used to study the genetic diversity in parasite populations of rodent or falciparum malaria (Carter & Walliker, 1977; Walliker, 1983; Fenton *et al.*, 1985). The use of monoclonal antibodies in serological typing of different strains of malaria

parasite extends the range of methods for identification of different strains of this malaria parasite (McBride *et al.*, 1982; 1985).

1.1.3 Genetic Crosses & Genetic Recombinations

Genetic crosses provide powerful tools in studying conventional genetics in the malaria parasite. However, few genetic studies have been performed on malaria parasites due to the technical difficulty in manipulating organisms with a complex life cycle in the laboratory. Precise genetic analysis relies on the use of genetically pure clones which can be isolated by limiting dilution (Rosario, 1981) or micro-manipulation (Trager *et al.*, 1981). It also requires the technique of making crosses between parent clones displaying well-defined characteristics.

To date, a number of crosses have been successfully made in rodents due to the comparative ease of passaging the parasites through rodents (Walliker *et al.*, 1971; 1975; Rosario, 1976; Knowles *et al.*, 1981). The first genetic cross of a human malaria parasite was not made until recently (Walliker *et al.*, 1987). The method for making crosses is depicted in Fig. 1.2. Basically, a cross begins by mixing gametocytes from two cloned lines of parasites *in vitro* which are then fed to mosquitoes to allow cross-fertilization of gametes to take place. Because of the presence of male and female gametocytes of each parent line in the initial blood mixture, it is possible to form three types of zygotes: those of each parent line from self-fertilization and the hybrid form from cross-fertilization. Assuming that fertilization occurs randomly, equal numbers of parent-type and hybrid zygotes will be obtained. The resultant zygotes develop into sporozoites inside the mosquito vectors which can then infect mammalian hosts (i.e. chimpanzee, rodent) in which the asexual blood stages developed. Parasitized red blood cells obtained from the hosts are cloned to obtain an array of progeny for analysis

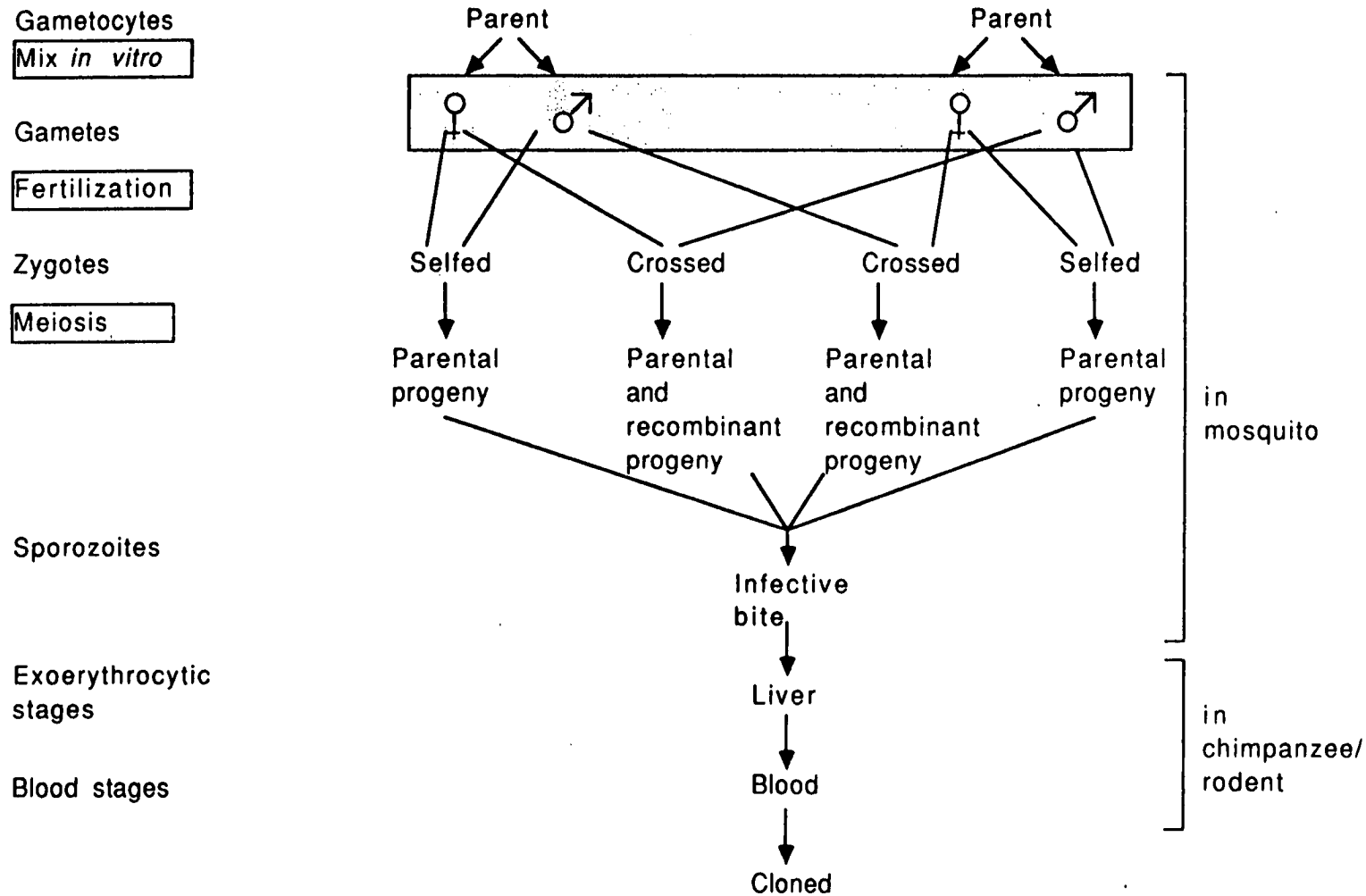


Fig. 1.2 Genetic cross of *Plasmodium*

of recombination of various markers, thereby providing an insight into the genetics of malaria parasites.

Studies on the inheritance pattern of variant forms of enzymes and drug resistance characters in crosses suggest a eukaryotic life cycle with haploid blood forms (Walliker *et al.*, 1971; 1975; Walliker, 1983; 1989). The haploid nature of the blood form chromosomes was established by the observation of one kind of enzyme in each cloned progeny. Genetically determined characters are inherited in a simple Mendelian pattern with segregation of various enzyme markers and drug resistance characters. The presence of a normal eukaryotic pattern of chromosomal segregation is also evident by electron microscopic studies on meiotic division in *Plasmodium berghei* (Sinden & Hartley, 1985).

The detection of novel-sized chromosomes in 2/3 of the progeny from the human malaria cross suggests that some kind of chromosomal rearrangement had taken place during the cross (Walliker *et al.*, 1987). This may be due to crossing over, reciprocal translocation or gene conversion during pairing at meiosis. Indeed, a novel-sized chromosome 4 in one such clone was produced by crossing-over between the parental chromosomes during meiosis (Sinnis & Wellems, 1988).

1.1.4 Restriction-fragment-length-polymorphism (RFLP) in P. falciparum

The advance in genetic studies of *P. falciparum* has been accompanied by the availability of an increasing number of cloned genes and the feasibility of using the restriction-fragment-length-polymorphisms (RFLP) as physical markers to tag corresponding genes. RFLP describes the size variations in specific restriction enzyme fragments often found closely linked to alleles of genes. The mutations responsible for the sickle cell anaemia and β-thalassaemia create new restriction enzyme sites and hence new RFLP's (Kan

et al., 1980; Orkin *et al.*, 1982). The RFLP markers were therefore originally used in prenatal diagnosis of a number of human genetic diseases. It is usually very accurate, providing an exact diagnosis of sickle cell status to greater than 80% (Kan & Dozy, 1978a; b; Philips III *et al.*, 1980) and carriers of haemophilia A to >96% (Harper *et al.*, 1984; Oberle *et al.*, 1985).

RFLP's have been used in several attempts to study various aspects of *Plasmodium* genetics such as the inheritance pattern of certain phenotypes, the establishment of linkage groups and the correlation of drug resistance with a particular target gene (Wellems *et al.*, 1987; 1990; 1991; Peterson *et al.*, 1988; Foote *et al.*, 1990b).

The mutations giving rise to RFLP's in the *Plasmodium* genes have not been investigated. In the case of human genetic diseases, the RFLP is often associated with point mutation(s) leading to the creation or destruction of a restriction enzyme site (Fig. 1.3a) as in the case of the β-globin genes (Kan *et al.*, 1980; Orkin *et al.*, 1982). It is also possible that a large insertion/deletion of sequence is involved (Fig. 1.3b) as is the case of the human α-globin gene (Higgs *et al.* 1981). The nature of the RFLP associated with the dihydrofolate reductase-thymidylate synthetase (DHFR-TS) alleles of *P. falciparum* will be studied and discussed in detail in CHAPTER 5.

1.2 THE WORLD MALARIA SITUATION

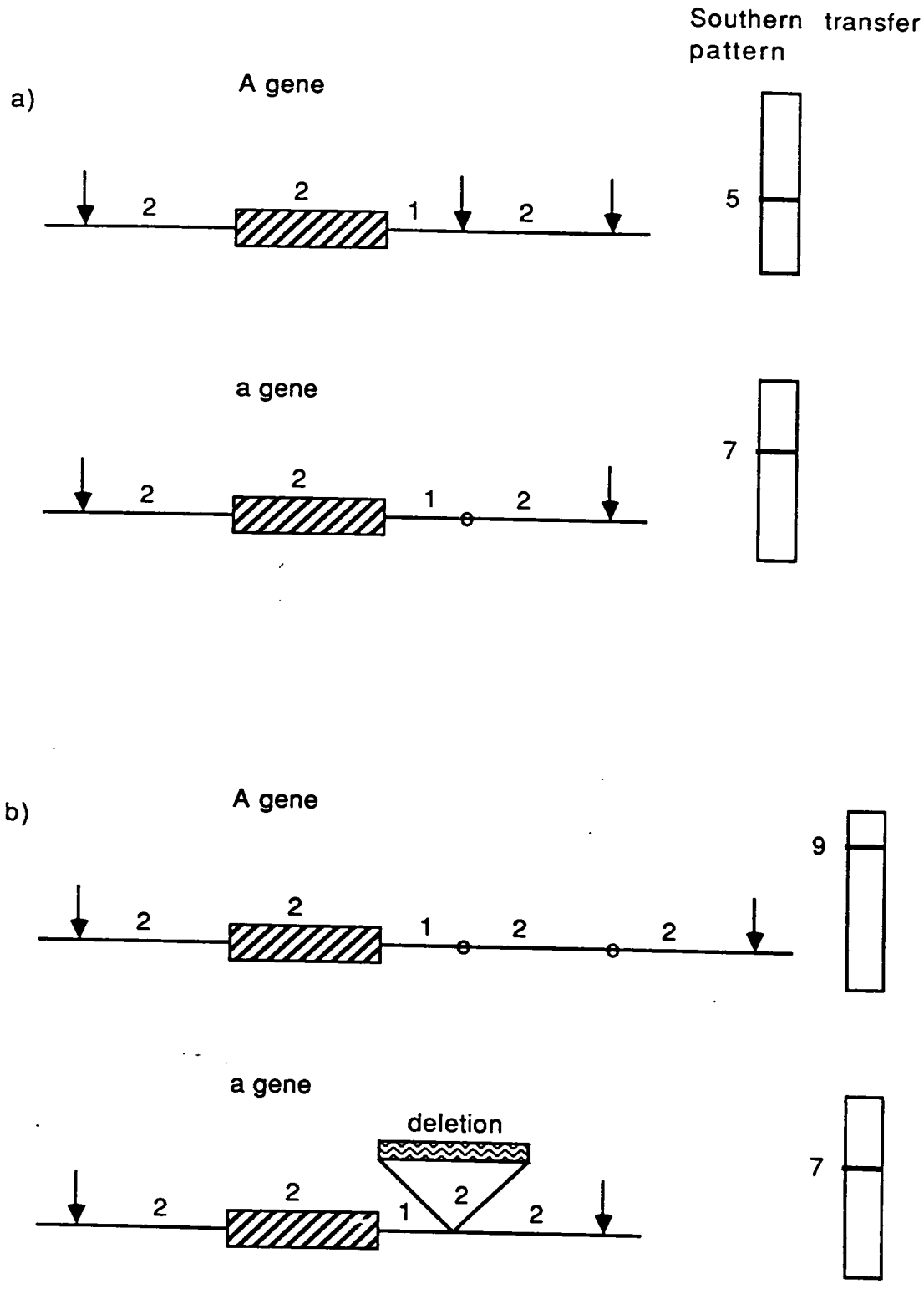
1.2.1 Global Distribution: Past and Present

Human malaria is endemic in the tropics and sub-tropics and is probably as old as mankind itself. There are records suggesting its origin from Egypt dating from 1,600 BC and cases were known in ancient China and Arabian countries. The first accurate clinical description of malarial fevers was given by Hippocrates in 400 BC, who mentioned the symptoms of the

Fig. 1.3 Examples of mutations causing restriction-fragment-length-polymorphism (RFLP).

a) Changes of endonuclease recognition sites caused by point mutations. Endonuclease recognition sites are designated by arrows. Arabic numbers give distances in kilobases (kb). The A gene, which is 2kb long, has an additional endonuclease restriction site 1kb from the right end of the gene. The mutant a gene is closely linked to a DNA sequence change 1kb downstream from the right end of the gene resulting in an altered sequence which no longer functions as a recognition site. Endonuclease digestion of DNA containing the A gene followed by Southern transfer will result in detection of a 5kb fragment. However, digestion of DNA containing the a gene will result in a longer 7kb fragment.

b) Deletion/insertion of DNA sequences. In A gene, an endonuclease restriction site is located 5kb from the right end of the gene. The mutant a gene is linked to a deletion of 2kb downstream from the right end of the gene. Endonuclease digestion of DNA containing the A gene followed by Southern transfer will result in detection of a 9kb fragment. However, digestion of DNA containing the a gene will result in a shorter 7kb fragment.


Southern transfer pattern
a)
A gene
2
2
1
2
5
a gene
2
2
1
2
7
b)
A gene
2
2
1
2
2
9
a gene
deletion
2
2
2
1
2
7

disease as periodic chills, fevers and sweating. Malaria was probably introduced into the Americas in the 16th century with the Spanish and Portuguese invasions and later with the advent of the African slave trade. The global distribution of the disease was then maintained largely unchanged until after the end of World War II when an intensive anti-malaria programme was put into operation. A concerted effort of vector control, use of insecticides and drug prophylaxis brought the population living in malarious areas under protection between 1957-1972. However, a resurgence of malaria has occurred throughout the tropics since the mid 1970s. By contrast, Europe, Australia and the United States have remained malaria-free despite cases imported from other countries (for review see Wernsdorfer, 1980).

According to the World Health Organization Tropical Disease Research 7th Programme Report, Jan 1983-31 Dec 1984 (World Health Organization, 1985), nearly 365 million people, representing 1/12th of the world's population, are living in highly endemic areas where no specific anti-malaria measures are being applied e.g. tropical Africa. A further 2,217 million, 46% of the world's population, are living in endemic areas where control measures have been undertaken. It is estimated that the disease is responsible for the deaths of one million infants and young children each year in Africa. In areas other than tropical Africa the incidence of the disease is estimated to be around 20 million cases a year. In Africa south of the Sahara some 200 million people are believed to be chronically infected and of these about 1/3 suffer acute manifestations of the disease in the course of a year.

Among the four human malaria parasites, *P. falciparum* and *P. vivax* account for most of the human suffering and economic loss due to malaria in the world today whereas *P. ovale* and *P. malariae* are of regional

importance only (World Health Organization, 1985). *P. falciparum* is the prevailing species in tropical Africa. *P. vivax* occurs most commonly in the Americas and Asia. However, with the recent success of malaria control in Southern Asia, the incidence of *P. vivax* infection has fallen although that of *P. falciparum* has remained virtually unchanged, apparently due to drug resistance.

1.2.2 Control Measures and Drug Prophylaxis

A concerted effort of drug chemotherapy and intensive vector control programmes has been carried out to eradicate the disease. Insecticides have been widely used to kill the mosquito vectors. The most extensively used insecticide, dichloro-diphenyl-trichloroethane (DDT), exerts its residual action for up to six months. However, the widespread use of this insecticide has selected populations of vector resistant to it. This has prompted a shift from chlorinated hydrocarbon insecticides to organophosphorous compounds or the more expensive carbamides (Wernsdorfer, 1980).

Apart from controlling the vectors, chemotherapeutic and chemoprophylactic measures have been undertaken as means of treatment and protection. A number of antimalarial drugs are available with different modes of action on specific stages of the malaria life cycle. Currently used drugs include quinine, chloroquine, primaquine, antifolates and sulfonamides (Dietrich & Kern, 1981). Their chemical formulae are displayed in Fig. 1.4.

Quinine: Quinine is primarily schizontocidal for vivax as well as for falciparum infections with some gametocidal activity against *P. vivax, P. ovale* and *P. malariae*. It is effective as a suppressive drug. When used in combination with sulfonamides, antifolates, tetracyclines or other compounds, it is able to control or avoid clinical attacks especially of chloroquine-resistant falciparum malaria. Resistance to quinine was described very early this

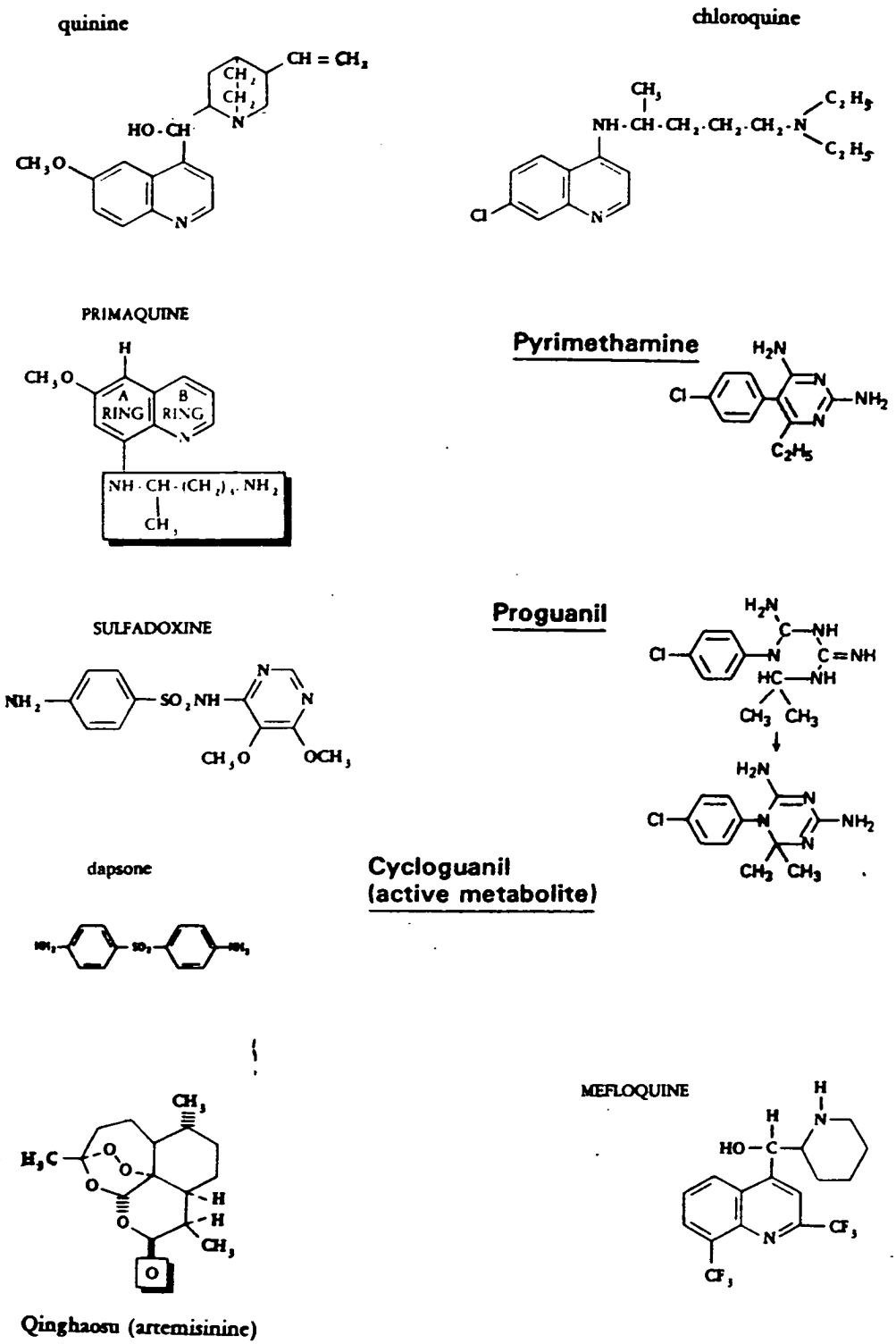


Fig. 1.4 Chemical formulae of commonly used antimalarial drug.

century.

Chloroquine: Chloroquine is a 4-aminoquinoline used in acute malaria attack against *P. vivax, P. ovale, P. malariae* and chloroquine-sensitive *P. falciparum*. Being a blood schizontocide, it causes rapid control over parasitaemia and clinical symptoms. In the case of chloroquine resistant falciparum malaria, a combination of sulfadoxine and pyrimethamine is usually used to treat the patients.

Primaquine: Primaquine is an 8-aminoquinoline currently available for the radical treatment of vivax and ovale infections. It is the only tissue schizontocide. Its gametocytocidal effect makes it a suitable candidate to be used in combination with blood schizontocides in combating spread of drug resistant parasites.

Antifolates: A class of drugs called antifolates provide important antimalarial drugs. They derive their name from their target of action, the enzyme dihydrofolate reductase (DHFR). Proguanil and pyrimethamine are two well known examples of antifolates. They are normally used in combination with other drugs in prophylaxis. In addition, proguanil is a causal prophylactic antimalarial agent by rendering gametocytes non-infectious to mosquitoes.

Sulphonamides: Sulphonamides are long-acting blood schizontocides. They exert their antimalarial effect by inhibiting the enzyme dihydropteroate synthetase, which is one of the key enzymes in folate metabolism. They exert prophylactic activities especially when used in combination with other drugs, most commonly antifolates.

Sulphones: Sulphones are schizontocides acting against *P. falciparum* only. They are suppressive when combined with other drugs.

Drug combinations: Drugs are used together to give either an additive effect or a potentiating effect. In the first case, a suppressive drug is usually

used with a causal prophylactic or anti-relapse drug e.g chloroquine and the DHFR inhibitor, pyrimethamine. In the second case, inhibitors of DHFR and sulfonamides or sulfones are usually used as these drugs affect the same pathway and act synergistically.

1.2.3 The Spread of Drug-resistant Parasites

Drug resistance in malaria has been defined as the "ability of a parasite strain to multiply or to survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication. Such resistance may be relative (yielding to increased doses of the drug tolerated by the host) or complete (withstanding maximum doses tolerated by the host)". Three levels of resistance denoted RI (low), RII (medium) and RIII (high) are generally recognized, according to the response of parasites in patients undergoing a standard course of drug treatment.

Resistance to almost all major antimalarial compounds has been reported. Among these, resistance to chloroquine has the greatest impact on malaria chemotherapy since chloroquine is the mainstay of malaria treatment. Simultaneous emergence of *P. falciparum* resistant to chloroquine was reported from Thailand and Colombia in the late 1950s whereas chloroquine resistant *P. falciparum* did not make its first appearance in East Africa until 1978. By the end of 1984, chloroquine resistance was widespread in Thailand, Venezuela, Colombia, eastern Asia, South America and East Africa and continued to spread outwards from these countries (Spencer, 1985). To date, chloroquine resistance remains the most serious problem in malaria chemotherapy. The distribution of chloroquine-resistant *P. falciparum* is shown in Fig. 1.5.

A combination of pyrimethamine/sulphadoxine is extensively used for the treatment of chloroquine resistant falciparum malaria. However,

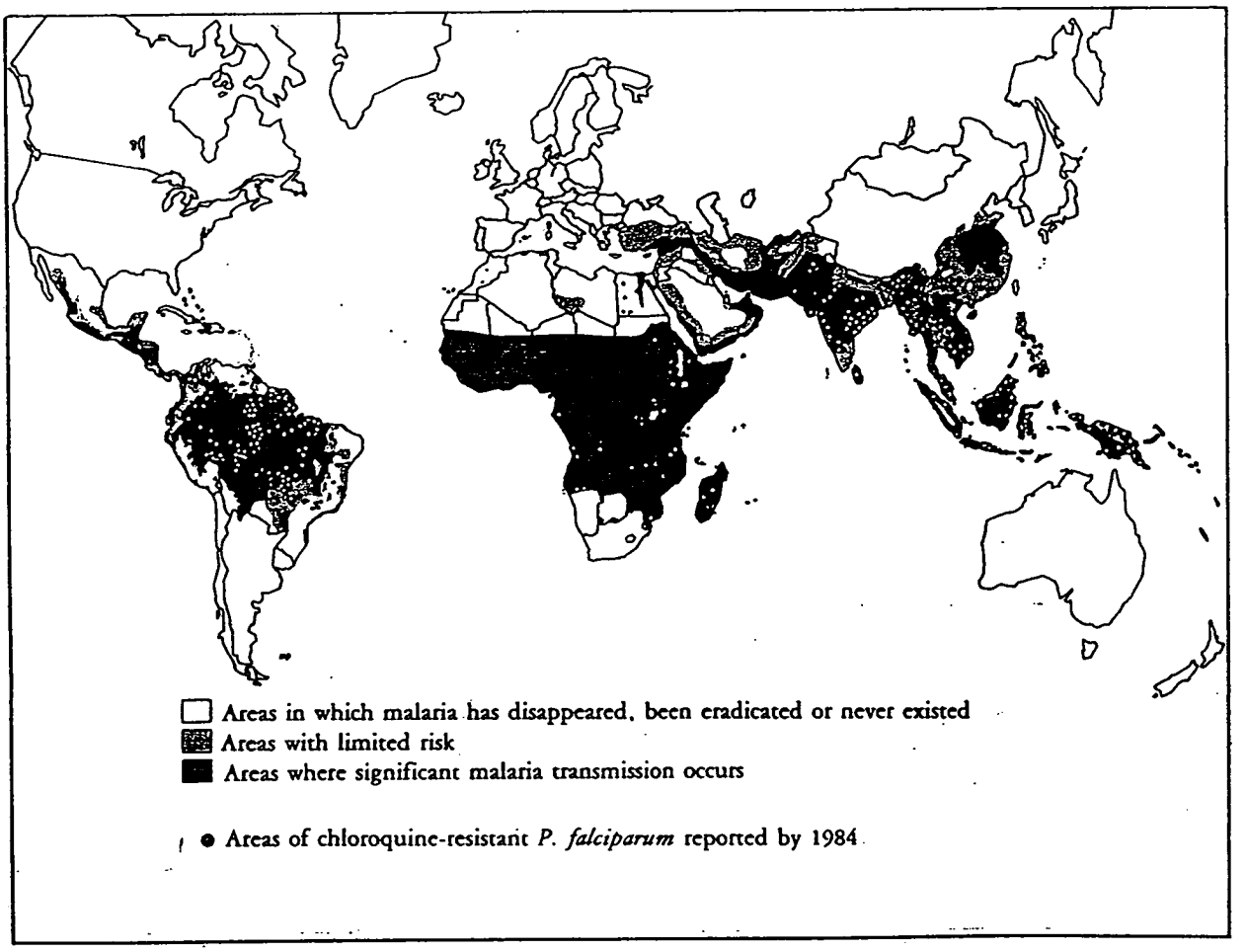


Fig. 1.5 Epidemiological assessment of status of malaria and chloroquine-resistant *P. falciparum* (adapted from World Health Organization, 1985).

resistance to this combination is already widespread in the 'hard-core' areas of Southeast Asia and South America and cases of resistance have also been reported in East Africa (Hurwitz *et al.*, 1981; Markwalder & Meyer, 1982; Stahel *et al.*, 1982; Farraroni *et al.*, 1983). Resistance to pyrimethamine/sulphadoxine seems to be directly related to pyrimethamine resistance (Spencer *et al.*, 1984).

The situation is complicated by the failure of some malaria patients to respond to multiple drugs. Multi-drug-resistance in plasmodia has become a common clinical problem. Isolates obtained from these patients showed resistance to multiple drugs (Thaithong *et al.*, 1983). Moreover, a number of cloned lines have been tested to be resistant to multiple drugs.

1.2.4 Strategies for Combatting Drug-resistant Parasites

Considerable attention has been paid to the design of new drugs. Among those tested, mefloquine and qinghaosu are the most promising. Mefloquine, a quinolinemethanol (Fig. 1.4), is a potent blood schizontocide active against multi-drug-resistant falciparum malaria. This new compound is registered for prophylaxis and treatment (Trenholme *et al.*, 1975). However, there is fear of cross-resistance between mefloquine and other structurally similar drugs i.e. quinine or chloroquine though no clear instance of such cross resistance seem to have been reported. Isolated cases of mefloquine-resistant falciparum infection have been documented (Boudreau *et al.*, 1982; Bygberg *et al.*, 1983) and parasites resistant to mefloquine have been cloned from these patients (Webster *et al.*, 1985). Stable resistance has also been successfully induced in the laboratory (Oduola *et al.*, 1988). To delay the widespread appearance of mefloquine resistance, this new compound is now used in combination with sulfadoxine and pyrimethamine.

Qinghaosu (artemisinine) (Fig. 1.4), the active principle of the Chinese

medicinal herb *Artemisia annua,* and its derivatives have been widely studied in China (China Cooperative research group on qinghaosu and its derivatives as antimalarials, 1982a) and shown to have a rapid action in treating chloroquine resistant falciparum malaria (China Cooperative research group on qinghaosu and its derivatives as antimalarials, 1982b; c). Presumably they are effective against the erythrocytic stages of malaria parasites by affecting protein synthesis and/or membrane function (Jiang *et al.*, 1982; Xiao & Fu, 1986). Their novel structures and different modes of action make them potential new drugs in reducing the risk of emergence of cross-resistant parasites. The drugs have already been put into clinical trials (Jiang *et al.*, 1982; Xiao & Fu, 1986). There has been no marked evidence of cross-resistance between artemisinine and chloroquine or sulfadoxine/pyrimethamine (World Health Organization, 1986) although artemisinine-resistant mutants has been induced in the laboratory (Inselburg, 1985).

The effect of artemisinine is markedly potentiated when used in combinations with mefloquine, primaquine or chloroquine whereas an antagonistic effect is obtained when combined with the antifolate compounds.

Increasing failure rates of drug chemotherapy has prompted research into the new area of vaccine development (for reviews see Anders, 1985; Nussenzweig & Nussenzweig, 1985; Ravetch *et al.*, 1985; Spriggs, 1985; Targett & Sinden, 1985; Wernsdorfer, 1985; Heidrich, 1986; Miller *et al.*, 1986). However, the design of the first antimalarial vaccine will be a complicated process which requires improvement of an expression system for large scale production of the vaccine (Smith *et al.*, 1984; Sharma & Godson, 1985; Young *et al.*, 1985) and a series of pre-clinical and clinical trials (Hall *et al.*, 1984; Collins *et al.*, 1986; Herrington *et al.*, 1987; Patarroyo *et al.*, 1988). It is hoped that some day a vaccine will become available, but until

then we will rely on improved chemotherapy.

1.2.5 The Potential for Developing a New Drug in Treating Chloroquine-resistant Malaria

Association of chloroquine resistance with increased efflux of the drug from the cell (Krogstad *et al.*, 1987) and the reversal of this efflux phenomenon and the chloroquine resistance phenotype by the calcium channel blocker, verapamil (Martin *et al.*, 1987) may point to the possible use of calcium-channel blockers as a new generation of drugs in treating chloroquine-resistant malaria.

An initial step to a better design of this new class of drugs will require an understanding of the modes of action of chloroquine and the mechanisms of chloroquine resistance. The mode of action of chloroquine is still controversial. It may act by forming a complex with ferriporphyrin IX (FP) which prevents the sequestration of FP into malaria pigment, thereby allowing FP and the FP-chloroquine complex to exert their intrinsic cellular toxicity and causing lysis of malaria parasites (Chou *et al.*, 1980; Orjin *et al.*, 1981; Fitch *et al.*, 1983; Fitch, 1986). Alternatively, the elevation of the vacuolar pH (Krogstad *et al.*, 1985; Warhurst, 1986; Krogstad & Schlesinger, 1987; Choi & Mego, 1988) resulting from accumulation of chloroquine inside the malarial food vacuoles (Aikawa, 1972) may interfere with the digestion of haemoglobin and hence starve the parasite of amino acids for protein synthesis (Homewood *et al.*, 1972; Yayon *et al.*, 1984; 1985). Another possibility is that intercalation of chloroquine into DNA (Gutteridge & Trigg , 1972) may result in impairment of DNA function such as DNA-dependent polymerase reaction in DNA synthesis (O'Brien *et al.*, 1966; Ciak & Hahn, 1966) and gene function (Kwakye-Berke & Meshnick, 1990).

Despite the yet unclear mode of action of chloroquine, there are

considerable studies on the mechanisms of chloroquine resistance. In analogy to the multi-drug-resistance (MDR) phenomenon in cancer cells which can also be reversed by verapamil (Slater *et al.*, 1982; Rogan *et al.*, 1984), it was speculated that a mechanism similar to that in cancer cells was operating in *P. falciparum* (for review see Cowman, 1991). MDR in cancer cells involves increased expression and amplification of a so-called *mdr1* gene (Shen *et al.*, 1986) encoding a membrane P-glycoprotein (Juliano & Ling, 1976; Akiyama *et al.*, 1985; Cornwell *et al.*, 1986) which functions as an efflux pump (Fojo *et al.*, 1985; Chen *et al.*, 1986; Marx, 1986). This idea became more evident by the isolation of genes from *P. falciparum* (*pfmdr1*, *pfmdr2*) homologous to the mammalian *mdr1* gene (Foote *et al.*, 1989; Wilson *et al.*, 1989). Circumstantial evidence also relates amplification and sequence variation of one of the genes with chloroquine resistance in some field isolates (Foote *et al.*, 1990b). However, there are exceptions in which the expected sequence variations do not occur. Moreover, genetic analysis showed that there is no linkage between the rapid efflux phenomenon, chloroquine resistance phenotype and either of the *pfmdr* genes or their amplification in *P. falciparum* (Wellems *et al.*, 1990). The same study also suggests that chloroquine resistance is governed by a single genetic locus, in contrast to chloroquine resistance in *Plasmodium chabaudi* which is multigenic (Rosario, 1976; Padua, 1981). This leads to the suggestion of the involvement of genes other than *pfmdr* in chloroquine resistance (Newbold, 1990). More thorough studies will be needed to fully understand the mechanism of chloroquine resistance. Recently, the gene governing chloroquine resistance in a chloroquine resistant strain has been mapped to a 400kb locus on chromosome 7 (Wellems, 1991).

1.3 RESISTANCE TO PYRIMETHAMINE AND RELATED COMPOUNDS IN P. FALCIPARUM

This section will be devoted to a review of the mechanism of resistance to pyrimethamine, which is one of the most commonly used antimalarial drugs. In addition, resistance to proguanil and sulphonamides will be described since these compounds are normally used in combination with pyrimethamine in malaria chemotherapy and chemoprophylaxis.

Antifolates exert their effects by inhibiting the key enzymes in the pathway of folate metabolism. A folate derivative is an essential co-factor in the biosynthesis of deoxythymidylate (dTMP) (Fig. 1.6) which is one of the building blocks in DNA synthesis. Since malaria parasites do not incorporate exogenous thymine or thymidine (Ferone, 1977), thymidylate must be synthesized *de novo* from deoxyuridylate (dUMP), in a reaction catalysed by thymidylate synthetase (TS) and mediated by the folate co-factor 5,10-methylenetetrahydrofolate which serves as both a one-carbon donor and reducing agent (Blakley, 1984). The 5,10-methylenetetrahydrofolate is itself converted into dihydrofolate. The dihydrofolate produced must be reduced immediately to avoid depletion of the intracellular pool of tetrahydrofolate which is the precursor for the co-factor. The reduction reaction is catalysed by dihydrofolate reductase (DHFR) (Blakley, 1984). The tetrahydrofolate is subsequently methylated to 5,10-methylenetetrahydrofolate by serine hydroxymethyltransferase (Blakley, 1984).

Thus, inhibition of folate metabolism will subsequently inhibit DNA synthesis. Pyrimethamine and proguanil exert their inhibitory effects by inhibiting dihydrofolate reductase. Sulphonamides, on the other hand, prevent the production of dihydrofolate by inhibiting dihydropteroate synthetase which catalyses the conversion of pteridines to dihydrofolate

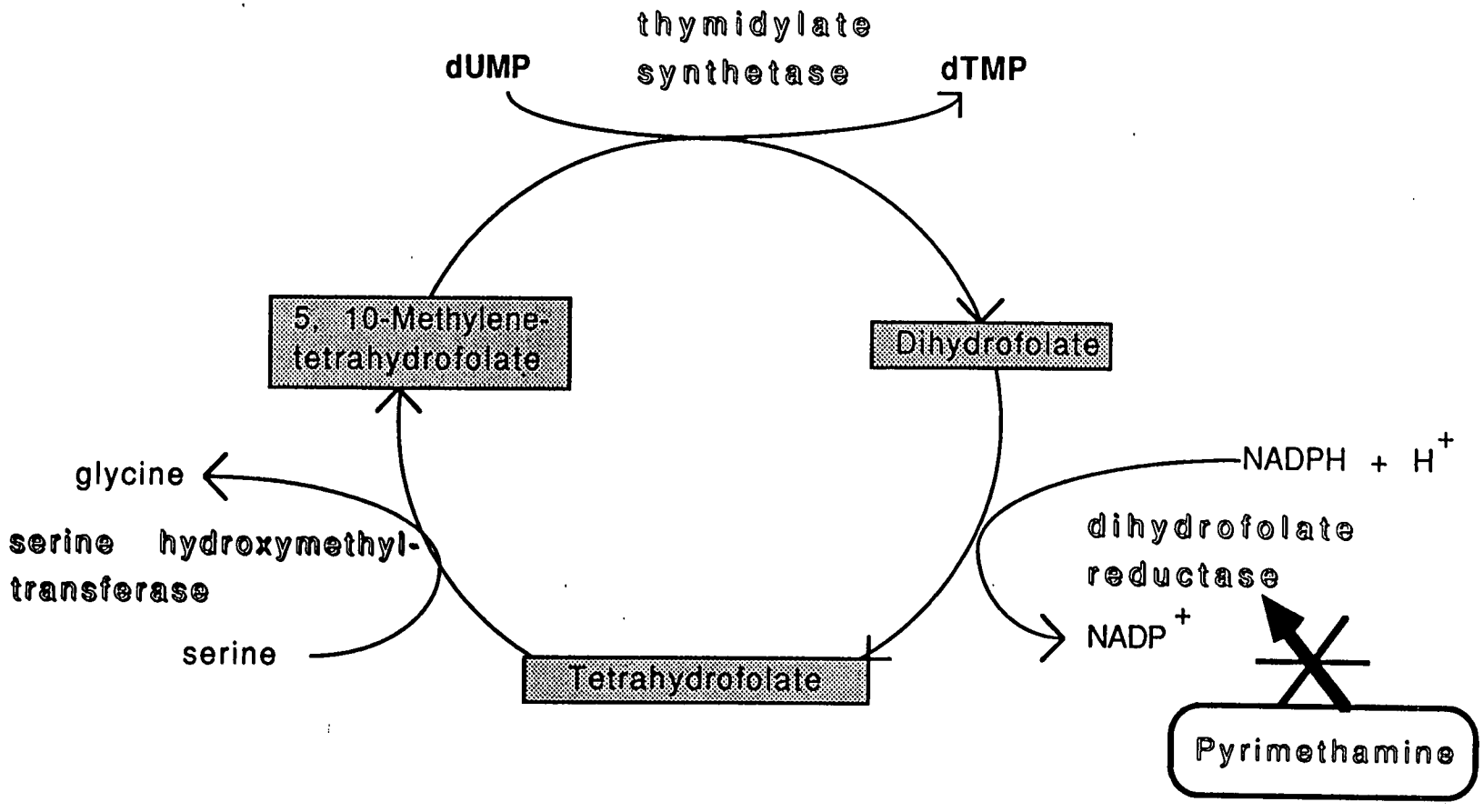


Fig. 1.6 Biochemical pathway for the synthesis of deoxythymidylate.

further back in the metabolic pathway (see Section 1.3.1).

To date, all the key enzymes for thymidylate synthesis have been identified and characterized, including dihydrofolate reductase, thymidylate synthetase and serine hydroxymethyltransferase in all the *Plasmodium* species so far examined (Ferone *et al.*, 1969; Reid & Friedkin, 1973; Ferone, 1977; Ruenwongsa *et al.*, 1989).

1.3.1 Folate Metabolism- De novo Pathway

There are two methods of generating the folate precursors for this reaction: *de novo* and salvage. The relationship of different folate related pathways is illustrated in Fig. 1.7. The part concerning *de novo* synthesis of folate from pteridine precursor is blown up and depicted in Fig. 1.8 which also shows the chemical formulae of various substrates.

The *de novo* pathway has long been thought to be the sole pathway occurring in malaria parasites. In this pathway, the 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine is converted into 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphate which then condenses with *p*-aminobenzoate (PABA) into dihydropteroate (Fig. 1.7 & Fig. 1.8). A glutamate residue is then incorporated into dihydropteroate to form dihydrofolate which is subsequently reduced to tetrahydrofolate. The existence of this *de novo* pathway is presumed by the identification of the corresponding enzymes 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase, dihydropteroate synthetase and dihydrofolate reductase (McCullough & Maren, 1974; Ferone, 1977; Sherman, 1979; Walter & Konigk, 1980). However, dihydrofolate synthetase, the enzyme catalysing the conversion of dihydropteroate to dihydrofolate has yet to be discovered.

This pathway can be inhibited by sulphonamides and pyrimethamine/proguanil which inhibit dihydropteroate synthetase and

Fig. 1.7 Folate related metabolic pathways in P. falciparum. The *de novo* and salvage synthesis of tetrahydrofolate is illustrated in the upper part of the diagram. This pathway is inhibited at one stage by sulphonamides and at the other stage by pyrimethamine and proguanil. The conversion of tetrahydrofolate to the methylated co-factors and their roles in the biosynthesis of deoxythymidylate and methionine is illustrated in the lower part of the diagram.

Key and Abbreviations:

*	may be salvaged from host plasma
GTP	guanosine triphosphate
PABA	*p*-aminobenzoate
PABG	*p*-aminobenzoylglutamate
dUMP	deoxyuridylate
dTMP	deoxythymidylate

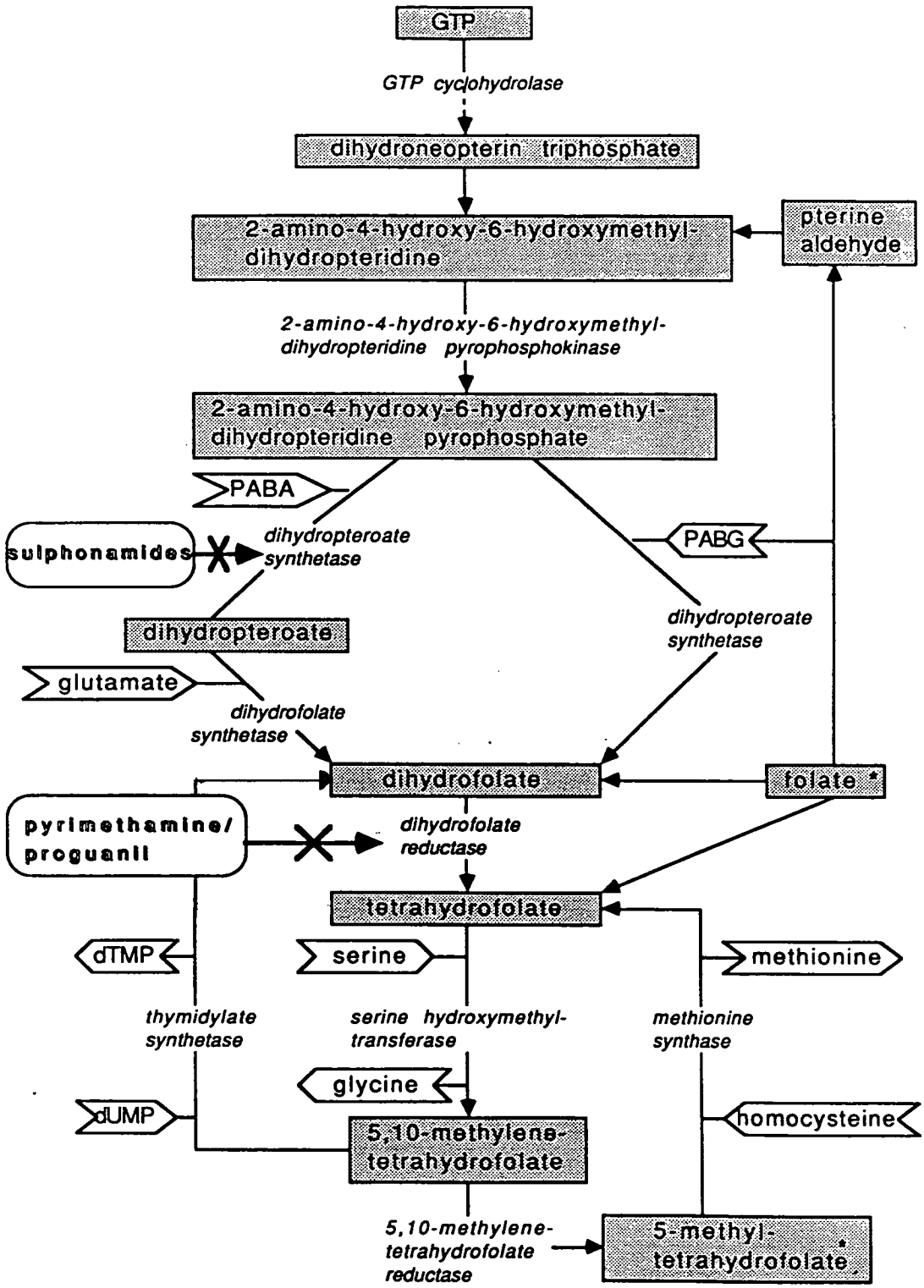
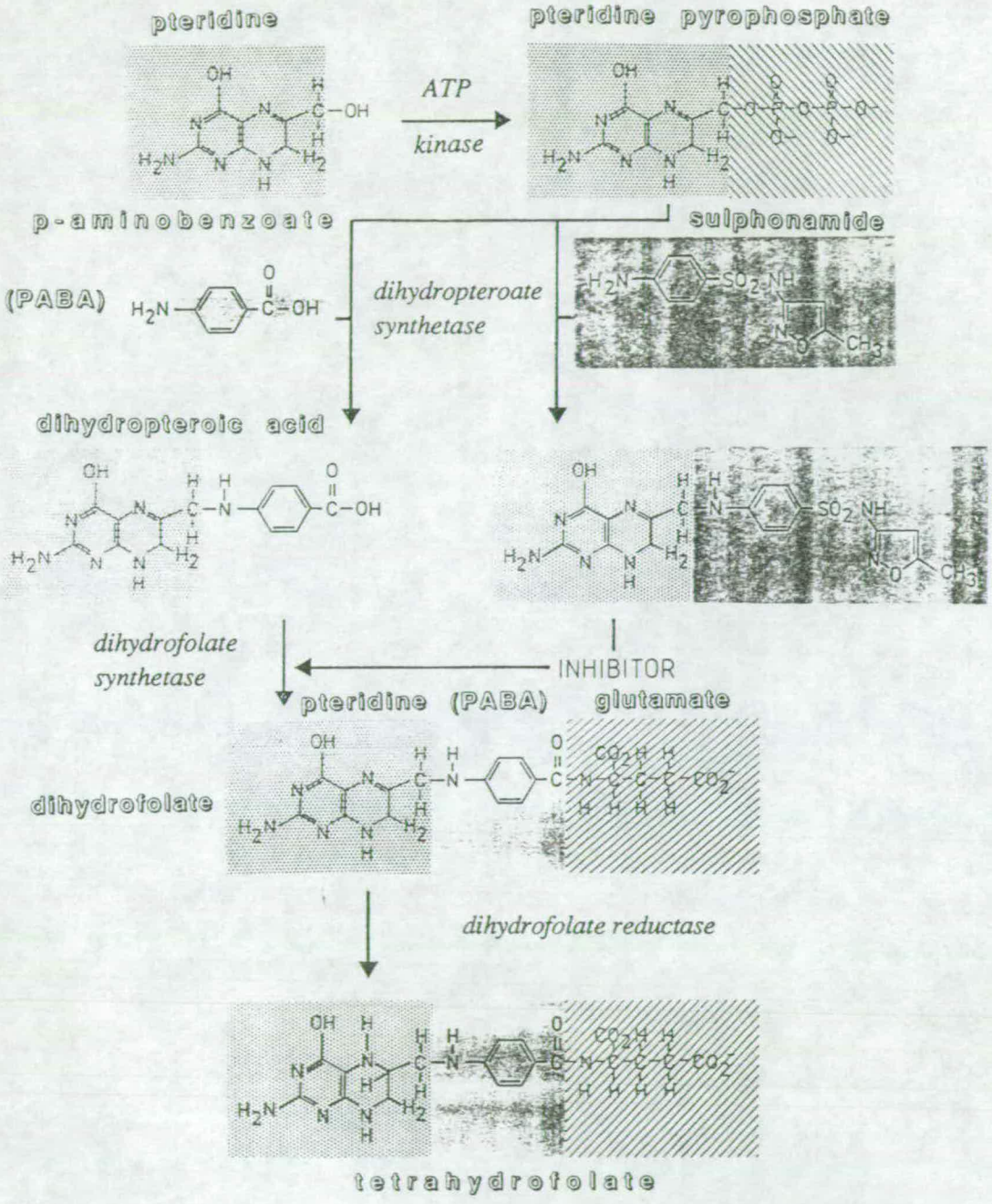

GTP
GTP cyclohydrolase
dihydroneopterin triphosphate
2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine
pterine aldehyde
2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine pyrophosphokinase
2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine pyrophosphate
PABA
sulphonamides
dihydropteroate synthetase
PABG
dihydropteroate
dihydropteroate synthetase
glutamate
dihydrofolate synthetase
dihydrofolate
folate
pyrimethamine/ proguanil
dihydrofolate reductase
tetrahydrofolate
dTMP
serine
methionine
thymidylate synthetase
serine hydroxymethyl-transferase
methionine synthase
glycine
dUMP
homocysteine
5,10-methylene-tetrahydrofolate
5,10-methylene-tetrahydrofolate reductase
5-methyl-tetrahydrofolate

Fig. 1.8 De novo synthesis of tetrahydrofolate from pteridine, p-aminobenzoate and L-glutamate. The pterin precursor is condensed with *p*-aminobenzoate (PABA) into dihydropteroate in a reaction catalysed by dihydropteroate synthetase. A glutamate residue is then incorporated into dihydropteroate to form dihydrofolate, catalysed by dihydrofolate synthetase. The dihydrofolate is then reduced to tetrahydrofolate in a reaction catalysed by dihydrofolate reductase.


pteridine
pteridine pyrophosphate
ATP
kinase
p-aminobenzoate
(PABA)
dihydropteroate
synthetase
sulphonamide
dihydropteroic acid
dihydrofolate
synthetase
INHIBITOR
pteridine (PABA) glutamate
dihydrofolate
dihydrofolate reductase
tetrahydrofolate

dihydrofolate reductase respectively. They are effective by being substrate analogues of *p*-aminobenzoate (PABA)/*p*-aminobenzoylglutamate (PABG) and dihydrofolate respectively.

The source of the pteridines is believed to be guanosine 5'-triphosphate (GTP). The enzyme, GTP cyclohydrolase which catalyses the conversion of GTP to dihydroneopterin triphosphate has already been isolated from *P. falciparum* (Krungkrai *et al.*, 1985). Furthermore, a number of the pterin intermediates from the pteridine synthesis pathway have been demonstrated (Krungkrai *et al.*, 1989a).

1.3.2 Folate Metabolism- Salvage Pathway

There is growing recognition of the importance of a salvage pathway in folate metabolism in plasmodia. It is evident from the ability of exogenous folate to reverse the antagonistic effects of pyrimethamine and sulphonamides (Chulay *et al.*, 1984; Sixsmith *et al.*, 1984; Milhous *et al.*, 1985; Watkins *et al.*, 1985) and the direct demonstration of incorporation of ^{3}H-labelled folate into 5-methyltetrahydrofolate (Krungkrai *et al.*, 1989a; 1990). The salvage pathway is shown to be preferentially utilized *in vitro* (Krungkrai *et al.*, 1989a; 1990). It is proposed that folate can either be used intact or in degraded forms, as pterin aldehyde or as *p*-aminobenzoylglutamate (PABG) (Fig. 1.7). PABG is known as an alternative but less efficient substrate than PABA for dihydropteroate synthetase (Ferone, 1973; Shiota, 1984) which, when using PABG as substrate, converts pteridine to dihydrofolate without the intermediate step of dihydropteroate synthesis (Shiota, 1984).

The bulk of the intraerythrocytic folate pool is comprised of 5-methyltetrahydrofolate (Blakley, 1969). It is speculated that malaria parasites can salvage the substrate 5-methyltetrahydrofolate from the host plasma in methionine synthesis (Fig. 1.7). The enzyme, methionine synthase, catalysing

the conversion of homocysteine to methionine has been identified (Krungkrai *et al.*, 1989b). This may be an alternative route by which salvaging pre-formed 5-methyltetrahydrofolate provides the folate co-factors needed by the parasites (Krungkrai *et al.*, 1990).

1.3.3 Mechanism of Resistance to Pyrimethamine- Single Base Changes

Biochemical studies on *P. falciparum* provide direct evidence that pyrimethamine blocks DHFR *in vivo* (Sirawaraporn & Yuthavong, 1984; Walter, 1986). From these studies, it is also evident that plasmodial DHFR differs from mammalian DHFR both in kinetic properties and structure (Ferone, 1977; Kan & Siddiqui, 1979). Thus, it is thought that pyrimethamine exerts its anti-malaria action by selective inhibition of DHFR since plasmodial DHFR binds to pyrimethamine 10^2-10^3 times more tightly than mammalian DHFR.

The greater concentration of pyrimethamine needed to inhibit the DHFR activity in strains resistant to pyrimethamine has led to speculation that resistance to pyrimethamine arose from a structurally altered enzyme with reduced affinity for pyrimethamine (McCutchan *et al.*, 1984b, Dieckmann & Jung, 1986a; Walter, 1986; Chen *et al.*, 1987; Zolg *et al.*, 1989).

To understand the mechanism of pyrimethamine resistance at the molecular level, the *P. falciparum* gene encoding DHFR was cloned and sequenced (Bzik *et al.*, 1987). In contrast to the DHFR in vertebrates, bacteria, yeast, bacteriophages and mammalian viruses which exist as distinct and separable proteins (Baccanari *et al.*, 1975; Blakley, 1984; Trimble *et al.*, 1988), DHFR is translated with thymidylate synthetase (TS) uninterruptedly from a single reading frame in *P. falciparum* (Bzik *et al.*, 1987). The first group of 228 amino acids denotes an amino-terminal DHFR-domain (≈27kDa) which is joined to a carboxyl-terminal TS-domain (residues 323-

608) by a junctional domain (residues 229-322).

The bifunctional proteins are dimers of identical subunit (Chen & Zolg, 1987). Both DHFR and TS are more related to their monofunctional counterparts in eukaryotes than in prokaryotes or bacteriophage T4. The TS domain is more conserved than the DHFR domain. The bifunctional nature of the protein is common to all protozoa studied so far (Ferone & Roland, 1980; Garrett *et al.*, 1984; Beverley *et al.*, 1986; Grumont *et al.*, 1986). The bifunctional enzyme offers the advantage of more efficient channeling of dihydrofolate produced in the TS reaction to the substrate binding site of DHFR (Meek *et al.*, 1985). On the other hand, the two enzymes retain a measure of independence as indicated by the possession of autonomous folate binding sites which are susceptible to different metabolic inhibitors (Meek *et al.*, 1985). In addition, expression of DHFR activity independently of the TS activity has been achieved in *Escherichia coli* from a recombinant plasmid carrying the DHFR region only (Hall *et al.*, 1991).

Sequence comparison of the dihydrofolate reductase-thymidylate synthetase (DHFR-TS) gene from geographically isolated strains resistant or sensitive to pyrimethamine has pinpointed conserved base changes in all the resistant strains (Table 1.1). The most highly conserved change is a serine/threonine residue to an asparagine residue at codon 108 (Cowman *et al.*, 1988; Peterson *et al.*, 1988; Hyde, 1989; Snewin *et al.*, 1989; Zolg *et al.*, 1989). Additional changes of Asn-51 to Ile-51 or Cys-59 to Arg-59 were found associated with increased levels of resistance (Cowman *et al.*, 1988; Peterson *et al.*, 1988). Substitutions in all three positions confer high resistance to pyrimethamine for the parasites. In *P. chabaudi*, *in vitro* selected pyrimethamine resistant (pyrR) mutants show a single mutation from serine to asparagine at position 106 which is equivalent to the mutation at codon 108 in *P. falciparum* (Cowman & Lew, 1990).

Table 1.1 A list of parasites, the levels of resistance to pyrimethamine, cycloguanil and the associated amino acid changes in the dihydrofolate domain

Parasite	Origin	Level of pyrimethamine resistance	Level of cycloguanil resistance	Amino Acid residues							References
				16	51	54	59	108	164	223	
Csl-2	Thailand	40	R	Ala	Asn	Asp	Arg	Asn	Leu	Phe	1
V1/S	Vietnam	R	R	Ala	Ile	Asp	Arg	Asn	Leu	Phe	8
Dd2	Indochina	25	n.r.	Ala	Ile	Asp	Arg	Asn	Ile	Phe	2
V-1	Vietnam	20	S	Ala	Asn	Asp	Arg	Asn	Ile	Phe	1
K1	Thailand	20	S	Ala	Asn	Asp	Arg	Asn	Ile	Phe	1,3
7G8	Brazil	12	S	Ala	Ile	Asp	Cys	Asn	Ile	Phe	1,2,4
It.D12	Brazil	4	S	Ala	Ile	Asp	Cys	Asn	n.r.	n.r.	2
HB3	Honduras	1.5	S	Ala	Asn	Asp	Cys	Asn	Ile	Phe	1,2,4
Hond.-1	Honduras	R	n.r.	Ala	Asn	Asp	Cys	Asn	Ile	Phe	5
Camp	Malaysia	1.0	n.r.	Ala	Asn	Asp	Cys	Asn	n.r.	n.r.	2
Palo Alto 1	Uganda	S	n.r.	Val	Asn	Asp	Cys	Thr	Ile	Phe	1
Palo Alto 2	Uganda	R	S	Ala	Asn	Asp	Cys	Asn	Ile	Phe	6
3D7	Netherlands	0.025	S	Ala	Asn	Asp	Cys	Ser	Ile	Phe	1,2,4
UPA	Uganda	S	R	Val	Asn	Asp	Cys	Thr	Ile	Phe	8
FCB	Columbia	S	R	Val	Asn	Asp	Cys	Thr	Ile	Phe	8
W2	Indochina	R	S	Ala	Ile	Asp	Arg	Asn	Ile	Phe	8
It.G2.F6	Brazil	0.006	R	Ala	Asn	Asp	Cys	Thr	n.r.	n.r.	2
SL/D6	Sierra Leone	0.004	S	Ala	Asn	Asp	Cys	Ser	Ile	Phe	2
L.E5	Liberia	0.004	n.r.	n.r.	Asn	Asp	Cys	Ser	n.r.	n.r.	2
FC27	Papua-NG	S	S	Ala	Asn	Asp	Cys	Ser	Ile	Phe	6
FAC8	Brazil	S	R	Val	Asn	Asp	Cys	Thr	Ile	Phe	6
FCR3	Gambia	0.020	R	Val	Asn	Asp	Cys	Thr	Ile	Phe	2,4,5,6
FCR3/D4-D7	} Expt. mutants	R	n.r.	Val	Asn	Asp	Cys	Thr	Ile	Ser	5
FCR3/D8i		R	n.r	Val	Asn	Asn	Cys	Thr	Ile	Ser	7,5
FCR3/D8ii		R	n.r.	Val	Asn	Asp	Cys	Thr	Ile	Ser	5

Legend to the table

n.r. not reported
S = sensitive, R = resistant
V1/S is a cloned line of V-1
FAC8 is a subclone of It.G2.F6
FCR3/D8i is the originally isolated mutant
FCR3/D8ii is the mutant maintained in culture in the absence of pyrimethamine
References: 1 = Cowman *et al.*, 1988; 2 = Peterson *et al.*, 1988; 3 = Snewin *et al.*, 1989; 4 = Zolg *et al.*, 1989; 5 = Tanaka *et al.*, 1990b; 6 = Foote *et al.*, 1990; 7 = Inselburg *et al.*, 1988; 8 = Peterson *et al.*, 1990.

Adapted from Hyde (1990)

I will outline the relevance of each of these changes in turn. Thus far there is no structural analysis on *Plasmodium* DHFR-TS protein. The functional significance of the nucleotide changes could only be deduced from the role of equivalent residues in substrate/inhibitor binding in other organisms. Residue 108 in *P. falciparum* is conserved as a threonine in all other organisms examined and occurs in a region analogous to the C α-helix bordering the active site cavity of bacterial, avian and mammalian enzymes (Blakley, 1984). In *Lactobacillus casei*, it was shown that residues lying in this helix interact with methotrexate- another antifolate drug (Blakley, 1984). It is therefore likely that replacement of serine/threonine with asparagine in *P. falciparum* may affect inhibitor binding due to the different spatial and chemical characteristics acquired by the side chain groups.

The residue Cys-59 is equivalent to a glutamine residue in eukaryotes and a lysine/arginine residue in prokaryotes. These residues lie in the αB helix which contributes to the substrate binding pocket. The residue is apparently functionally unimportant in prokaryotes since the side chain of the residue is directed out towards the solvent. However, when the mouse DHFR interacts with the antifolate drug trimethoprim, glutamine is in contact with highly conserved neighbouring residues directly involved in inhibitor binding (Stammers *et al.*, 1987). Thus, conversion of cysteine to a bigger and more polar arginine would significantly modify such interactions with a concomitant effect on inhibitor binding.

The functional significance of Ile-51 is uncertain since the equivalent leucine residue found in most other DHFR molecules is predicted to lie at the amino-terminal extremity of the αB helix at some distance and directed away from the active site cavity.

Recently, the significance of Asn-108 and Ile-51 mutations has been confirmed by the inhibition properties of pyrimethamine toward the

recombinant enzymes purified from an expression system (Sirawaraporn *et al.*, 1990). Mutations in these two positions *in vitro* alter the sensitivities of the enzymes to pyrimethamine.

1.3.4 Mechanism of Resistance to Pyrimethamine- Other Possible Mechanisms

The existence in nature of a wide range of resistance to pyrimethamine, from very low level of resistance to as much as 10^6-fold more resistant than the sensitive organism suggests a multitude of factors operating together, in different combinations to confer different degrees of resistance on different parasites. In the rodent malaria parasite, *P. berghei*, both increased enzyme levels and decreased affinity for inhibitor are important factors in determining the degree of resistance in both field isolated pyrimethamine resistant (pyrR) strains and *in vitro* selected mutants (Diggens *et al.*, 1970; Ferone, 1970; Ferone *et al.*, 1970). There is already a documented case in *P. falciparum* in which the specific activity of DHFR increases 30- to 80-fold (Kan & Siddiqui, 1979). Other possible mechanisms such as gene amplification and altered membrane permeability have not been detected in field isolates (Dieckmann & Jung, 1986a; Cowman *et al.*, 1988). In the kinetoplastids, reduction of uptake of folate antagonists has been implicated as one mechanism for drug resistance (Dewes *et al.*, 1986; Ellenberger & Beverley, 1987). This, in conjunction with gene amplification, contributes resistance to another DHFR inhibitor, methotrexate in *Leishmania major* (Coderre *et al.*, 1983; Beverley *et al.*, 1984; 1986; Washtien *et al.*, 1985; Ellenberger & Beverley, 1987). Gene amplification is also one of the mechanisms contributing to methotrexate resistance in cultured mammalian cells (Schimke *et al.*, 1978).

Analysis of pyrR mutants selected *in vitro* from *P. falciparum* (Inselburg, 1984; Banyal & Inselburg, 1986; McCutchan, 1988) revealed several possible novel mechanisms of pyrimethamine resistance which have not been reported to operate *in vivo* in this organism. The change at codon 108 found in all pyrR field isolates has not occurred in *in vitro* selected mutants. In one set of experiments a nucleotide change resulting in a novel amino acid change from phenylalanine to serine at codon 223 was detected (Table 1.1) (Tanaka *et al.*, 1990b). This point mutation may be significant in conferring resistance to some of the mutants since other changes such as the enzyme content and activity, the accumulation of drug, gene copy numbers and chromosome sizes have not been detected in them. However, it is impossible to predict the effect of this mutation on substrate/inhibitor binding because the equivalent residues in prokaryotic/eukaryotic systems are not known to be involved in binding these ligands (Hyde, 1990).

An additional mutation of Asp-54 to Asn-54 was detected in one of the mutants (Tanaka *et al.*, 1990b; Inselburg *et al.*, 1988) which may be responsible for the higher degree of resistance in this mutant. However, this mutation is unstable and revertants were found in prolonged culturing in the absence of drug pressure (Tanaka *et al.*, 1990a).

Chromosomal size polymorphisms and gene amplification may be partly responsible for the tolerance to a higher level of pyrimethamine in some mutants (Tanaka *et al.*, 1990a; b). In one case mutants were associated with a 10-fold over-production of the enzyme (Inselburg *et al.*, 1987). Gene duplication and chromosomal rearrangement have also been observed in *in vitro* selected pyrR *P. chabaudi* (Cowman & Lew, 1989).

One mutant accumulates about 5 times more drug than wild type with a normal level of DHFR activity (Banyal & Inselburg, 1986). It is speculated that resistance to pyrimethamine in this mutant is partly due to titration of

the drug by the higher level of protein expressed in this mutant (Banyal & Inselburg, 1986; Inselburg *et al.*, 1987).

1.3.5 Proguanil Resistance and Its Relationship to Pyrimethamine Resistance

Proguanil (Fig. 1.4) is an antifolate with a known target DHFR. It has been used as an antimalarial drug in combination with pyrimethamine and sulphonamides in prophylaxis and treatment of *P. falciparum* infection. Resistance to cycloguanil (Fig. 1.4), the active metabolite of proguanil and pyrimethamine occur independently of each other (Thompson & Bayles, 1968; Vestergaard, 1983; Milhous *et al.*, 1985).

Analysis of field isolates or clones from geographically distinct areas shows no linkage between pyrimethamine and cycloguanil resistance (Table 1.1). Parasites resistant to cycloguanil only show a change of Ser-108 to Thr-108 and another change of Ala-16 to Val-16 (Foote *et al.*, 1990a; Peterson *et al.*, 1990). In parasites that are highly resistant to both drugs, there is a unique change of Ile-164 to Leu-164 in conjunction with the changes associated with pyrimethamine resistance at other positions. These mutations are located in conserved regions bordering the active site cavity of the enzyme. Equivalent residues have been shown to be involved in the binding of various inhibitors to avian, bacterial and mammalian DHFR. It is postulated that the degree of resistance to these drugs is due to accumulation of changes in the DHFR gene leading to increased resistance to pyrimethamine or cross-resistance to cycloguanil.

1.3.6 Sulphadoxine (Sulphonamide) Resistance

Sulphonamides are substrate analogues competing with *p*-aminobenzoate (PABA) for binding to dihydropteroate synthetase. Metabolism of sulphonamides to the sulpha-analogues of dihydropteroate will subsequently

inhibit the synthesis of dihydropteroate which is the precursor of dihydrofolate. Three mechanisms have been proposed to operate together to account for the sulphonamide resistance in *P. falciparum* (Dieckmann & Jung, 1986b).

P. falciparum does not metabolically inactivate sulphadoxine (Fig. 1.4). However, metabolism of sulphadoxine to the toxic analogue of dihydropteroate is reduced in resistant plasmodia.

Uptake of sulphadoxine is markedly decreased in resistant parasites. Contrary to the sensitive parasites which acquire PABA exogenously, PABA is not an essential nutrient for sulphadoxine resistant parasites. The potential for the parasite to synthesize PABA *de novo* is highlighted by the demonstration of four of eight enzymes for the *de novo* synthesis of PABA in both sensitive and resistant parasites. It is therefore possible that by shifting to *de novo* synthesis of PABA, exogenous supply of PABA is no longer required and hence the uptake of PABA which may be coupled to uptake of sulphonamide will be markedly reduced.

1.4 THE PLASMODIUM GENOME

The haploid genome size of *Plasmodium* is in the range of 2-4x10^7bp (Goman *et al.*, 1982). The complexity of the *Plasmodium* genome is similar to that of yeast (2x10^7) and slightly greater than that of *E. coli* (5x10^6) but is only one hundredth that of man (3x10^9).

It is estimated that the parasite possesses in the range of 2,700-5,400 genes, based on the calculation from the size of the genome, the large difference in base composition between coding and non-coding regions and the average length of *P. falciparum* mRNA transcripts (Hyde *et al.*, 1984).

The *Plasmodium* genome exhibits a number of features characteristic of the genus. They are summarized in the following sections.

1.4.1 Nucleotide Composition

Analysis of a number of *P. falciparum* genes revealed an average AT content of 69% for the coding region flanked by even more AT rich regions of 86% (Hyde & Sims, 1987; Weber, 1987; Saul & Battistutta, 1988). The A residue is predominant over T in the mRNA synonymous strand. The coding sequence preference for A and T increases in the order of 1st, 2nd and 3rd bases of the codon (Weber, 1987). In addition, A and G are preferred in the first position whereas T is preferred in the last position. The C residue is under-represented throughout the coding region. The frequencies of the dinucleotides TG and CC are elevated while those of CG, TA and AC are suppressed. No CGG codon has been found in any of the *P. falciparum* genes sequenced so far (Weber, 1987). The stop codon TAA is preferred among the genes sequenced to date although the other two stop codons are also found.

In the non-coding region, there is no consistent purine or pyrimidine asymmetry. The 5' flanking region shows dinucleotide preferences involving C and G residues. All four C/G containing doublets GG/CC/GC/CG are over-represented with nearly half the C's and G's in these highly AT rich regions being in a C/G pairing (Hyde & Sims, 1987).

Analysis of sequences surrounding the ATG start codon revealed a consensus sequence AAAAATG (Saul & Battistutta, 1990). A functional significance of the A residues in the initiation of translation is suggested by the significantly more frequent occurrence of A in the four positions preceding the initiation ATG than in adjacent non-translated DNA.

Methylation of the cytosine residues has not been detected in the

Plasmodium genome (Pollack *et al.*, 1982; McCutchan *et al.*, 1984a), thus it is unlikely that methylation of the cytosine residues will play a role in gene regulation in *Plasmodium*.

1.4.2 Introns

Most of the *Plasmodium* genes examined so far are devoid of introns. Even when present, the number is small, usually restricted to one, which separates a short 5' exon from the main body of the 3' exon (Favaloro *et al.*, 1986). Genes containing more than one intron appear to be rare, although an α-tubulin gene (Holloway *et al.*, 1989) and a β-tubulin gene (Delves *et al.*, 1989) containing two introns and antigen genes containing two (Simmons *et al.*, 1987) and three introns (Knapp *et al.* 1989) have been described. DNA sequence analysis of the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene revealed the presence of three introns (A. Khan, personal communication). The sizes of the introns are small, usually in the range of a few hundred bases. The intron-exon boundary contains the consensus splice sites sequence GT----AG (Kemp *et al.*, 1987a). An unusual case is denoted in an aldolase gene which is predicted to have a 5' exon encoding a single methionine residue (Knapp *et al.*, 1990).

1.4.3 Repeats

Considerable interest in vaccine development in recent years together with progress in recombinant DNA technology allowed the isolation and cloning of a number of genes from *P. falciparum* (for review see Scaife, 1988). Surprisingly, repeats are common features in these antigen-determining genes e.g. the genes encoding for the circumsporozoite protein (CSP) (Dame *et al.*, 1984; Enea *et al.*, 1984), the S-antigen (Coppel *et al.*, 1983; Cowman *et al.*, 1985; Stahl *et al.*, 1985a), the glycophorin-binding

protein (Perkins, 1984), the merozoite surface antigens (Mackay *et al.*, 1985; Tanabe *et al.*, 1987), the knob-associated histidine-rich protein (KAHRP) (Triglia *et al.*, 1987), the ring-infected erythrocyte surface antigen (RESA) (Cowman *et al.*, 1984; Favaloro *et al.*, 1986) and the small histidine-alanine rich protein (SHARP) (Stahl *et al.*, 1985b). Since the repeats are immunodominant parts of the molecule, they may play an important role in evasion of the immune system. By stimulating the immune system to over-produce antibodies against these regions, they may act as a 'smokescreen' to camouflage essential parasite proteins from the host immune system (Kemp *et al.*, 1987b). It is also likely that the tandem repeats actually mimic host protein epitopes to avoid their immunological recognition as foreign proteins (Kobayashi *et al.*, 1986).

1.4.4 Chromosomal Heterogeneity of the Genome

At least 14 chromosomes ranging in size from 800-3,500kb are detected in the *P. falciparum* genome (Kemp *et al.*, 1987b; Langsley *et al.*, 1987; Wellems *et al.*, 1987; Prensier & Slomianny Ch., 1986). Alteration in the molecular karyotypes of geographically different isolates and clones is a common feature of *P. falciparum* in natural infections (van der Ploeg *et al.*, 1985; Kemp *et al.*, 1985; Corcoran *et al.*, 1986). Chromosome size polymorphisms are most commonly attributed to differences in the subtelomeric regions encompassing repetitive DNA (Foote & Kemp, 1989), which probably arise by chromosomal rearrangement during meiotic or mitotic divisions e.g. polymorphism in the subtelomeric regions containing repetitive DNA can occur in progeny of a genetic cross or in cloned parasites grown in culture (Oquendo *et al.*, 1986; Walliker *et al.*, 1987; Corcoran *et al.*, 1988; Patarapotikul & Langsley, 1988).

In some cases, polymorphisms arose from the loss of protein-coding

genes. The three histidine-rich-protein (HRP) genes can each be deleted without loss of parasite viability *in vitro* (Kilejian *et al.*, 1986; Pologe & Ravetch, 1986; Kemp *et al.*, 1987b; Wellems *et al.*, 1987; Biggs *et al.*, 1989), so can the RESA gene (Pologe & Ravetch, 1988; Cappai *et al.*, 1989).

Drug resistance may be an important factor contributing to chromosome size polymorphisms. Changes in karyotypes were documented in *P. falciparum* and *P. chabaudi* after pyrimethamine selection. The alterations putatively involve duplication of the targeted genes together with subsequent chromosomal rearrangements (Tanaka *et al.*, 1990a; b; Cowman & Lew, 1989; 1990). In addition, size heterogeneity of chromosome 5 has been observed in some multi-drug resistant isolates. This is thought to be due to the variation in numbers of different-sized *pfmdr1*-containing amplicons which are arranged in a tandem array (Foote *et al.*, 1989).

1.5 REGULATION OF GENE EXPRESSION IN EUKARYOTIC CELLS

Most genes, when in an inert state, are tightly packaged with histones into chromatin. Transcriptional activation involves decondensation of this inert structure, making transcriptional control sequences available to regulatory proteins. The mechanism for this decondensation is unclear but it may involve binding to the nucleoskeleton through DNA sequences that function as attachment sites. Active genes are characterized by chromosomal changes that include undermethylation (Bird, 1986; Yisraeli *et al.*, 1988) and a looser packing of the nucleosomal DNA (Weisbrod, 1982; Reeves, 1984); the latter change was revealed by the increased nuclease sensitivity of the active genes.

The onset of transcription takes place through the interaction of defined proteins (transcription factors) with specific DNA sequences

(promoters) adjacent to the gene which is analogous to the regulation of gene expression in prokaryotes. In bacteria conserved RNA polymerase-binding sites are located 10 and 35bp upstream of the initiation point (Rosenberg & Court, 1979). In contrast to prokaryotes where a single RNA polymerase enzyme is responsible for the transcription of DNA into RNA, three enzymes are involved in eukaryotes. RNA polymerase II is utilized to direct transcription of all protein-coding genes and some small nuclear RNA involved in RNA splicing whereas RNA polymerase I and III are used to transcribe ribosomal RNA and tRNA. There is no reason to suggest a classical eukaryotic system is not operating in *Plasmodium*. The gene encoding the largest subunit of RNA polymerase II was isolated and it shows homology to the enzyme from higher eukaryotes (Li *et al.*, 1989). Therefore, the following sections will only describe control of transcription by RNA polymerase II.

1.5.1 Promoters Involved in Regulation of Transcription by RNA Polymerase II

Transcription begins at a certain distance 5' to the translation start site. The mRNA start sites do not display extensive homology of sequence. However, there is a tendency for the start site to be 'A' flanked on either side by pyrimidines (Breathnach & Chambon, 1981).

Promoter elements 'TATA', 'CAAT' boxes and GC-rich elements are generally required for specific transcription by RNA polymerase II. They can occur in different combinations in different genes. The 'TATA' boxes occur more proximally and the other two are often contained within sequences from 40-110bp from the transcriptional start site. The main characteristics of promoter motifs are distance-dependent activation and the requirement for location 5' to the transcriptional start site. Some motifs e.g. 'CAAT'

boxes and GC-rich motifs are functional in both orientations. It is postulated that the distal control region comprising the 'CAAT' box and the GC elements facilitates initial entry of RNA polymerase II onto the gene to make contact with the proximal 'TATA' sequence. The interaction of RNA polymerase II and the 'TATA' motif results in specific initiation.

The 'TATA' motif is shared by many but not all promoters, having a consensus TATAA/TAA/T sequence surrounded by GC rich sequence (Breathnach & Chambon, 1981). The 'TATA' boxes are required for accurate initiation of transcription 25-30bp downstream. In the absence of the 'TATA' sequence, mRNA with heterogeneous 5' termini would be synthesized (Grosschedl & Birnstiel, 1980; Benoist & Chambon, 1981).

The 'CAAT' boxes are found upstream of virtually all globin genes and in several other genes (Efstratiadis *et al.*, 1980; Grosveld *et al.*, 1982; McKnight *et al.*, 1985). GC-rich elements containing the consensus GGGCGG sequence have been found in a number of genes including the thymidine kinase gene of herpes simplex virus (McKnight & Kingbury, 1982; McKnight *et al.*, 1984), the SV40 early promoter region (Byrne *et al.*, 1983; Everett *et al.*, 1984) and many 'housekeeping' genes. This GGGCGG sequence has been shown to bind to the promoter-specific transcription factor Sp1 (Dynan & Tjian, 1983).

1.5.2 Enhancers of Eukaryotic Genes

An enhancer is a short, cis-acting regulatory sequence. The activity of a promoter is enormously increased by the presence of an enhancer sequence. An enhancer is functionally independent of distance and orientation and can be located either upstream or downstream of a promoter. Moreover, an enhancer can activate a heterologous promoter of a remote gene (Banerji *et al.*, 1981; Khoury & Gruss, 1983; Serfling *et al.*, 1985). The role of enhancer

elements in stimulating transcription is unknown. They may serve as an initial entry site for transcription factors or may bring about structural changes of the DNA to facilitate binding of RNA polymerase with the DNA.

Enhancer elements are found in the 72bp repeat of the SV40 early promoter (Banerji *et al.*, 1981) and also in cellular genes such as immunoglobulin gene (Banerji *et al.*, 1983; Serfling *et al.*, 1985; Wirth *et al.*, 1987). In the latter case, the enhancer stimulates transcription in a cell-specific manner.

1.5.3 The Putative Role of an Altered DNA Configuration on Gene Activation

DNA molecules having an unusual base composition can adopt configurations other than the conventional right-handed B-form e.g. Z-DNA which, in crystals, is left-handed. Formation of Z-DNA is favoured in nucleotide sequences that have alternating purines and pyrimidines i.e. poly d(GC), poly d(CA), poly d(GT), d(CGCG), d(CGCGCG), poly d(CA/TG) (Hamada *et al.*, 1982; Dickerson *et al.*, 1982; Nordheim & Rich, 1983a; Rich *et al.*, 1984) and short stretches (8-13) of alternating purine-pyrimidine base pairs and also in sequences in which some base pairs are found out of purine-pyrimidine alternations (Nordheim & Rich, 1983b).

Z-DNA forming sequences are found in a number of viral system transcriptional enhancers, the 5' flanking sequence of some genes in yeast and in the promoter region of other eukaryotic genes (Cantor, 1981; Nordheim & Rich, 1983b; Karin *et al.*, 1984; Rich *et al.*, 1984). This class of sequence may be involved in some fundamental functions such as activation or inactivation of the gene by directing interconversion between the B- and Z-forms. One piece of evidence comes from the adoption of a Z-form by poly (dG.d^{m}5C) at near physiological salt concentration when the C residue is methylated (Cantor, 1981). Simultaneously, methylation of cytosine *in vivo*

is associated with gene inactivation in eukaryotes (Bird, 1986; Yisraeli *et al.*, 1988). It is therefore possible that these two phenomena are related.

Association of the formation of Z-DNA with transcriptional activity is evident from the staining of the heat-inducible puffs in *Drosophila* polytene chromosomes (Nordheim *et al.*, 1981; Pardue *et al.*, 1983) and the transcriptionally active macronucleus of the ciliated protozoan *Stylonichia* by Z-DNA specific antibodies (Lipps *et al.*, 1983). Similarly, Z-DNA forming sites are found closely associated with DNase I hypersensitive regions within the enhancer element that regulates transcription of the eukaryotic virus SV40 (Cantor, 1981; Nordheim & Rich, 1983b; Rich *et al.*, 1984). DNase I hypersensitive regions are often associated with transcriptionally active chromatin. Regulation through specific interactions with Z-DNA binding proteins is suggested by the isolation of such proteins from SV40 (Nordheim & Rich, 1983b) and *Drosophila* (Nordheim *et al.*, 1982).

1.5.4 Regulation of DHFR Expression in Mammalian Species

DHFR belongs to a class of 'housekeeping' enzymes whose products are necessary for growth of all cell types. Unlike tissue-specific genes, the 'housekeeping' genes are transcribed at a low level. Expression of the DHFR gene is cell cycle regulated at the transcriptional level. A low rate of transcription from a constitutive promoter is maintained throughout the cell cycle apart from a very brief increase in transcription rate at the onset of DNA synthesis (Farnham & Schimke, 1985).

Presence of multiple mRNA species is a common feature of the mammalian DHFR genes. This results from utilization of different 3' polyadenylation sites and heterogeneous 5' termini (McGrogan *et al.*, 1985;

Mitchell *et al.*, 1986). The major transcriptional start sites have been mapped to -55, -63 and -71 from the translation start sites in mouse, hamster and human DHFR genes (Chen *et al.*, 1984; Mitchell *et al.*, 1986; Sazer & Schimke, 1986). Utilization of minor start sites further upstream has been observed in mouse and hamster DHFR genes *in vitro* and *in vivo* giving rise to bigger transcripts (Mitchell *et al.*, 1986; Sazer & Schimke, 1986).

The DHFR promoter regions do not contain consensus 'TATA' or 'CAAT' boxes as in other eukaryotic genes. However, GC-rich elements containing the consensus Sp1 binding site sequence GGGCGG and another consensus sequence CACAAATA are represented in different copies in tandem repeat in the upstream region in all three genes mentioned above (Chen *et al.*, 1984; McGrogan *et al.*, 1985; Farnham & Schimke, 1986; Mitchell *et al.*, 1986; Ciudad *et al.*, 1988). Binding of the transcription factor Sp1 to the GC elements (Dynan *et al.*, 1986) is shown to be essential for DHFR promoter activity in the mouse and in Chinese hamster ovary cells (Dynan *et al.*, 1986; Swick *et al.*, 1989). Functional analysis indicated that the sequence surrounding the GC elements could specify transcription 40-60 nucleotides downstream. One GC element is required for accurate transcription from the major start site in mouse DHFR gene (Farnham & Schimke, 1986) whereas three are needed for transcription of the hamster DHFR gene *in vitro* (Swick *et al.*, 1989).

Another transcription factor E_2F binds 3' to the major transcription start site and is required for efficient expression of the hamster DHFR gene *in vitro* and *in vivo* (Blake & Azizkhan, 1989).

Apart from the GC elements, the striking homology between the 5' flanking sequences of the three mammalian DHFR genes up to 900 bases from the coding region may imply a functional significance of this region in regulation of gene expression in these species (Chen *et al.*, 1984; McGrogan

et al., 1985; Azizkhan *et al.*, 1986). A functional region is located 700bp upstream of the coding region of the mouse DHFR gene which contains a CAACT sequence separated by 50bp from a TAATAA sequence. However, it is unclear whether they are functional *in vivo* since they are about 200bp upstream from the longest transcript detected by S1 mapping.

A class of small, nuclear, poly A^- RNA orientated in the opposite direction to that of DHFR mRNA has been identified (Farnham *et al.*, 1985; Mitchell *et al.*, 1986). They are short, 180-240 nucleotides in length with heterogeneous 5' termini mapped adjacent to the DHFR promoter region. Probably they arise by bi-directional transcription from the promoter region.

The functional significance of these opposite-strand RNAs is untested but they may be involved in translational regulation of DHFR expression as in the *E. coli ompC* and *ompF* system involving expression of outer membrane proteins (Mizuno *et al.*, 1984). The OmpF production is regulated by the transcription of an opposite-strand micRNA (mRNA-interfering complementary RNA) from an independent transcriptional unit of the *ompC* locus which shows homology to the 5' end of the *ompF* mRNA. Hybridization of micRNA to *ompF* mRNA may inhibit translation of the *ompF* mRNA. This type of antisense regulation may be plausible in mouse DHFR since the opposite-strand RNA sequence shows complementarity to the first ten nucleotides of the major DHFR transcript and a short region of the DHFR mRNA immediately following the translation stop codon.

1.5.5 Regulation of DHFR Expression in Kinetoplastids

Transcription of *L. major* DHFR gene starts at major sites of -245/-375 and minor sites of -195/-295/-440. The 5' end profile remains unchanged in amplified or wild type genes and in different growth and developmental stages (Kapler *et al.*, 1987).

Examination of the 5' flanking sequence does not reveal any homologies with other trypanosomal genes, eukaryotic consensus promoter elements or mammalian DHFR promoter elements. Immediately 5' of the -375 sites there are two positions displaying sequence homology with *L. major* mini-exon; otherwise, sequences upstream of the start sites do not exhibit significant homology to each other.

It is possible that the *L. major* DHFR gene utilizes different regulatory signals which may be cryptic or may be located far away from the body of the mRNA. One feature of the flanking sequence is the common occurrence of dinucleotide repeats. A $(CT)_4$ tract followed by a $(CA)_{20}$ tract is located at -301 to -348. Poly (CA) has the capacity to adopt a Z-DNA configuration (Nordheim & Rich, 1983a) and has been shown to enhance gene expression in cultured mammalian cells (Hamada *et al.*, 1984).

In contrast to *L. major*, the 5' flanking sequence of the DHFR-TS gene of a closely related kinetoplastid protozoan, *Crithidia fasciculata* displays striking homology to mammalian DHFR promoter element including a 70% identity with a repetitive sequence element that functions as the mouse DHFR promoter and a potential Sp1 binding site CGCCCC (Hughes *et al.*, 1989). The putative promoter element is contained within -366 to -272 with no apparent 'TATA' or 'CAAT'-like elements.

It is possible that while *C. fasciculata* uses promoter elements similar to those of the mammalian system, *L. major* uses completely different control signals. Alternatively, the consensus CGCCCC may not represent a true promoter for *C. fasciculata*. The 90% amino acid identity in the coding regions of the two kinetoplastid DHFR genes suggests that they may utilize similar regulatory elements such as the conserved sequence PyrPyrCCCTCTC found in both species.

As with the mammalian system, in *C. fasciculata*, divergent

transcription from similar promoters of the DHFR-TS gene generates opposite-strand RNA. A sequence downstream from the promoter consensus region is complementary to the 3' end of the *C. fasciculata* mini-exon-derived RNA, suggesting a possible role of trans-splicing to form the mature RNA.

1.6 REGULATION OF GENE EXPRESSION IN SPOROZOA

1.6.1 Transcriptional regulation

Little is known about regulation of gene expression in sporozoa at the transcriptional level. Although functional transcripts can be mapped by techniques such as S1 mapping and primer extension, identification of putative promoters relies on the development of functional tests and consensus analyses of a number of upstream sequences. Thus far only the transcriptional unit of the circumsporozoite (CS) gene of *Plasmodium knowlesi* is well characterized. The transcriptional start sites for a few *Plasmodium* genes and the α-tubulin gene of *Toxoplasma gondii* have been mapped (Nagel & Boothroyd, 1988).

The *P. knowlesi* CS gene (Ozaki *et al.*, 1983) is the first and most thoroughly studied *Plasmodium* gene at the transcriptional level (Ruiz I Altaba *et al.*, 1987). CS protein constitutes 5-20% of the total protein product synthesized by the mature sporozoite (Cochrane *et al.*, 1982). The gene is developmentally regulated and is only expressed in the sporozoite stage as a 2,050 bases transcript (Ruiz I Altaba *et al.*, 1987) which cannot be detected in the blood stage (Ellis *et al.*, 1983). However, the transcriptional unit is maintained intact in these two stages with identical DNA sequences across 1.5kb on the 5' side, 0.5kb on the 3' side and the entire coding region. There is no DNA rearrangement within 15kb of chromosomal DNA and there is no obvious gene amplification (Ruiz I Altaba *et al.*, 1987).

Regulation is therefore likely at the level of transcription or RNA stability.

S1 nuclease and primer extension analyses have identified intronless transcripts with multiple start sites spanning a 100-base region. Consensus analysis of the 5' upstream region with prokaryotic, eukaryotic and viral systems has identified several potential regulatory elements including the 'TATA' and 'CAAT' boxes, repeat sequences resembling prokaryotic attenuators and terminators and sequences highly homologous to SV40 enhancer and reiterated core sequences (Laimins *et al.*, 1982; Weiher & Botchan, 1984).

The abundance of CS protein synthesized in a stage-specific manner suggests that the promoter is strong and tightly regulated. Its regulation may be similar to mechanisms regulating tissue-specific gene expression in higher eukaryotes.

Similarly, the expression of the precursor to the major surface antigens (gp195) gene in *P. falciparum* is stage-specific (Myler, 1990). The mRNA is detected most abundantly at the late trophozoite and early schizont stages but at much reduced level at the ring stage. This implies that the gene is most likely regulated at the level of transcription or mRNA stability. The major transcriptional start site is mapped to about 520bp upstream within a long oligo dT tract. Potential transcription regulatory sequences ('TATA' and 'CAAT' boxes) have been identified upstream of this region.

In analogy to the gp195 gene in *P. falciparum*, it is thought that transcription of the equivalent gene in *Plasmodium yoelii*- the gene of the precursor to the major merozoite surface antigens (PMMSA) (Lewis, 1989)- is regulated in a stage-specific manner during the asexual erythrocytic cycle. Primer extension analysis has mapped multiple 5' termini to 377-417 bases from the AUG codon (Lewis, 1990). Consensus analysis with eukaryotic and viral systems has identified potential promoter regulatory elements including

the 'TATA' boxes and an immunoglobulin octamer sequence. Possession of several Z-DNA forming regions may imply a role of enhancer function since formation of Z-DNA may be related to transcriptional activation (see Section 1.5.3).

The transcriptional start sites of two other *P. falciparum* genes were also mapped. The 5' non-coding region of the mRNA extends to at least 322bp from the coding region of the aldolase gene (Knapp *et al.*, 1990). However, the calmodulin mRNA has a major start site 62bp upstream of the initial AUG codon (Robson & Jennings, 1991). Consensus eukaryotic 'TATA' and 'CAAT' boxes are found upstream of the major transcriptional start site in *P. falciparum* calmodulin gene.

The transcriptional unit of the α-tubulin gene of *T. gondii*, a parasite closely related to *P. falciparum*, has been examined (Nagel & Boothroyd, 1988). The gene contains multiple introns which are spliced to form the mature mRNA with a 5' terminus at -269 from the coding region as mapped by primer extension. Trans-splicing is not involved in the formation of the mature α-tubulin transcript. Examination of the sequence upstream of the start site does not reveal any homologies to known eukaryotic promoter elements.

1.6.2 Differential Expression, RNA Processing and RNA Stability

The small subunit rRNA genes of *P. berghei* and *P. falciparum* occur as low copy number genes dispersed throughout the genome, without any amplifications of the genes. There are two classes of structurally distinct rRNA genes 'A' and 'C' (Dame & McCutchan, 1983; Gunderson *et al.*, 1986; 1987; McCutchan *et al.*, 1988) that are developmentally regulated (Waters *et al.*, 1989). Developmental regulation of the two gene units illustrates a combination of differential expression of the genes, RNA processing and

RNA stability.

Transcription of the two genes is stage-specific with transcript from the 'A' gene predominating in the asexual blood stage and transcript from the 'C' gene found almost exclusively in the mosquito stage. Studies on *P. falciparum* demonstrated that a switch from 'A' to 'C' gene expression resulted in accumulation of the 'C' gene precursor RNA in gametocytes (Waters *et al.*, 1989). Increased transcription associated with accelerated processing of precursor RNA to mature RNA of the 'C' gene occurs in the zygote and the early ookinete stages. This is accompanied with the breakdown of the 'A' gene rRNA so that by the late oocyst stage, the 'A' gene transcripts are virtually replaced by mature 'C' gene transcripts.

1.6.3 Gene Amplification

Certain genes exist in multiple copies to meet the great demand for protein synthesis e.g. the rDNA gene is amplified in number from 50-10,000 in some eukaryotic organisms (Long & Dawid, 1980). Likewise, increased expression and amplification of the human *mdr* 1 gene is related to multi-drug-resistance in cancer cells (Shen *et al.*, 1986). In *Plasmodium*, gene amplification accompanied by over-production of the gene product has also been found associated with drug resistance both naturally occurring and under *in vitro* conditions (Inselburg *et al.*, 1987; Cowman & Lew, 1989; Foote *et al.*, 1989; Tanaka *et al.*, 1990a). It is tempting to speculate that parasite strains containing certain amplified genes may be selected under the pressure of drugs.

1.7 SCOPE OF THE PRESENT STUDY

Resistance to antimalarial drugs has become a common clinical problem in malaria endemic areas. Despite this, little is understood about the mechanisms underlying drug resistance in malaria. In the present study our interest focuses on pyrimethamine which is thought to have a defined drug target, the enzyme DHFR. The gene for DHFR-TS has been cloned from *P. falciparum* and sequenced. A genetic cross between a pyrimethamine sensitive cloned line 3D7 and a pyrimethamine resistant cloned line HB3 has also been carried out and was made available for this work. Knowledge of the gene sequence together with the availability of genetic cross data allows us to decide whether or not pyrimethamine acts on DHFR and may help us formulate models for the mechanisms underlying resistance to pyrimethamine.

The objective of the first part of the study was to define an RFLP associated with the DHFR-TS gene. This RFLP was then used as a marker in a genetic study.

The objective of the second part of the study was to investigate over-production of the enzyme as a possible mechanism of pyrimethamine resistance, employing an assay to measure the amount of DHFR-TS enzyme in different parasite strains.

The consequent results prompted the initiation of a study of the significance of transcriptional regulation in pyrimethamine resistance. This involved sequencing the 5' non-coding regions of the DHFR-TS genes from 3D7 and HB3 and mapping of the transcriptional start sites.

CHAPTER TWO
MATERIALS AND METHODS

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2.4.11 Southern Blotting

2.4.12 Dot-blots

2.4.13 Labelling of DNA

2.4.13.1 Nick Translation

2.4.13.2 Random Priming Method

2.4.13.3 Separation of Unincorporated Nucleotides from Labelled DNA

2.4.13.4 Priming from M13 Single-stranded Templates

2.4.13.5 End-labelling of Oligonucleotides

2.4.14 Hybridization of DNA

2.4.14.1 Hybridization with Homologous DNA Probes

2.4.14.2 Hybridization with Oligonucleotide Probes

2.4.14.3 Autoradiography

2.4.14.4 Removal of Probes and Re-use of Blots

2.4.15 Polymerase Chain Reactions (PCR)

2.4.16 Preparation of Plating Cells

2.4.17 Lambda Phage Titration

2.4.18 Preparation of Competent Cells

2.4.19 Ligation of DNA

2.4.20 Plaque Hybridization

2.4.21 Colony Hybridization

2.4.22 Identification of Recombinants

2.4.23 Construction of Genomic Library

2.4.23.1 Amplification and Storage of Library

2.4.24 Screening of Library

2.4.24.1 Preparation of Stock Culture of λ Recombinant Clones

2.4.25 Subcloning of DNA Fragments into M13 Vectors

2.4.26 Subcloning of DNA Fragments into pUBS1

2.4.27 DNA Sequencing

2.4.27.1 Preparation of Single-stranded M13 Templates

2.4.27.2 Sequencing Reactions Using Single-stranded M13 DNA as Templates

2.4.27.3 Sequencing Reactions Using Double-stranded DNA as Templates

2.4.27.4 Primers

2.4.27.5 Sequencing Gel Electrophoresis

2.4.27.6 DNA Sequence Data Compilation and Analysis

2.4.28 Alkaline Denaturing Gels

2.4.28.1 Dephosphorylation of DNA Fragment

2.4.28.2 End-labelling of Dephosphorylated DNA

2.4.28.3 Alkaline Denaturing Gel

2.5 RNA METHODS

2.5.1 Preparation of RNA from P. falciparum

2.5.2 Separation of Poly A^+ RNA from Poly A^- RNA

2.5.3 Measurement of RNA Concentrations

2.5.4 PCR from RNA

2.5.5 Primer Extension

2.5.6 Northern Blotting

2.6 PROTEIN METHODS

2.6.1 Protein Preparation

2.6.2 Estimation of Protein Concentration (Bradford's Method)

2.6.3 Thymidylate Synthetase (TS) Assay

2.6.3.1 Preparation of Methylenetetrahydrofolate (CH_2FAH_4)

2.6.3.2 TS Quantification

2.6.3.3 SDS-PAGE (Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis)

2.6.3.4 Detection of the Reaction Products

2.1 MATERIALS

2.1.1 Bacterial Strain, Bacteriophages and Plasmid

All *Escherichia coli* strains, bacteriophages and plasmid used in this study are listed below with their genotypes.

E. coli strain	Genotype and Reference
NM494	*hfl hsd*MSΔ
	(N.E. Murray, personal communication)
TG1	K12, Δ(*lac-proAB*), *sup*E, *thi*, *hsd*Δ5,
	F'[*tra*D36,*pro*A$^+$B$^+$*lac*I^q*lac*ZΔM15]
	(Gibson, 1984)
JM83	*ara*, Δ(*lac-pro*A,B), *rps*L, ϕ80, *lac*Z, ΔM15;
	(Vieira & Messing, 1982)
BHB2690	Lysogen of λ2690.*imm*434cI$_{ts262}$*red*3*D*am15*S*am7
	(Hohn & Murray, 1977; Hohn, 1979)
BHB2688	Lysogen of λ2688.*imm*434cI$_{ts262}$*red*3*E*am4*S*am7
	(Hohn & Murray, 1977; Hohn, 1979)

Bacteriophage	Genotype and Reference
λNM1149	λ*Imm*^{434}, b(538); (Murray, 1983)
M13mp18/mp19	(Yanisch-Perron *et al.*, 1985)

Plasmid	Reference
pUBS1	(G. Murphy, personal communication)

2.1.2 Media and Solutions

All bacteriological media were sterilized by autoclaving.

L Broth:	Difco Bacto Tryptone 10g; Difco Bacto yeast extract 5g; NaCl 5g; per litre adjusted to pH 7.2.
L Agar:	Difco Bacto Tryptone 10g; Difco Bacto yeast extract 5g; NaCl 10g; Difco agar 15g; per litre adjusted to pH 7.2.
BBL Agar:	Baltimore Biological Laboratories trypticase 10g; NaCl 5g; Difco agar 10g; per litre (pH unaltered).
BBL Top Agar:	As for BBL Agar, but only 6.5g Difco agar per litre.
Phage Buffer:	KH_2HPO_4 3g; Na_2HPO_4 (anhydrous) 7g; NaCl 5g; 1mM $MgSO_4$; 0.1mM $CaCl_2$; 1ml 1% gelatin solution per litre.
5x(Spitizizen salts):	$(NH_4)_2SO_4$ 2g; K_2HPO_4 14g; KH_2PO_4 6g; trisodium citrate 1g; $MgSO_4$ 0.2g/ per litre.

Minimal Medium:	Difco Bacto Agar 15g; 100ml Spitizizen salts (5x); 10ml 20%(w/v) glucose; 50mg Vitamin B1/ per litre.
Antibiotics:	L-Broth or molten L-Agar was supplemented with an appropriate antibiotic when necessary. Ampicillin was added to 50μl-100μg/ml, kanamycin to 430μg/ml and tetracycline to 10μg/ml.
X-Gal Indicator Plates:	Molten L-Agar, supplemented with an appropriate antibiotic if necessary, was poured into a plate and allowed to solidify. After drying at 37°C, 200μl of L-Broth containing 30μl of X-Gal (20mg/ml, 5-bromo-4-chloro-3-indoyl β -galactosidase in dimethylformamide) and 20μl of IPTG (100mM, isopropylp-β-D -thiogalactoside in double distilled water) was spread onto the plate.
TE(1x):	10mM Tris-HCl pH 8.0;

1mM EDTA pH 8.0.

20xSSC: 175.3g NaCl;
88.2g sodium citrate;
pH to 7.0 with concentrated HCl;
adjust volume to 1 litre.

PBS: NaCl 8.76g;
$Na_2HPO_4.2H_2O$ 17.8g;
$NaH_2PO_4.2H_2O$ 7.8g/ per litre;
pH 7.2.

TBE(10x): 108g Tris base;
55g boric acid;
40ml 0.5M EDTA pH 8.0.

TAE(50x): 242g Tris base;
57.1ml glacial acetic acid;
100ml 0.5M EDTA pH 8.0.

2.2 PARASITOLOGY METHODS

2.2.1 Culture Media

Two types of medium were used depending upon the purpose of the experiment. The standard RPMI medium (Trager & Jensen, 1976), consisting of 10.39g/l RPMI 1640 (Gibco), 5.94g/l HEPES buffer (Sigma), 100mg/l gentamycin sulphate (Sigma) and 2.5g/l $NaHCO_3$ (BDH), was used to culture

parasites before they were tested for drug sensitivity.

The modified RPMI medium (Zolg *et al.*, 1982; 1984) was used for maintaining stock cultures and large scale cultivation. It consists of 10.4g/l RPMI 1640 (Gibco), 6.87g/l TES buffer (Sigma), 2g/l glucose (Sigma), 50mg/l gentamycin sulphate (Sigma), 50mg/l hypoxanthine (Sigma) and 2.25g/l $NaHCO_3$ (BDH). The pH was adjusted to 7.25 with 1M NaOH. The media were filter sterilized using Millipore 0.22μm filters and then stored at 4°C until required.

2.2.2 Preparation of Human Red Blood Cells and Human Serum

Human red blood cells obtained from Edinburgh Blood Transfusion Service were washed 3 times in modified RPMI medium with centrifugation (3,500rpm/15min./4°C). Washed red blood cells could be kept at 4°C for up to two weeks. Pooled human serum obtained from Edinburgh Blood Transfusion Service was kept at -20°C. Before use, the serum was heat treated at 56°C for 30min.

2.2.3 In vitro Cultivation of Plasmodium falciparum

Stock cultures of *P. falciparum* were maintained at low parasitaemia in 5ml of modified RPMI medium supplemented with 10% pooled human serum and 0.25ml packed human red blood cells in 50ml culture flasks (Falcon) under an atmosphere of 95% N_2, 3% O_2 and 2% CO_2. The cultures were incubated at 37°C with daily medium changes. The growth of the parasites were monitored by making thin smears on microscopic slides which were then examined by light microscopy after fixing in methanol and staining with 10% Giemsa stain (BDH). At least 1,000 red blood cells were counted and the percentage infected gave the parasitaemia. The cultures were diluted with fresh uninfected red blood cells when the parasitaemia reached 5% or

higher.

Parasite cultures were expanded from small 50ml flasks into large 250ml flasks for large scale cultivation. To start off with, each 250ml flask contained 50ml of modified RPMI medium supplemented with 10% of human serum and 1ml of packed red blood cells. An equal volume of fresh red blood cells was added to the culture once the parasitaemia reached 5% or higher. This procedure was repeated until there were 5ml of packed red blood cells in each flask. The parasites were usually harvested when the parasitaemia reached 10%.

2.2.4 Saponin Lysis of Red Blood Cells

Parasites used as the source for preparation of DNA and RNA were released from red blood cells by saponin lysis. All the washing were performed in 1xSSC (standard saline citrate 20x: 3M NaCl, 0.45M sodium citrate, pH 7.0) or modified RPMI medium. Parasitized red blood cells were washed 3 times with centrifugation (3,500rpm/10min./4°C) and lysed with 0.1% saponin in RPMI medium at room temperature for 10min. The pelleted cells were washed once and lysed once again with 0.1% saponin. The free parasites obtained by centrifugation (3,500rpm/10min./4°C) were washed 3 times before being used in nucleic acid preparation.

2.2.5 Freezing of P. falciparum Culture

Cultures of *P. falciparum* were frozen down and stored in liquid nitrogen as stocks. Since schizonts would be destroyed in the freezing process, cultures of approximately 5% parasitaemia with a high proportion of ring-forms were chosen. Deep-freeze solution (28% glycerol/3% sorbitol or mannitol/0.65% NaCl) was prepared by adding 28ml glycerol to 72ml of 4.2% sorbitol or mannitol in 0.9% NaCl. Infected red cells were pelleted

(2,000rpm/5min.) and an equal volume of sterile deep-freeze solution was added to the packed blood cells slowly, drop by drop, with mixing. The preparation was left at room temperature for 5min. to allow the glycerol to penetrate the cells. Aliquots of 0.5ml were frozen down as stocks in liquid nitrogen.

2.2.6 Thawing of Cryopreserved P. falciparum

Frozen ampoules were returned to culture by rapidly thawing at 37°C and pelleting the blood cells (2,000rpm/5min.). The packed cells were mixed slowly with one-fifth volume of sterile 12% NaCl and left at room temperature for 3min., without stirring, before mixing slowly with ten volumes of sterile 1.6% NaCl. The blood cells were pelleted and again mixed slowly with ten volumes of sterile 0.2% dextrose and 0.9% NaCl. After centrifugation at 2,000rpm/5min., the pellet was resuspended in modified RPMI medium and cultured under the usual conditions except that initially the culture medium was supplemented with 15-20% human serum until the culture had well established.

2.2.7 Synchronization of Parasite Stages

Sorbitol treatment destroys trophozoite and schizont stages leaving the ring forms intact. An asynchronous culture of *P. falciparum* with a high percentage of rings was synchronized by sorbitol treatment as in Lambros & Vanderberg (1979). Infected red blood cells were pelleted (2,000rpm/5min.) and resuspended in five volumes of sterile 5% D-sorbitol for 5min. at room temperature. After removing the sorbitol by centrifugation and washing once in modified RPMI medium, the culture was re-established under usual conditions.

2.2.8 Drug Sensitivity Tests

Drug sensitivity tests were performed by Professor David Walliker. They were carried out using parasites cultured in standard RPMI medium. The haematocrit of the culture was adjusted to 5% with fresh red blood cells. Dilution to the desired parasitaemia (1%/0.3%/0.1%) was made with 5% haematocrit medium. The final cell suspension was dispensed into microtitre plates (100μl/well). After 1hr, the medium was replaced by medium containing the test drug at serial dilutions of 10^{-5}M, 10^{-6}M, 10^{-7}M, 10^{-8}M, 10^{-9}M etc. Control wells without addition of the drug were included in each assay. Incubation was continued at 37°C in a gassed container for 72hrs with daily changes of medium with drug before thin blood smears were taken from each well for microscopic examination. The growth of the parasites was graded from -(no growth) to + + + +(very healthy growth with high number of parasites in good morphology). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of drug that will kill all or nearly all the parasites after exposure to drug containing media for 72hrs.

2.3 CHEMISTRY OF THE ENZYME REACTIONS

A brief account is given concerning the chemistry of the various enzyme reactions which were used in the method sections.

Restriction Endonucleases (Type II) obtained chiefly from prokaryotes cut double-stranded DNA at or near to their particular recognition sequences, which typically are 4-6 nucleotides in length with a two-fold axis of symmetry, and thus create sticky or blunt ends to be used in molecular cloning.
T4 DNA Ligase obtained from T4-infected *E. coli* is used to catalyse the formation of a phosphodiester bond between adjacent 3'OH and 5'-phosphate termini in DNA during ligation of cohesive or blunt ends in molecular cloning.
DNA Polymerase I is obtained from *E. coli*. Its 5'-3' polymerase and 5'-3' exonuclease activities are utilized to catalyse labelling of DNA by nick translation which involves the elimination of nucleotides from the 5' side and the sequential addition of nucleotides to the 3' side.
Klenow is a large fragment of DNA polymerase I. It possesses 5'-3' polymerase activity which is used to catalyse the synthesis of a labelled DNA from single-stranded template with 3'OH. It is commonly used in labelling of randomly primed DNA and in synthesis of DNA probe from primed M13 templates.
Taq Polymerase is isolated from the thermophilic bacteria *Thermus aquaticus*. It is extensively used in amplification of DNA segments in polymerase chain reactions by virtue of its 5'-3' DNA polymerase activity and its thermostability.
Sequenase derives from bacteriophage T7 DNA polymerase. It is a 5'-3' DNA polymerase, used in catalysing sequencing reactions.

DNase I (RNase-free) obtained from bovine pancreas is a double-stranded specific endonuclease. It degrades DNA by catalysing hydrolysis of double-stranded DNA to a complex mixture of mono- to oligonucleotides.
RNase A obtained from bovine pancreas is an endo-ribonuclease. It degrades RNA to oligonucleotides.
T4 Polynucleotide Kinase obtained from T4-infected *E. coli* catalyses the transfer of γ-phosphate of ATP to a 5'OH terminus in single-stranded or double-stranded DNA. It is commonly used in end-labelling of oligonucleotides.
Alkaline Phosphatase (CAP) obtained from calf intestine catalyses the removal of 5'-phosphate residues from DNA prior to labelling the 5' end with [γ-^{32}P].
Reverse Transcriptase is obtained from *E. coli* expressing a portion of the *pol* gene of M-MLV (Moloney Murine Leukemia Virus) on a plasmid. It has an RNA-dependent 5'-3' DNA polymerase activity which uses single-stranded RNA or DNA template in the presence of a primer to synthesize a cDNA strand.

2.4 DNA METHODS

2.4.1 Phenol Extraction and Precipitation of DNA Preparations

DNA preparations were usually extracted in phenol to remove contaminating protein which would otherwise degrade the DNA or inhibit enzymatic reactions. The extraction process was usually carried out in eppendorf tubes. Unless otherwise stated, phenol was made up as phenol:

chloroform: isoamyl alcohol (25:24:1). An equal volume of phenol was mixed with the aqueous DNA solution and then separated by centrifugation in a microfuge for 2min. The top aqueous phase was collected and extracted a further 2-3 times. This was followed by extraction 1-2 times with chloroform to remove the phenol and then extraction once by ether to remove the chloroform. Chloroform was normally made up as chloroform: isoamyl alcohol (24:1). Traces of ether were allowed to evaporate from the surface by opening the lid of the eppendorf for a few min. The DNA in the bottom aqueous phase after the ether wash could now be precipitated by adding 1/10 volume of 3M sodium acetate pH5.2 and 2 volumes of absolute ethanol. This was quick frozen in liquid nitrogen and the DNA collected by centrifugation (microfuge/20min.). It was then washed once by resuspending in 70% ethanol and collected by centrifugation in a microfuge for 15min., the DNA pellet was dried under vacuum and then resuspended in TE (10mM Tris-HCl pH 8.0; 1mM EDTA pH 8.0).

2.4.2 Proteinase K Treatment of DNA Preparations

Proteinase K was used to digest contaminating protein from DNA preparations which would otherwise inhibit enzymatic reactions.

To 1ml of the DNA sample was added 40μl 1M Tris-HCl pH 8.0, 25μl 20% SDS and 10μl 50mg/ml Proteinase K (Boehringer). After incubation at 37°C for 1hr, the Proteinase K was removed by phenol extraction and the DNA recovered by ethanol precipitation (Section 2.4.1).

2.4.3 Estimation of DNA Concentration

The DNA sample was diluted in 1ml of distilled water and the OD at wavelengths 260nm and 280nm was measured by a spectrophotometer. An OD_{260} of 1.0 is equivalent to a concentration of 50μg/ml for DNA and

20μg/ml for oligonucleotides. The ratio OD_{260}/OD_{280} provides an estimate for the purity of the nucleic acid. A value around 1.8 indicates pure preparations of DNA.

2.4.4 Preparation of DNA from P. falciparum

2.4.4.1 Maxi-prep of P. falciparum DNA: Packed red blood cells (50ml) at 10% parasitaemia were used for one DNA preparation. The parasites obtained by lysing the red blood cells as described in Section 2.2.4 were lysed with 2ml of 20% sodium lauroyl sarcosine (Sigma) in 20ml of 1xSSC. The parasite DNA was then banded in a caesium chloride (CsCl) gradient. To the preparation was added 20.9g CsCl and 0.2ml of 10mg/ml ethidium bromide. The whole mixture was centrifuged at 38,000rpm, 18°C for 48hrs (Ti50 rotor, Beckman). The DNA band was collected and the ethidium bromide removed by extracting several times with water-saturated iso-propanol. After dialysing against several changes of TE, the DNA was recovered by phenol extraction and ethanol precipitation (Section 2.4.1).

2.4.4.2 Mini-prep of P. falciparum DNA: Genetic analysis of *P. falciparum* involves screening a large number of progeny for certain genotypes which in turn requires preparation of DNA from them. Extraction of DNA from parasites by the conventional maxi-prep method is a time consuming and laborious process, involving weeks of large scale cultivation with daily medium changes. At least 50ml of packed red blood cells at 10% parasitaemia is needed for one preparation. It was therefore very important to work out a simple and rapid protocol for the isolation of DNA from a small quantity of blood. I thereby devised the following mini-prep method which was frequently used instead of the maxi-prep method throughout this study.

Parasitized human red blood cells (100μl) were washed 3 times with

0.5ml of buffer (150mM NaCl, 25mM EDTA) and resuspended in 0.4ml of the same buffer. The parasites were lysed by adding 10% SDS (5μl) and incubated with 50μg Proteinase K (Boehringer) at 37°C overnight. Parasite DNA was obtained after phenol extraction and ethanol precipitation (Section 2.4.1). The final DNA was resuspended in 50μl of TE.

2.4.5 Preparation of DNA from λ

2.4.5.1 Maxi-prep of λ DNA: This method was used for large-scale preparation of DNA from recombinant clones. An exponential culture of NM494 was obtained by growing 10ml of an overnight culture in 500ml L-broth containing 10mM $MgSO_4$ and 0.2% maltose at 37°C for 2-3hrs. The cells were mixed with 2ml of phage stock and stood at 37°C for 10min. to allow adsorption of the phage particles to the bacteria. Incubation at 37°C was then continued with shaking for 4-6hrs to lyse the bacteria. The bacterial debris was removed by centrifugation at 9,000rpm, 19°C for 20min. (Sorvall GSA rotor) before pelleting the phage at 19,000rpm, 19°C for 3hrs (Type 19 rotor, Beckman). The phage was resuspended in 10ml of phage buffer by shaking at 4°C overnight. To this was added 8.03g CsCl and the phage were then banded by centrifugation at 38,000rpm, room temperature for 24hrs (Ti50 rotor, Beckman). The band containing the phage was collected and dialysed against several changes of TE at 4°C overnight. The DNA was treated with Proteinase K (Section 2.4.2) and phenol extracted and ethanol precipitated as described in Section 2.4.1.

2.4.5.2 Mini-prep of λ DNA: This method adapted from Grossberger (1987) was used to prepare DNA from a number of putative positive clones from a library as a quick check of the size and nature of the cloned inserts by restriction analysis. A plaque was picked into 0.3ml of adsorption buffer (10mM $MgCl_2$ and 10mM $CaCl_2$) and incubated with 0.2ml of an exponential

culture of NM494 grown in L-broth containing 0.4% maltose at 37°C for 10min. Then, 10ml of L-broth containing 10mM $MgCl_2$ and 0.1% glucose was added and incubation was continued overnight. The bacterial debris was removed by centrifugation at 3,000rpm for 15min. The supernatant containing the phage was collected. DNase and RNase were added to the supernatant at concentrations of 10μg/ml and incubation was continued at 37°C for 30min. The phage were pelleted by centrifugation at 27,000rpm for 2.5hrs, resuspended in 200μl of phage buffer and treated with 200μl of 1mg/ml Proteinase K at 37°C for 2hrs. The mixture was extracted once with phenol and once with chloroform (Section 2.4.1) and the DNA precipitated by adding 100μl of 7.5M ammonium acetate and 1ml of absolute ethanol. After washing once with 100% ethanol, the DNA was dried and resuspended in 100μl of TE.

2.4.6 Preparation of DNA from Plasmid

DNA was prepared from an overnight culture of bacteria which was prepared by inoculating a single colony into L-broth (250ml for maxi-prep, 5ml or 50ml for mini-prep) containing 50μg/ml ampicillin.

2.4.6.1 Maxi-prep of Plasmid DNA: The bacterial cells were pelleted (6,000rpm/10min./4°C, Sorvall GSA rotor), then washed with 50ml cold 10mM Tris-HCl, 1mM EDTA pH 8.0 with centrifugation. The pellet was resuspended in 18ml ice-cold 50mM glucose, 25mM Tris-HCl, 10mM EDTA pH 8.0. The cells were lysed with 2.5ml fresh cold lysozyme (20mg/ml) at room temperature for 10min., then 0°C for 5min. A further incubation with 40ml fresh cold 0.2M NaOH, 1% SDS at 0°C for 5min. was used to disrupt the chromosomes. The preparation was then mixed with 20ml 5M potassium acetate (pH 4.8), left at 0°C for 15min., and centrifuged (10,000rpm/10min./4°C, Sorvall SS-34 rotor). The supernatant was collected.

The product was precipitated by adding 45ml (0.6 volume) of propan-2-ol and leaving at -20°C for 30min. The precipitate was spun down (8,000rpm/5min./4°C, Sorvall SS-34 rotor), washed with cold 95% ethanol and resuspended in TE. The nucleic acids were re-precipitated once with 1/10 volume of potassium acetate and 2.5 volumes of absolute ethanol at -20°C overnight. After centrifugation and drying under vacuum, the nucleic acids were redissolved in 20ml 0.1M Tris-HCl, 0.01M EDTA pH 8.0. CsCl (19g) was added followed by 1ml of 10mg/ml ethidium bromide and the DNA purified on a CsCl gradient at 38,000rpm for 48hr/18°C (Ti50 rotor, Beckman). After banding, the lower band which contains the supercoiled forms was collected and the ethidium bromide removed by extraction with water-saturated butan-1-ol. The DNA was dialysed against several changes of TE and the DNA precipitated with ethanol.

2.4.6.2 Mini-prep of Plasmid DNA- Boiling Method: Small quantities of plasmid DNA were prepared to allow a rapid screen of the insert size of cloned DNA fragments.

The cells from a 5ml overnight culture were pelleted by centrifugation in a microfuge for 1min. After removing the medium by aspiration, the pellet was resuspended in 0.35ml of 8% sucrose, 0.5% Triton X-100, 50mM EDTA, 10mM Tris HCl pH 8.0 and lysed by vortexing with 25μl of lysozyme (10mg/ml in 10mM Tris HCl pH 8.0) for 3 seconds. The preparation was boiled for 2min. and centrifuged in a microfuge for 10min. After removing the slimy pellet with a toothpick, the supernatant was incubated with 50μg/ml RNase A at 37°C for 30min. The DNA was recovered by the standard procedures of phenol extraction and ethanol precipitation (Section 2.4.1). After resuspending in TE, the DNA preparation was treated with Proteinase K (Section 2.4.2), re-extracted with phenol and re-precipitaed with ethanol as before.

2.4.6.3 Mini-prep of Plasmid DNA for Double-stranded Sequencing: This method was used to generate DNA in sufficient quality and quantity to be used in double-stranded sequencing without going through the laborious steps of the maxi-prep method.

Pelleted cells from a 50ml overnight culture were resuspended in 3.5ml of 25mM Tris-HCl pH 8.0, 10mM EDTA and 15% sucrose. After addition of 8mg of lysozyme in 0.5ml of the same buffer, the preparation was left on ice for 20-40min. Chromosomal DNA was disrupted by adding 8ml of fresh 0.2M NaOH, 1% SDS on ice for 30min. and then 5ml of 3M sodium acetate pH5.2 on ice for 10min. The bacterial debris was removed by centrifugation at 15,000rpm for 30min (Sorvall SS-34 rotor). After incubation with 20μl of 10mg/ml RNase A at 30°C for 20min., the supernatant was extracted once with phenol: chloroform. The DNA was precipitated with 1/10 volume of 3M sodium acetate pH 5.2 and 2 volumes of absolute ethanol at -20°C overnight. The pellet obtained, after centrifugation at 15,000rpm/30min., was resuspended in 0.5ml water and treated with 5μl of 10mg/ml RNase at 37°C for 20min. The DNA preparation was phenol extracted (Section 2.4.1) and re-precipitated as before. Dried DNA pellet was resuspended in TE.

2.4.7 Preparation of Replicative Forms (RF) from M13 Vectors

M13 is a male-specific filamentous bacteriophage of *E. coli*. It is composed of circular DNA, which can exist in an infectious single-stranded form or a double-stranded replicative form. The replicative forms are those used as cloning vectors. Usually, DNA of high purity is required for cloning purposes and this is done by the maxi-prep method. For a simple and quick check of the subcloned insert size, DNA is prepared by the mini-prep method.

2.4.7.1 Maxi-prep of M13 RF DNA: A phage stock was prepared by

incubating a single plaque of M13mp vectors with 5ml TG1 cells at logarithmic phase of growth for 6hrs at 37°C. The cells were spun down (5min., microfuge) and the supernatant was stored at 4°C as stock.

The phage stock (1ml) was added to 250ml TG1 cells in logarithmic phase of growth and incubated for 4.5hrs at 37°C. Preparation of the replicative form DNA from the phage-infected cells then proceeded as for the maxi-prep method of plasmid DNA in Section 2.4.6.1.

2.4.7.2 Mini-prep of M13 RF DNA: An aliquot (10μl) of the phage stock was incubated with an overnight culture of TG1 diluted 1/50 in 5ml L-broth at 37°C for 4-6hrs, with shaking. Preparation of the replicative form DNA from the phage then proceeded as for the boiling method for preparation of plasmid DNA (Section 2.4.6.2).

2.4.8 Restriction Enzyme Digestion

Restriction enzyme digestion of DNA was carried out in the appropriate buffer at the optimal temperatures for times varying from a couple of hours to overnight. The Boehringer Mannheim's incubation buffer system for restriction enzymes was followed (Table 2.1). Double or multiple digestions were performed simultaneously in compatible buffers. The reactions were terminated by heating at 65°C for 10min.

2.4.9 Agarose Gel Electrophoresis

2.4.9.1 Mini-gels: A rapid way to check the integrity and the purity of DNA preparations, the completion of enzymatic digestions etc. was to run the samples on mini-gels which were prepared as 0.7% agarose in 1xTBE (0.089M Tris-borate; 0.089M boric acid; 0.002M EDTA). Ethidium bromide was added at a final concentration of 1μg/ml to the gel and the running buffer (1xTBE). An appropriate amount of DNA was mixed with 1μl of

Table 2.1 The Boehringer Mannheim incubation buffer system for restriction enzymes.

a) Buffer composition

Buffer components	Final concentration in (mmol/l) (1 : 10 diluted set buffer)				
	A	B	L	M	H
Tris acetate	33	–	–	–	–
Tris-HCl	–	10	10	10	50
Mg-acetate	10	–	–	–	–
$MgCl_2$	–	5	10	10	10
K-acetate	66	–	–	–	–
NaCl	–	100	–	50	100
Dithioerythritol (DTE)	–	–	1	1	1
Dithiothreitol (DTT)	0.5	–	–	–	–
2-Mercaptoethanol	–	1	–	–	–
pH at 37 °C	7.9	8.0	7.5	7.5	7.5

b) Correct buffer to be used with each restriction enzyme.

A	B	L	M	H
Acc I *Alu* I *Apa* I *Ava* II *Dpn* I *Eae* I (*Cfr* I) *Hae* II *Hpa* I *Nae* I *Sac* I (*Sst* I) *Sau* 3A *Sau* 96 *Sma* I (25 °C)	*Acy* I (50 °C) *Asp* 700 (*Xmn* I) *Asp* 718 *Ava* I *Bam* HI *Ban* II *Bst* EII *Eco* RV *Hind* III *Nru* I *Sau* I *Scr* FI *Stu* I *Taq* I (65 °C)	*Apy* I (30 °C) *Cfo* I (*Hha* I) *Dra* II *Hpa* II *Kpn* I *Msp* I *Nci* I *Rsa* I *Xho* II	*Bcl* I *Bgl* II *Dra* I (*Aha* III) *Fok* I *Hae* III *Hind* II (*Hinc* II) *Mvn* I (*Tha* I) *Nhe* I *Pvu* II *Sna* BI *Sph* I	*Bgl* I *Cla* I *Dde* I *Dra* III *Eco* RI *Hin*f I *Ksp* 6321 *Mae* II (50 °C) *Mlu* I *Nco* I *Nde* I *Not* I *Nsi* I *Pst* I *Pvu* I *Sal* I *Sca* I *Spe* I *Ssp* I *Sty* I *Xba* I *Xho* I

All enzymes should be incubated at 37°C unless otherwise stated in brackets after the enzyme name.

c) Percentage activity in the 5-buffer system.

Enzyme	A	B	L	M	H
Aat II	75–100	0–10	75–100	0–10	0–10
Acc I	100	0–10	10–25	0–10	0–10
Acy I	10–25	100	10–25	50–75	25–50
Alu I	100	50–75	25–50	25–50	0–10
Apa I	100	10–25	50–75	50–75	0–10
Apy I	75–100	50–75	100	50–75	0–10
Asp 700	50–75	100	10–25	50–75	0–10
Asp 718	75–100	100	0–10	25–50	50–75
Ava I	100	100	10–25	50–75	10–25
Ava II	100	50–75	75–100	100	10–25
Bam HI	100	100	75–100	100	25–50
Ban I	25–50	0–10	25–50	0–10	0–10
Ban II	75–100	100	50–75	50–75	25–50
Bcl I	100	100	25–50	100	100
Bgl I	25–50	50–75	10–25	25–50	100
Bgl II	100	100	25–50	100	100
Bst EII	75–100	100	25–50	50–75	50–75
Cfo I	75–100	50–75	100	50–75	25–50
Cla I	100	100	75–100	100	100
Dde I	50–75	75–100	25–50	25–50	100
Dpn I	100	75–100	50–75	75–100	75–100
Dra I	100	75–100	100	100	50–75
Dra II	100	50–75	100	50–75	0–10
Dra III	50–75	75–100	50–75	75–100	100
Eae I	100	25–50	75–100	50–75	10–25
Eco RI	100	100	25–50	50–75	100
Eco RV	25–50	100	0–10	25–50	50–75
Fok I	100	50–75	75–100	100	25–50
Hae II	100	50–75	25–50	50–75	10–25
Hae III	50–75	50–75	75–100	100	25–50
Hind II	100	100	25–50	100	50–75
Hind III	50–75	100	25–50	100	10–25
Hinf I	100	100	50–75	75–100	100
Hpa I	100	25–50	25–50	50–75	25–50
Hpa II	50–75	25–50	100	50–75	10–25
Kpn I	75–100	10–25	100	25–50	0–10
Ksp 6321	75–100	0–10	50–75	75–100	100

Enzyme	A	B	L	M	H
Mae I	25–50	25–50	0–10	0–10	10–25
Mae II	0–10	25–50	0–10	25–50	100
Mae III	0–10	10–25	0–10	0–10	10–25
Mlu I	10–25	25–50	0–10	10–25	100
Msp I	100	100	100	100	50–75
Mvn I	50–75	0–10	50–75	100	10–25
Nae I	100	0–10	100	0–10	0–10
Nci I	75–100	50–75	100	10–25	0–10
Nco I	50–75	50–75	50–75	50–75	100
Nde I	25–50	100	10–25	50–75	100
Nde II	10–25	10–25	0–10	0–10	10–25
Nhe I	100	25–50	100	100	10–25
Not I	10–25	50–75	0–10	25–50	100
Nru I	10–25	100	0–10	10–25	75–100
Nsi I	50–75	100	10–25	50–75	100
Pst I	25–50	25–50	10–25	25–50	100
Pvu I	50–75	75–100	25–50	50–75	100
Pvu II	25–50	25–50	25–50	100	25–50
Rsa I	100	50–75	100	50–75	0–10
Sac I	100	0–10	100	50–75	0–10
Sal I	0–10	25–50	0–10	10–25	100
Sau I	50–75	100	0–10	50–75	10–25
Sau 3A	100	25–50	25–50	75–100	0–10
Sau 96	100	50–75	25–50	25–50	25–50
Sca I	0–10	100	0–10	75–100	100
Scr FI	10–25	100	10–25	10–25	50–75
Sma I	100	0–10	0–10	0–10	0–10
Sna BI	75–100	25–50	100	100	10–25
Spe I	75–100	75–100	75–100	100	100
Sph I	50–75	75–100	25–50	100	75–100
Ssp I	75–100	100	10–25	100	100
Stu I	100	100	100	75–100	50–75
Sty I	50–75	100	10–25	75–100	100
Taq I	50–75	100	25–50	50–75	50–75
Xba I	100	75–100	100	100	100
Xho I	25–50	75–100	10–25	25–50	100
Xho II	50–75	25–50	100	75–100	0–10

Boehringer Mannheim's recommended incubation buffer is indicated in bold type.

loading buffer (95% fromamide; 20mM EDTA pH 8.0; 0.05% bromophenol blue; 0.05% xylene cyanol) and the volume was made up to 10μl with distilled water. Electrophoresis was carried out at 200V constant voltage. The gel was then checked or photographed upon UV illumination.

2.4.9.2 Horizontal Slab Gels: Horizontal slab gels were used to fractionate enzyme restricted DNA before Southern blotting or for their purifications. The gels were made up in 1x TAE or in 1/2x TBE.

TAE gels (28x 14x 0.5cm) were prepared by dissolving 0.7%-1.0% agarose in 1x TAE. Ethidium bromide was added to a final concentration of 1μg/ml in both the gel and the running buffer (1x TAE). The samples were mixed with 1/10 volume of loading buffer, which were then heated at 65°C for 10min. before loading. Electrophoresis was carried out at low voltage (70-120V) overnight with the gel connected by wicks at each end of 500ml tanks containing gel buffer (1x TAE). The two tanks were also connected by a salt bridge. At the end of the run, the gel was photographed upon UV illumination. Fractionated DNA could then be blotted for Southern hybridization or isolated for purification.

TBE gels (20x 14cmx 0.5cm) were prepared as 0.8% agarose in 1/2x TBE. Ethidium bromide was added at a final concentration of 1μg/ml to the gel and the running buffer (1/2x TBE). The samples were mixed with 1/10 volume of loading buffer which were then heated at 65°C for 10min. before loading. Electrophoresis was carried out at 300V for several hours with the gel submerged in 1/2x TBE running buffer. At the end of the run, the gel was photographed upon UV illumination and processed similarly to the TAE gel.

2.4.9.3 DNA Molecular Weight Markers: Different DNA molecular weight markers were used according to the size range of the fractionated DNA (Table 2.2). One set were either *Hin*dIII or *Eco*RI/*Hin*dIII digests of

Table 2.2 Sizes of DNA fragments in Molecular Weight Markers

Sizes of DNA fragments in Molecular Weight Markers (bp)			
λcI_{857} *Hin*dIII	λcI_{857} *Eco*RI/*Hin*dIII	123bp ladder	1kb ladder
23,130	21,226	4182	12,216
9,416	5,148	4059	11,198
6,562	4,973	3936	10,180
4,368	4,268	3813	9,162
2,322	3,530	3690	8,144
2,027	2,027	3567	7,126
564	1,904	3444	6,108
125	1,584	3321	5,090
	1,375	3198	4,072
	947	3075	3,054
	831	2952	2,036
	564	2829	1,635
	125	2706	1,018
		2583	516/506
		2460	394
		2337	344
		2214	298
		2091	211/210
		1968	
		1845	
		1722	
		1599	
		1476	
		1353	
		1230	
		1107	
		984	
		861	
		738	
		615	
		492	
		369	
		246	
		123	

phage λcI_{857} DNA. Another consisted of a synthetic 123bp ladder (Bethesda Res. Lab.) or 1kb ladder (Bethesda Res. Lab.).

2.4.9.4 Gel Photography: Agarose gels were photographed under short wave UV light and a red filter using Ilford HP5 professional 5" x 4" film. Exposure times varied from 30 seconds to a few min. depending on the intensity of the bands. The film was developed for 5min. in Ilford Microphen developer, stopped and fixed for 5min. followed by immediate flushing in cold water to remove excess fixer. The film was then dried.

2.4.10 Purification of DNA Fragments

2.4.10.1 Electroelution: DNA fragments could be purified from TAE gel (Section 2.4.9.2) by electroelution. After the DNA was fractionated by electrophoresis, a trough of 2-3mm wide was cut right in front of the DNA band to be purified. The far side of the trough was lined with dialysis tubing and the trough was filled with 1x TAE. Electrophoresis was continued at 300V until all the DNA had run into the trough and stuck onto the dialysis tubing. This was checked by UV illumination. After reversing the current at 300V for 30 seconds to detach the DNA from the dialysis tubing, the buffer in the trough containing the DNA was collected and extracted 3-6 times with butanol to remove the ethidium bromide. The DNA was recovered by phenol extraction and ethanol precipitation as described in Section 2.4.1.

2.4.10.2 GENECLEAN II Kit (BIO 101 Inc.): DNA fragments could be purified from TAE or TBE gel using a GENECLEAN II kit (BIO 101 Inc.). After the DNA was fractionated by electrophoresis, the appropriate DNA band was excised from the gel. A maximum of 0.4g gel was put in an eppendorf tube and dissolved by heating in 2.5 volumes of NaI solution at 55°C for 5min. For gel slices obtained from TBE gels, 1 volume of TBE

modifier was added in addition. Sufficient quantity of Glassmilk- a suspension of porous silica (5μl to bind the first 5μg DNA, then 1μl for each additional 0.5μg of DNA) was added to adsorb the DNA at 4°C for 5min. The pelleted Glassmilk was washed 3 times in 0.5ml of NEW WASH with centrifugation (microfuge, 5 seconds). The adsorbed DNA was eluted in 5-10μl of TE at 55°C/5min. The Glassmilk was spun down (microfuge, 30 seconds) to obtain the supernatant which contained the eluted DNA. The elution procedure was repeated once and the eluted DNA was pooled.

2.4.11 Southern Blotting

This technique was used to transfer DNA from agarose gel onto filters for the detection of specific sequences among DNA fragments (Southern, 1975). The method of Smith & Summers (1980) was adopted with modifications.

DNA fractionated on slab gels was denatured twice for 15min. in denaturation solution (0.5M NaOH; 1.5M NaCl), then neutralized twice for 20min. in neutralizing solution (1M ammonium acetate; 0.02M NaOH). The gel was put on a clean glass plate and overlaid with a Hybond-N membrane (Amersham) followed by three sheets of filter paper saturated with neutralizing solution, five sheets of dry filter paper and finally a stack of paper towels. Another glass plate was put on top which supported a heavy weight. Transfer under a heavy weight was completed in 1-2hrs. The Hybond-N membrane was recovered, washed for 2min. in 2xSSC and air dried for an hour. After fixing the DNA upon UV illumination for 5min., the membrane was ready for hybridization.

2.4.12 Dot-blots

Dot-blot analysis was used to compare the number of copies of a gene

in different isolates of parasite, following the method of the Bio-Dot Microfiltration Apparatus Instruction Manual (Bio-Rad). The Bio-Dot Apparatus was assembled according to the manufacturer's instruction. A Hybond-N membrane (Amersham), pre-wetted in 1M ammonium acetate for 30min., was aligned into the apparatus. Vacuum was applied to dry the membrane. The membrane was washed once with 1M ammonium acetate, 100μl/well. Unused wells were closed off with masking tape. DNA diluted in TE was denatured with 1/10 volume of 3M NaOH for 5min. and neutralized with an equal volume of 2M ammonium acetate before it was applied to the wells. After the solution had passed through the filter and the DNA stuck to the membrane, the wells were washed once in 1M ammonium acetate. The membrane was removed from the apparatus and air dried for an hour. The DNA was fixed by UV illumination (5min.) after which the membrane was ready for hybridization.

2.4.13 Labelling of DNA

DNA was labelled with radioactive nucleotides for use as probes in hybridization experiments. Several methods are available for labelling DNA. End-labelling with polynucleotide kinase has been employed with short oligonucleotides, while longer DNA molecules have been labelled by nick translation and random priming methods. DNA subcloned into M13 vectors can be labelled directly from the single-stranded templates.

2.4.13.1 Nick Translation: The method follows essentially the same as described (Rigby *et al.*, 1977) using a nick translation reagent kit (Bethesda Research Lab.). In this reaction 10μCi (1μl) of radioactive labelled nucleotide [α-^{32}P] dCTP or [α-^{32}P] dATP (Amersham) of specific activity 3,000 Ci/mM was used to label 1μg of DNA in a reaction mix containing 5μl of solution A (which contains all nucleotides except the one used in

radioactive form in a suitable reaction buffer) and water to a total volume of 45μl. Nick translation was initiated by adding 5μl of solution C (DNA polymerase I and DNaseI) and proceeded at 15°C for 1hr. Unincorporated nucleotides were separated from the labelled DNA by chromatography on a column of Sephadex G-50 (see Section 2.4.13.3 below). The activity incorporated into the labelled DNA was typically $5x10^7$ cpm/μg.

2.4.13.2 Random Priming Method: DNA labelled to high activities was obtained using the randomly primed DNA labelling method (Feinberg & Vogelstein, 1983). This method enables the labelling of DNA available only in minimal amounts. It is based on the hybridization of a mixture of hexanucleotides to the DNA to be labelled. Many sequence combinations are represented in the hexanucleotide primer mixture, which leads to binding of primer to template in a statistical manner. The complementary strand is synthesized from the 3'OH termini of the random hexanucleotide primer using Klenow enzyme during which radiolabelled dNTP is incorporated into the newly synthesized DNA strand.

A reaction buffer, OLB, containing nucleotides and random primers is required for this method. It is made by mixing 50μl Solution A (1.25M Tris-HCl pH 8.0; 0.125M $MgCl_2$; 25mM β-mercaptoethanol; 0.5mM each of dGTP, dTTP, dCTP/dATP), 125μl Solution B (2M HEPES buffer adjusted to pH 6.6 with NaOH) and 75μl Solution C (random hexanucleotides OD_{260}=90 in TE).

The DNA (100ng and water to 7.5μl) was denatured by boiling for 2min., spun and chilled on ice briefly. After that, 11.4μl of OLB buffer, 2μl of 10mg/ml bovine serum albumin (BSA), 5μl of [α-^{32}P] dATP or [α-^{32}P] dCTP and 1 unit of Klenow (Pharmacia) were added to the denatured DNA at room temperature. The incubation was continued at room temperature overnight. Unincorporated nucleotides were separated from labelled DNA

by chromatography on a Sephadex G-50 column (see Section 2.4.13.3 below).

2.4.13.3 Separation of Unincorporated Nucleotides from Labelled DNA: A Sephadex G-50 (Pharmacia) column was used to remove unincorporated [α-^{32}P] dNTP from labelled DNA fragment (Maniatis *et al.*, 1982). The column was prepared by packing a 1ml syringe with Sephadex G-50 in 10mM Tris pH 7.5, 1mM EDTA, 100mM NaCl with centrifugation (1,500rpm/4min./4°C) until the packed volume was 0.7ml. The Sephadex was washed with 70μl of the same buffer with centrifugation. The labelling mix was applied to the column and eluted into an eppendorf tube by centrifugation. Incorporated counts were monitored by a mini-monitor. The elution was repeated by washing the column with 100μl aliquots of the buffer until insignificant counts were detected in the eluted fractions. Those fractions with high counts were pooled and boiled for 5min. to denature the DNA before they were used as probes in hybridization.

2.4.13.4 Priming from M13 Single-stranded DNA Templates: The M13 17-base sequencing primer (8ng) was boiled with 1μg of the single-stranded template DNA in a mixture containing 1μl of 10x polymerase reaction buffer (10x: 100mM Tris-HCl pH 8.0; 100mM $MgCl_2$; 300mM NaCl) and water in a total volume of 12μl. After 5min., the mixture was cooled at room temperature for 30min. for annealing of primer to the template DNA. At the end of the annealing step, 2μl of dNTP mix (0.4mM each of dGTP, dTTP, dCTP), 1μl of Klenow (1U/μl) and 1μl of [α-^{32}P] dATP were added and incubation was continued at room temperature for 30min. The labelled DNA was used directly in hybridization without further purification by chromatography. Before use, the probe was denatured by boiling for 5min.

2.4.13.5 End-labelling of Oligonucleotides: Oligonucleotide (8pmol) was end-labelled with 3.5μl [γ-^{32}P] dATP in a kinasing reaction containing 2μl 10x kinase buffer (10x: 500mM Tris-HCl pH 7.6; 100mM $MgCl_2$; 50mM DTT;

1mM spermidine; 1mM EDTA), 2μl T4 polynucleotide kinase (5U/μl) and water in a total volume of 20μl. After incubation at 37°C for 1hr, the end-labelled oligonucleotide was used, without further processing or boiling steps, directly in hybridization.

2.4.14 Hybridization of DNA

By adjusting the stringency of hybridization, it is possible to distinguish between closely- and distantly-related members of a family. In practice, to distinguish between the distantly-related members of a family of sequences, hybridization should take place under permissive conditions followed by washing under progressively more stringent conditions. To identify closely-related members, a stringent hybridization followed by a stringent wash is better. The stringency of hybridization depends on a number of factors, the most significant ones are listed below.

Definitions:

*T*m (melting temperature): the temperature at which the strands of a DNA duplex or an RNA.DNA hybrid are half dissociated or denatured. *T*m is dependent on ionic strength, base composition and denaturing agents.

$$Tm = 81.5 + 16.6(\log M) + 0.41(\%G+C) - 0.72(\%\text{ formamide})$$

where M is the molarity of the monovalent cation and (%G+C) is the percentage of guanine and cytosine residues in the DNA.

For oligonucleotides in aqueous 1M Na^+, the relationship of the G+C content on duplex stability is given by the parameter *T*d.

$$Td = 4(G+C) + 2(A+T)$$

T_i = incubation temperature

Temperature: The temperature of reaction affects the rate of hybridization which increases to reach a maximum at 20-25°C below T_m. Self-hybridization is favoured at higher temperature. At low temperatures, a high rate of cross-hybridization is attained. So, ideally, hybridization should be carried out at a T_i that is 20-25°C below T_m. In practice, for well-matched hybrids, the hybridization reaction is usually carried out at 68°C in aqueous solution and at 42°C for solutions containing 50% formamide. For poorly-matched hybrids, incubation is generally at 35-42°C in formamide containing solutions.

Formamide: Formamide can be used to alter the stringency of the reaction conditions. Formamide destabilises double-stranded nucleic acid. Thus, the T_i can be decreased whilst maintaining the stringency of the nucleic acid interaction. By including 30-50% formamide in the hybridization solution, the T_i can be reduced to 30-42°C.

Ionic strength: High salt concentrations stabilize mismatched duplexes, so to detect cross-hybridizing species, the salt concentration of hybridization and washing solutions must be kept fairly high.

Dextran sulphate: Addition of an inert polymer such as dextran sulphate increases the rate of hybridization. The effect is attributed to the exclusion of the DNA from the volume occupied by the polymer, effectively increasing the concentration of the DNA. This favours the formation of concatenates i.e. extensive networks of reassociated probe which by virtue of single-stranded regions, hybridize to filter-bound nucleic acid and so lead to an increase in hybridization signal.

2.4.14.1 Hybridization with Homologous DNA Probes: Hybridization with homologous DNA fragments was performed under stringent conditions

in 0.5M sodium phosphate (Na_2HPO_4/NaH_2PO_4 pH 7.5), 7% SDS at 65°C overnight with a 1hr pre-hybridization under the same conditions. Non-specifically bound nucleotides were removed by washing the membrane stringently in several changes of 0.2xSSC, 0.1%SDS at 65°C over a period of 2hrs. Filters were then autoradiographed.

2.4.14.2 Hybridization with Oligonucleotide Probes: Hybridization with oligonucleotides was performed under a less stringent condition. Pre-hybridization was carried out in 5x Denhardt's solution (100x: 2% BSA-Pentax Fraction V; 2% Ficoll; 2% polyvinylpyrollidone), 0.2%SDS, 6xSSC at 37°C from 2hrs to overnight. Hybridization to end-labelled oligonucleotides was performed under the same conditions overnight. The membrane was initially washed in several changes of 6xSSC at 37°C for 1hr before being autoradiographed. In case of high background, more stringent wash could be carried out in 6xSSC at 57°C or in 0.1xSSC, 0.1%SDS at 37°C for 1hr.

2.4.14.3 Autoradiography: Autoradiography was performed using CRONEX 4 X-ray film in a cassette with lightning plus intensifying screens (Du Pont). The cassettes were stored at -70°C during exposure as this results in a sharper signal.

2.4.14.4 Removal of Probes and Re-use of Blots: The blots could be re-used after dehybridization. This was achieved by boiling in 0.1xSSC for 1hr. The blots were autoradiographed to confirm that dehybridization was complete.

2.4.15 Polymerase Chain Reactions (PCR)

Polymerase chain reactions (PCR) are simple and powerful methods, used to amplify specific DNA segments *in vitro* through a succession of incubation steps at different temperatures (Saiki *et al.*, 1985; 1988). Typically, the double stranded DNA is heat denatured, the two primers complementary

to the 5' and 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. Repetition of this cycle can amplify DNA segments by at least 10^5 fold, and potentially as high as 10^9 fold. The process was made possible by the discovery of a DNA polymerase from *Thermus aquaticus* (*Taq*), which is thermostable.

Taq polymerase was originally purchased from Cetus. More recently, IBI and Boehringer enzymes were also used.

PCR was carried out in 0.5ml eppendorf tubes with the Techne heating block following the thermal profile listed in Table 2.3. Each 100μl reaction contained 100ng genomic DNA, 0.1μM of each 5' and 3' primers, 200μM of each dNTP and 2 units of *Taq* polymerase in a reaction buffer of 0.05M KCl; 0.01M Tris-HCl pH 8.3; 1.5mM $MgCl_2$) . For the Cetus enzyme, the buffer contained gelatin at 0.01%. For the IBI and Boehringer enzymes, the buffer contained gelatin, Tween 20 and NP-40 each at 0.1%. The reactions were overlaid with 100μl paraffin oil. The eppendorf lids were punctured with a needle before subjected to PCR. At the end of the reaction, paraffin oil was removed by extracting with chloroform. The PCR products were typically checked by agarose gel electrophoresis of 5μl samples. The PCR product was purified by gene-clean (Section 2.4.10.2) with or without prior separation by gel electrophoresis.

2.4.16 Preparation of Plating Cells

Plating cells were prepared by diluting an overnight culture of bacteria 1/50 in 5ml L-broth and incubating for 1.5-2.5hrs at 37°C until the cells reached logarithmic phase of growth. The L-broth was supplemented with 1% maltose when plating cells were prepared for bacteriophage λ. They were then collected by centrifugation and resuspended in 1/10 volume 10mM $MgSO_4$.

Table 2.3 The thermal profile for polymerase chain reactions (PCR)

Cycle	No. of Loops	Temperature (°C)	Time
1	1	95	5min.
2	1	92	5min.
		45	10 seconds
		70	1min.
3	1	92	3min.
		40	10 seconds
		70	1min.
4	1	92	1min.
		40	10 seconds
		70	1min.
5	27	92	10 seconds
		40	10 seconds
		70	1min.
6	1	70	5min.
7		30	hold

2.4.17 Lambda Phage Titration

The phage stock to be titrated was serially diluted in phage buffer. To 100μl of each dilution was added 100μl of plating cells and 3ml molten BBL Top Agar. The whole mixture was poured onto a BBL-agar plate. The plates were incubated at 37°C overnight.

2.4.18 Preparation of Competent Cells

An overnight culture of bacteria was diluted 1/50 in L-broth. Incubation was continued at 37°C with shaking until the culture had reached logarithmic phase of growth, normally 2hrs. The bacterial cells were pelleted (1,500rpm/15min./4°C) and resuspended in ice-cold 0.1M $MgCl_2$ to half of the original volume. The cells were pelleted once more (1,500rpm/15min./4°C), resuspended in ice-cold 0.1M $CaCl_2$ to 1/20 of the original volume. The calcium treated cells were put on ice for 30min. or longer before use.

2.4.19 Ligation of DNA

Both insert and vector DNA were cut with suitable restriction enzymes to create competent clonable sites. Ligation of insert to the vector was performed in a ratio of 3 molecules of insert DNA to 1 molecule of vector DNA in a 10μl reaction mixture containing 50mM Tris-HCl pH 7.5; 10mM $MgCl_2$; 10mM DTT; 10mM ATP and 1 unit of T4 DNA ligase at 15°C overnight. The ligated DNA was transformed into *E. coli* or *in vitro* packaged into λ phage.

2.4.20 Plaque Hybridization

Plaques were lifted by overlaying a pre-chilled agar plate with Hybond-N membrane for 2min. The orientation of the filters in respect to the agar

plates was marked. The DNA was denatured by putting the membrane, plaque-side-up, onto filter papers saturated with denaturation solution (1.5M NaCl, 0.5M NaOH) for 5min. and then neutralized by placing on filters saturated with neutralizing solution (1.5M NaCl, 0.5M Tris-HCl pH8.0) for a further 5min. The filters were finally transferred to filter papers saturated with 2xSSC for 5min. After drying at 37°C for 5min., the DNA was fixed onto the Hybond-N membrane by UV cross-linking. The membranes were now ready for pre-hybridization and hybridization (Section 2.4.14). Positive signals were identified and corresponding regions of plates were picked into phage buffer.

2.4.21 Colony Hybridization

Colonies were streaked onto a master L-amp plate and then duplicated onto a Hybond-N membrane overlaid a L-amp plate. The cells were allowed to grow for at least 6hrs to overnight. The filters were removed and processed as for plaque hybridization (Section 2.4.20). Pre-hybridization and hybridization were carried out under stringent conditions (Section 2.4.14). Positive signals were identified and corresponding regions of plates were picked into L-broth.

2.4.22 Identification of Recombinants

To identify recombinants from those putative positive clones, DNA was prepared from them by the mini-prep method (Section 2.4.5.2, Section 2.4.6.2 & Section 2.4.7.2). The DNA was digested with the appropriate enzyme to release the subcloned inserts. The sizes of the inserts were checked by agarose gel electrophoresis (Section 2.4.9) and the specificity of the inserts was checked by hybridization of a Southern blot (Section 2.4.11) with an appropriate probe (Section 2.4.14).

2.4.23 Construction of Genomic Library

A library of *Eco*RI fragments of HB3 DNA was constructed for the screening of recombinants containing the DHFR-TS inserts. Bacteriophage λNM1149 (Murray, 1983) was used as the cloning vector. The genetic map of λNM1149 is shown in Fig. 2.1. The vector contains both *Eco*RI and *Hin*dIII cloning sites though only the *Eco*RI site was used in this study. The strategy used to construct such libraries is illustrated in Fig. 2.2. The genomic DNA and the vector DNA were cleaved by *Eco*RI to generate compatible cloning sites for ligation to form a concatameric, recombinant DNA. Ligation was performed as in Section 2.4.19 using 0.5μg *Eco*RI cleaved HB3 genomic DNA and 1μg *Eco*RI cleaved λNM1149 DNA in a total volume of 20μl. After that, the phage particles were packaged *in vitro* during which a different recombinant DNA molecule was inserted into each bacteriophage particle. Phage infected *E. coli* cell extracts were used to supply the mixture of proteins and precursors required for encapsidating λ DNA. A sonicated extract from *E. coli* BHB2690 (SE) served as the prohead donor while a freeze-thaw lysate from *E. coli* BHB2688 (FTL) served as the packaging protein donor. Both SE and FTL were kindly provided by N. Somia. *In vitro* packaging was carried out by incubating 6μl of ligation mix with 7μl of buffer A (20mM Tris-HCl pH 8.0; 3mM $MgCl_2$; 0.05% β-mercaptoethanol; 1mM EDTA), 1μl of M1 buffer (110μl water; 6μl 500mM Tris-HCl pH 7.5; 300μl 50mM spermidine; 100mM putriscine 7.0; 9μl 1M $MgCl_2$; 75μl 100mM ATP; 1μl β-mercaptoethanol), 10μl of SE and 12μl of FTL at room temperature for 1hr. All the reagents were kept on ice before the packaging reaction started. After 1hr, the packaging mix was diluted with 200μl of phage buffer. For transfection into *E. coli*, aliquots (1μl and 10μl) of the diluted packaging mix were incubated with 100μl of NM494 plating cells at 37°C for 20min. The infected cells were then mixed with 9ml molten BBL top agar and plated

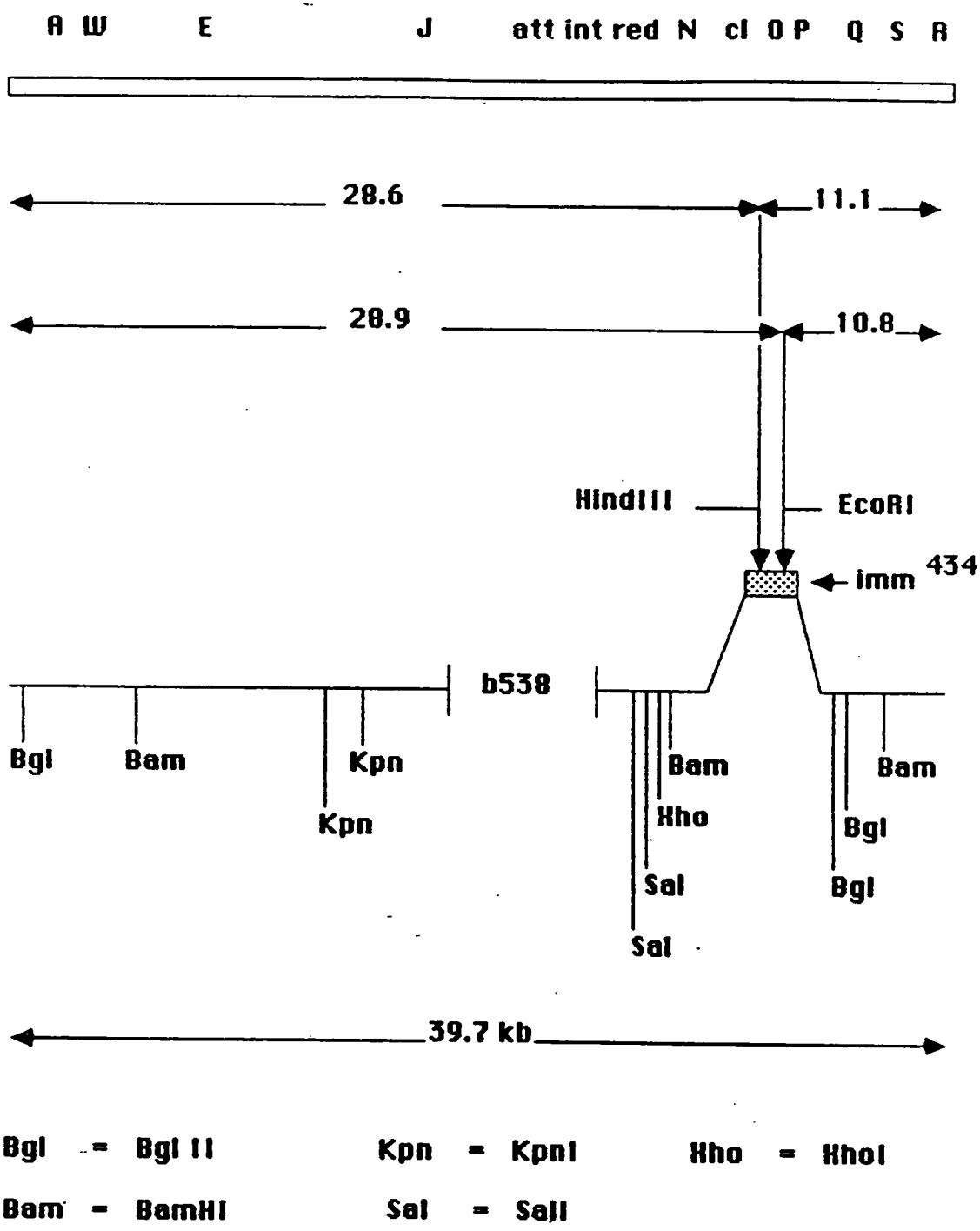
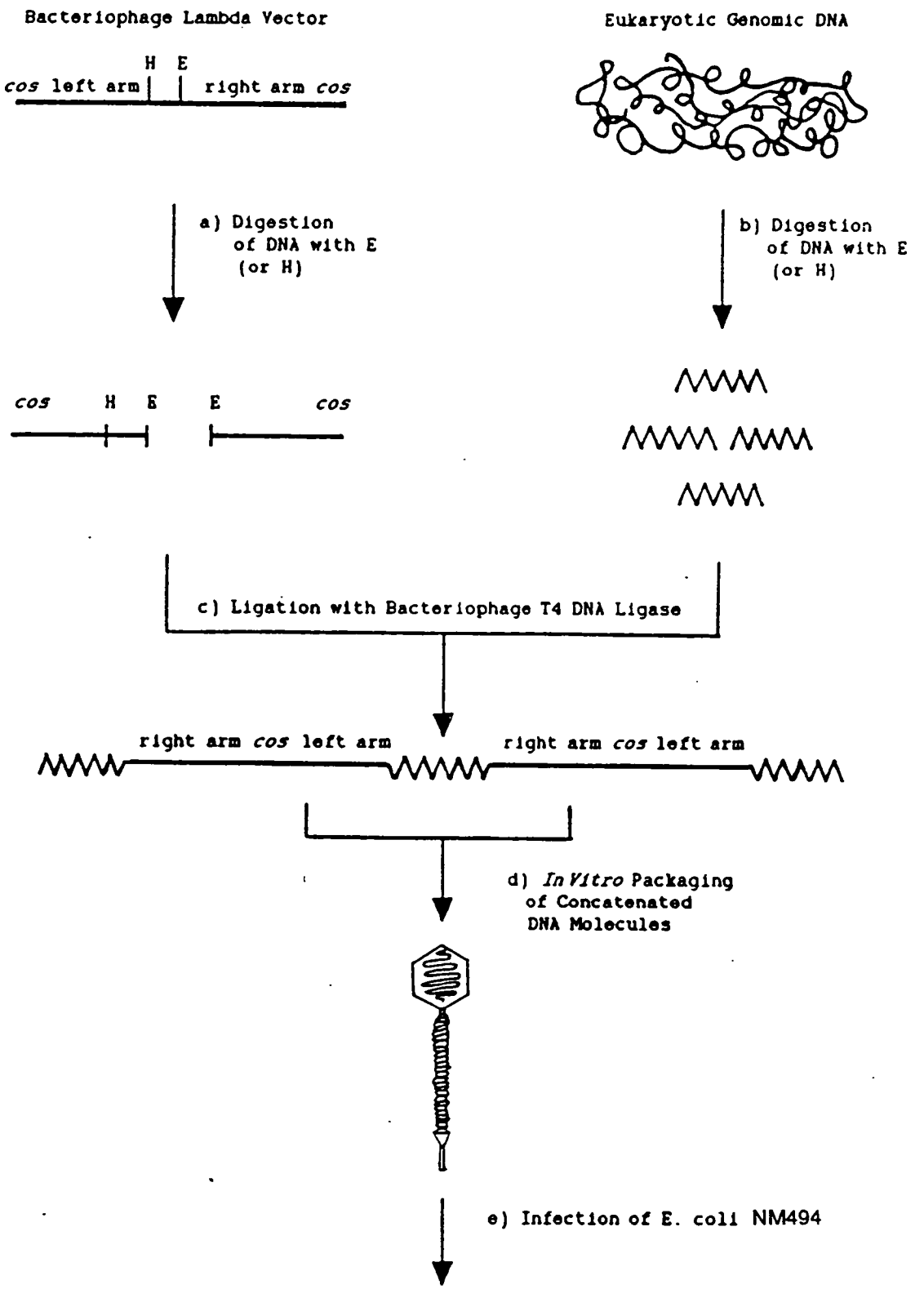


Fig. *2.1* A restriction enzyme map of Lambda NM1149 vector DNA illustrating the unique HindIII and EcoRI cloning sites.

FIG 2.2 The strategy used to construct libraries of random fragments of eukaryotic DNA. Left: preparation of the vector DNA fragments. Right: preparation of eukaryotic DNA fragments. A concatemeric recombinant DNA molecule is produced by the action of bacteriophage T4 DNA ligase. This concatemer is the substrate for the *in vitro* packaging reaction during which a different recombinant DNA molecule is inserted into each bacteriophage lambda particle. Following amplification by growth in *E. coli*, a lysate is obtained, consisting of a library of recombinant clones that, in aggregate, contain most of the sequences present in the eukaryotic genome. H=*Hin*dIII; E=*Eco*RI. The figure represents digestions with *Eco*RI.


Bacteriophage Lambda Vector
H E
cos left arm
right arm cos
Eukaryotic Genomic DNA
a) Digestion of DNA with E (or H)
b) Digestion of DNA with E (or H)
cos H E E cos
c) Ligation with Bacteriophage T4 DNA Ligase
right arm cos left arm
right arm cos left arm
d) In Vitro Packaging of Concatenated DNA Molecules
e) Infection of E. coli NM494

out on BBL-agar plate at a density of >10,000 plaques per 150-mm dish for primary screening (see below). Controls included packaging self re-ligated vectors and uncut vectors.

2.4.23.1 Amplification and Storage of Library: The plates containing the recombinants were overlaid with 12ml of phage buffer and stored at 4°C overnight. The bacteriophage suspension was recovered from each plate and transferred to a sterile polypropylene tube. The plates were then further rinsed with 4ml of phage buffer. After incubation for 15min. at room temperature with occasional shaking, the cells and agar debris were removed by centrifugation (3,500rpm/5min./4°C). The supernatant was collected into a glass bottle. A few drops of chloroform were added and the library was stored at 4°C.

2.4.24 Screening of Library

The library was screened by plaque hybridization (Section 2.4.20) with a homologous 5.65kb DHFR-TS gene fragment from the K1 isolate which had beeen cloned into the *Eco*RI sites of λNM1149 (Snewin *et al.*, 1989). To isolate the insert DNA for use as a probe, the phage DNA was digested with *Eco*RI and fractionated by agarose gel electrophoresis (Section 2.4.9.2). The insert DNA was purified from the gel (Section 2.4.10) and labelled (Section 2.4.13) for hybridization. However, some of the vector DNA might have co-purified with the insert DNA. This would give rise to cross-hybridization between the vector DNA resulting in a high background. This was prevented by pre-hybridizing the filters with 10μg of sheared λ DNA at 65°C for at least 4hrs before hybridization with the probe. The sheared λ DNA bound to the λ sequence in the library, thus blocking it from hybridizing to the vector DNA within the probe. Hybridization was then carried out under stringent conditions as described in Section 2.4.14.1. Plaques giving rise to positive

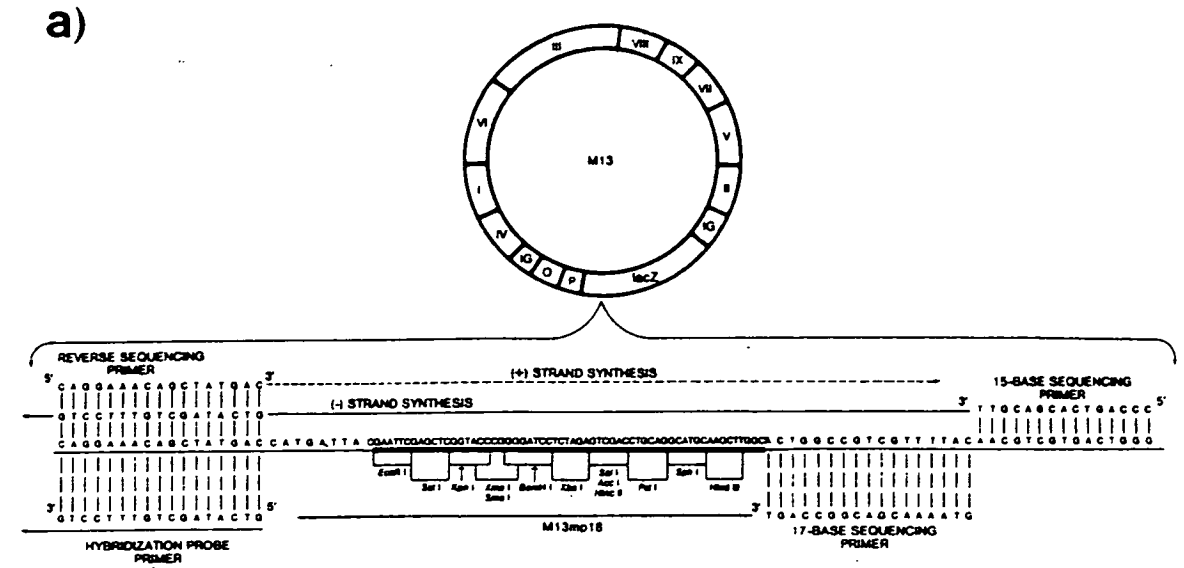
signals were picked and re-screened until plaque pure. Clones remaining positive were further characterized as in Section 2.4.22.

2.4.24.1 Preparation of Stock Culture of λ Recombinant Clones: Individual positive clones were picked, diluted and plated out on fresh 90-mm BBL-plate at near confluent lysis. Top agar from fresh BBL plate was transferred to 2.5ml phage buffer and left to extract for several hours with occasional shaking. The agar and cell debris were spun down by centrifugation at 3,500rpm/10min. A drop of chloroform was added to the supernatant which was stored at 4°C as stock.

2.4.25 Subcloning of DNA Fragments into M13 Vectors

The multiple cloning sites of the M13 vectors are depicted in Fig. 2.3. Insert DNA was subcloned into the multiple cloning sites, which have been inserted into the 5' end of the *lac*Z gene in M13. Recombinant phage with a lac$^-$ phenotype due to the insertion of DNA fragment into the 5' end of *lac*Z were selected as white plaques on indicator plates.

Both insert and vector DNA were cut with suitable restriction enzyme to create competent clonable sites. Ligation of insert to the replicative forms of M13mp18 and M13mp19 was performed as described in Section 2.4.19. The ligated DNA was transfected into competent *E. coli* cells. This was done by mixing aliquots (1μl and 0.5μl) of the ligation mix with 100μl of competent TG1 cells (Section 2.4.18). The preparation was left on ice for 30min., heat-shocked at 42°C for 2min. and then left on ice for another 30min. After that, 100μl of TG1 plating cells (Section 2.4.16), 20μl of 2% X-gal, 20μl of 100mM IPTG and 3ml of molten BBL Top Agar were added and the whole mixture was plated out on minimal plates. The plates were incubated at 37°C overnight. White plaques were picked onto a master plate which was then incubated at 37°C overnight to allow growth of the recombinants. Plaque lifts



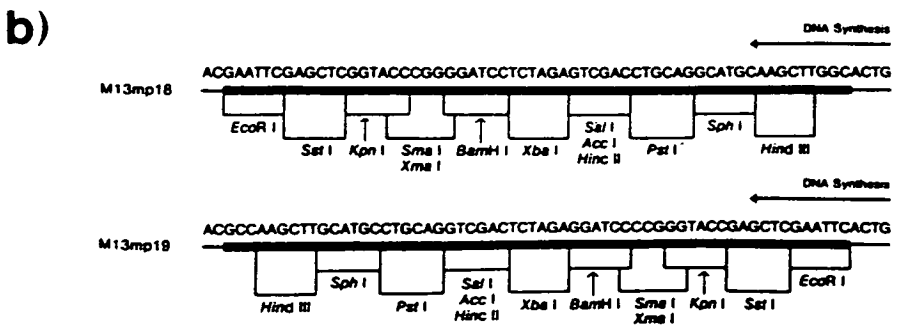


FIG. 2.3 Multiple cloning sites of M13 vectors. a) illustrates the position of the multiple cloning sites of M13mp18 in respect to the *lac*Z gene and the sequences and locations of the sequencing primers. b) shows the sequences of the multiple cloning sites in M13mp18 and M13mp19.

obtained from the master plate (Section 2.4.20) were then screened by probing with appropriate fragments. Putative positive clones were identified as described in Section 2.4.22.

2.4.26 Subcloning of DNA Fragments into pUBS1

The multiple cloning sites of pUBS1 are shown in Fig. 2.4. DNA fragments were subcloned into the multiple cloning sites of pUBS1 by transformation. Since pUBS1 does not contain a *lacZ* gene, no colour selection with X-gal was available. Screening for positive clones was therefore carried out by colony hybridization (Section 2.4.21).

After cutting the DNA fragments and the plasmid pUBS1 with appropriate restriction enzymes to generate compatible cloning sites, they were ligated as described in Section 2.4.19. Aliquots (1μl) of the ligation mix were mixed with 100μl of competent JM83 cells (see Section 2.4.18), kept on ice for 30min., heat-shocked at 42°C for 5min. To this was added 0.5ml L-broth and incubation was continued with rotation at 37°C for 1-2hrs. Different dilutions (100μl/50μl/20μl) of the mixture were spreaded onto L-amp plates (L-plate supplemented with 50μg/ml ampicillin) which were then incubated at 37°C overnight to allow the bacteria to grow. Positive clones were screened (Section 2.4.22.1) and identified (Section 2.4.22.2).

2.4.27 DNA Sequencing

DNA sequencing was performed with either M13 templates, plasmid DNA, λ DNA or PCR amplified fragments using a protocol according to the dideoxynucleotide chain termination principle of Sanger (Sanger *et al.*, 1977). Sequencing in M13 vectors requires cloning the DNA fragments into the M13mp series of phage vectors (see Section 2.4.25) from which the single-stranded DNA can be prepared (Messing *et al.*, 1981) which is the template

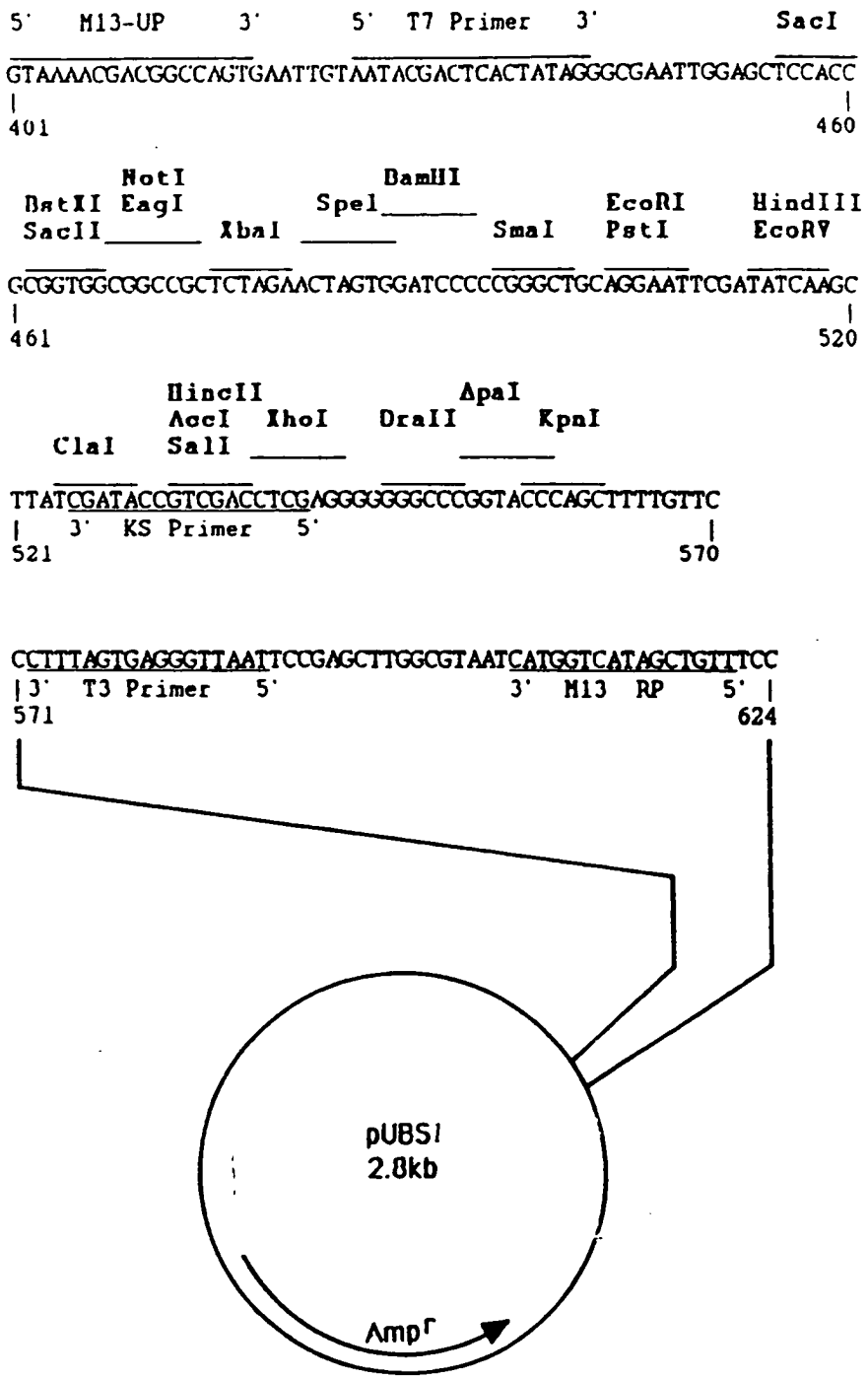


FIG 2.4 pUBS1 plasmid multiple cloning sites. M13-UP, T7 Primer, KS Primer, T3 Primer and M13 RP are oligonucleotide primers commonly used in sequencing reactions.

for DNA sequencing. For the others, the double-stranded DNA is the template for sequencing.

2.4.27.1. Preparation of Single-stranded M13 Templates: A 10μl aliquot of phage stock were added to 5ml L-broth containing 100μl of an overnight culture of TG1 cells. After shaking at 37°C for 4-6hrs, the cells were pelleted (microfuge, 5min.). To 1.5ml of the supernatant was added 200μl of PN (20% polyethylene glycol 6000, 2.5M NaCl) which precipitated the virus. After 15min. at room temperature, the phage were pelleted by spinning in a microfuge for 5min and the PN solution removed. The phage pellet was resuspended in 125μl TES (20mM Tris-HCl pH 7.5; 10mM NaCl; 0.1mM EDTA). The suspension was extracted once with 50μl phenol and once with phenol: chloroform: isoamyl alcohol (25:24:1). The aqueous phase was collected and the DNA was precipitated by adding 10μl of 3M sodium acetate pH4.5 and 275μl of absolute ethanol. After centrifugation (microfuge/20min.) and washing once in 1ml 95% ethanol, the DNA pellet was dried and resuspended in 40μl TE. The single-stranded DNA template preparations were stored at -20°C until required.

2.4.27.2 Sequencing Reactions Using Single-stranded M13 DNA as Templates: Single-stranded M13 clones were sequenced following the dideoxy chain termination protocols supplied by the United States Biochemical Corporation (USB) with modifications. An annealing mixture containing 2μl 5x sequencing buffer (5x: 200mM Tris-HCl pH 7.5; 100mM $MgCl_2$; 250mM NaCl), 1μl primer (0.5pmol) and 7μl template DNA was incubated at 55°C for 5min. and then put on ice for another 5min. to allow annealing of the primer to the DNA. Complementary DNA strand was synthesized and labelled by adding 1μl of 0.1M DTT, 2μl 1x labelling mix (1.5μM each of dGTP/dCTP/dTTP), 0.5μl [α-^{32}S] dATP and 2μl 2 units of T7 DNA polymerase (Sequenase™, USB) to the annealed DNA mixture. After

incubation at room temperature for 5min., 3.5μl of labelling reaction were transferred to pre-warmed termination tubes containing 2.5μl of G/A/T/C termination mixes (Table 2.4) respectively. Incubation of the termination reaction continued at 37°C for 5min. before 4μl of stop solution (95% formamide; 20mM EDTA; 0.05% bromophenol blue; 0.05% xylene cyanol FF) was added to stop the reaction. The samples were heated to 75°C for 2min. immediately before loading.

2.4.27.3 Sequencing Reactions Using Double-stranded DNA as Templates: Sequencing from double-stranded DNA templates followed essentially the same method of single-stranded sequencing except that it was performed in the presence of 10% dimethylsulphoxide (DMSO) to keep DNA denatured. This method has been successfully employed to sequence from plasmid, λ and PCR products in the present study.

An annealing mixture containing 2μl 5x sequencing buffer (5x: 200mM Tris-HCl pH 7.5; 100mM $MgCl_2$; 250mM NaCl), 1μl (8-10ng) primer, 6μl DNA and 1μl DMSO was boiled for 5min. to denature the DNA and then put on ice for 10min. to allow annealing of the primer to the denatured single-stranded templates. To this was added 1μl 0.1M DTT, 1μl labelling mix (0.375μM each of dGTP/dTTP/dCTP), 1μl (10μCi) [α-^{32}S] dATP and 1μl 2 units Sequenase^{TM} (USB). Immediately after that, 3.5μl were transferred to pre-warmed tubes containing 2μl G/A/T/C termination mixes respectively (Table 2.5). Incubation was continued at 37°C for 5min. before 4μl of stop solution was added. The samples were boiled for 5min. immediately before loading onto a 6% denaturing polyacrylamide gel for separation.

2.4.27.4 Primers: The primers used in single-stranded and double-stranded sequencing reactions were either the M13 sequencing primers or synthetic oligonucleotide primers synthesized by Oswell DNA Service, Department of Chemistry, University of Edinburgh.

Table 2.4 The composition of the termination mixes used in single-stranded sequencing reactions

Component	G	A	T	C
ddGTP	8μM	-	-	-
ddATP	-	8μM	-	-
ddTTP	-	-	8μM	-
ddCTP	-	-	-	8μM
dGTP	80μM	80μM	80μM	80μM
dATP	80μM	80μM	80μM	80μM
dTTP	80μM	80μM	80μM	80μM
dCTP	80μM	80μM	80μM	80μM
NaCl	50mM	50mM	50mM	50mM

Table 2.5 The composition of the termination mixes used in double-stranded sequencing reactions

Component	G	A	T	C
ddGTP	8μM	-	-	-
ddATP	-	8μM	-	-
ddTTP	-	-	8μM	-
ddCTP	-	-	-	8μM
dGTP	80μM	80μM	80μM	80μM
dATP	80μM	80μM	80μM	80μM
dTTP	80μM	80μM	80μM	80μM
dCTP	80μM	80μM	80μM	80μM
NaCl	50mM	50mM	50mM	50mM
DMSO	10%	10%	10%	10%

2.4.27.5 Sequencing Gel Electrophoresis: Sequencing reactions were separated on a 6% polyacrylamide gel (38cmx 17cmx 0.3mm) which was prepared by dissolving 28.8g urea, 3.6g acrylamide, 0.18g bis-acrylamide in 28ml distilled water and 6ml 10x TBE. Polymerization was initiated by addition of 360μl 10% ammonium persulfate and 50μl TEMED (NNN'N-tetra-methyl-1,2-diamino-ethane) immediately before pouring the gel. The gel was allowed to set for 2hrs to overnight. Sequencing reaction samples were loaded onto wells formed by a Sharkstooth comb. Electrophoresis was carried out in 1x TBE at 40W constant power. The gel was fixed in 10% acetic acid, 12% methanol for 15min. and dried under vacuum at 80°C before being exposed for autoradiography.

2.4.27.6 DNA Sequence Data Compilation and Analysis: Sequence information was read off the autoradiograph and compiled by version 2.0 of the programmes of Staden (1984) and analysed by version 4.0 of the programmes of the University of Wisconsin Genetic Computer Group (Devereux *et al.*, 1984).

2.4.28 Alkaline Denaturing Gels

Alkaline denaturing gels are usually used to check the size of the DNA strand in nuclease-S1-resistant DNA.RNA hybrids and the size of first and second DNA strands synthesized by reverse transcriptase etc. (Maniatis *et al.*, 1982). This method was employed in this study to check the actual size of denatured DNA fragments which presumably had a secondary structure.

The DNA fragments were dephosphorylated, end-labelled with [γ-^{32}P] dATP and fractionated on an alkaline denaturing gel.

2.4.28.1 Dephosphorylation of DNA Fragment: The terminal 5' phosphates were removed from DNA by treatment with 1μl of calf intestinal alkaline phosphatase (CAP) (Boehringer) in a reaction mixture containing 5μl

10x CAP buffer (10x: 0.5M Tris-HCl pH 9.0; 10mM $MgCl_2$; 1mM $ZnCl_2$; 10mM spermidine) and distilled water in a total volume of 49μl. After successive incubation at 37°C for 15min. and 56°C for 15min., another 1μl of CAP was added and the incubation cycle was repeated. The reaction was terminated by inactivating the enzyme at 68°C for 15min. in a solution containing 10μl of 10xSTE (10x: 100mM Tris-HCl pH 8.0; 1M NaCl; 10mM EDTA) and 2.5μl of 20% SDS and distilled water in a total volume of 100μl. The dephosphorylated DNA was purified using the gene-clean procedure (Section 2.4.10.2) and extracted into 10μl of TE.

2.4.28.2 End-labelling of Dephosphorylated DNA: The dephosphorylated DNA was end-labelled as described in Section 2.4.13.5. The labelling mix contained 10μl of dephosphorylated DNA, 2μl of kinase buffer, 2.5μl of distilled water, 3.5μl of [γ-^{32}P] dATP and 2μl of kinase (5U/μl). After incubation at 37°C for 1hr, the labelled DNA was purified using the gene-clean procedure (Section 2.4.10.2) and extracted into 20μl of TE.

2.4.28.3 Alkaline Denaturing Gel: The gel was prepared as 1.2% agarose in a neutral, unbuffered solution (50mM NaCl, 1mM EDTA) and then equilibrated in alkaline electrophoresis buffer (30mM NaOH, 1mM EDTA) for at least 30min. Excess buffer was removed to leave sufficient buffer to cover the gel to a depth of 1mm. DNA samples were mixed with 1/10 volume of 10x alkaline loading buffer (10x: 500mM NaOH, 10mM EDTA, 25% Ficoll type 400 (Pharmacia), 0.25% bromocresol purple) before being loaded onto the gel. Electrophoresis was carried out at 60V. After the dye had migrated out of the loading slot, a glass plate was put on top of the gel to prevent diffusion of the dye into the buffer. After 7hrs, the gel was recovered and processed by soaking in 7% trichloroacetic acid for 30min. at room temperature. The gel was dried for several hours under stacks of filter

papers and paper towels and a heavy weight and was autoradiographed.

2.5 RNA METHODS

All solutions for RNA works were prepared in diethylpyrocarbonate (DEPC)-treated water. DEPC was added to distilled water at a final concentration of 0.1% to remove contaminants which would otherwise degrade RNA. After 30min., the water was autoclaved twice to destroy the DEPC.

2.5.1 Preparation of RNA from P. falciparum

RNA was prepared according to Chomczynski & Sacchi (1987) from 40ml packed red blood cells obtained from 500ml of an asynchronized culture. The parasites were collected by saponin lysing the infected red blood cells as described in Section 2.2.4. Aliquots of the parasites (0.7ml) were transferred to an eppendorf tube and lysed in 0.5ml of solution D containing 4M guanidinium thiocyanate, 25mM sodium citrate, 0.5% Sarcosyl and 0.1M β-mercaptoethanol. To this was added 50μl 2M sodium acetate pH4.0, 0.5ml water-saturated phenol and 0.1ml chloroform:isoamyl alcohol (49:1) sequentially. After vortexing the contents in the eppendorf tube for 10 seconds, the lysate was left on ice for 15min. before centrifugation in a microfuge (20min./4°C). The aqueous phase was collected and the nucleic acids were precipitated with 0.5ml isopropanol at -20°C for at least 1hr. The pellet obtained (microfuge/20min./4°C) was resuspended in 0.3ml solution D and the RNA was re-precipitated with 0.3ml of isopropanol at -20°C for 1hr followed by centrifugation (microfuge/20min./4°C). The RNA pellet was washed once with 70% ethanol, dried and resuspended in 50μl water.

2.5.2 Separation of Poly A^+ RNA from Poly A^- RNA

Poly A^+ RNA can be separated from poly A^- RNA by passing through a column of oligo-(dT)-cellulose during which poly A^+ RNA will be adsorbed onto the oligo-(dT) substrate.

A column was rinsed with autoclaved water supplemented with 0.1% DEPC and then DEPC-treated water before packing with 0.5ml oligo-(dT)-cellulose. The cellulose was washed with 2.5ml of 0.1M NaOH and 5ml of application buffer (10mM Tris pH7.5, 1mM EDTA, 0.5M NaCl). Total RNA was made up to 1ml with 1xTE. After incubation at 65°C for 5min., the preparation was put on ice and 1ml of 1M NaCl was added. The mixture was then applied to the column at room temperature. The eluate was re-applied to the column which was then washed with 2.5ml application buffer. The poly A^- RNA was collected. The bound poly A^+ RNA was eluted into 100μl-200μl fractions with 1.5ml TE. Fractions containing RNA were checked by examining a mixture of 2μl from each fraction, 8μl water and 1μl (20μg/ml) ethidium bromide on Saran-wrap upon UV illumination. This purification step was then repeated. The eluate was pooled, incubated at 65°C for 5min., and then chilled on ice. To this was added an equal volume of 1M NaCl. The whole preparation was recycled through the column once. The poly A^- RNA and the poly A^+ RNA were collected as before.

2.5.3 Measurement of RNA Concentrations

The quantity of RNA was measured spectrophotometrically at wavelengths of 260nm and 280nm as in Section 2.4.3. An $OD_{260}=1$ was equivalent to about 40μg/ml RNA. A value of 2.0 for OD_{260}/OD_{280} indicates pure preparations of RNA.

2.5.4 PCR from RNA

The demonstration of the ability of *Taq* polymerase to directly transcribe RNA templates *in vitro* (Tse & Forget, 1990) makes it more convenient to amplify the RNA transcripts using the same procedure as DNA PCR (Section 2.4.15). Instead of DNA, 4μg of total RNA were used as templates. To remove contaminating DNA, the RNA was first treated with 1μl of RNase-free DNase (Boehringer) at 37°C for 1hr. The DNase was then denatured by boiling for 10min. before subjected to PCR.

A control was included in which a portion of the DNase-treated RNA sample was further treated with RNase at 37°C for 1hr before subjected to PCR. Since all RNA would be destroyed in this procedure, no fragments should be amplified from the samples. This would confirm that in previous experiments, the signals came from RNA but not contaminating DNA.

2.5.5 Primer Extension

This technique was used to map the 5' termini of mRNA. It involves hybridization of the test RNA with a synthetic oligonucleotide primer radiolabelled at its 5' end by phosphorylation, extension of the primer by reverse transcriptase to produce a cDNA which was analysed by denaturing polyacrylamide gel electrophoresis.

The oligonucleotide was labelled at its 5' end in a reaction mixture containing 1μl (20pmol) of oligonucleotide, 20μCi [γ-^{32}P] dATP, 3μl 10x kinase buffer (10x: 500mM Tris-HCl pH 8.0, 100mM $MgCl_2$), 3μl 100mM DTT, 2μl distilled water and 0.3μl T4 polynucleotide kinase (5units/μl). The labelling reaction proceeded at 37°C for 15min. and then terminated at 70°C for 10min.

Total RNA (30μg) was mixed with 0.5μl-2μl of the labelled oligonucleotide primer mix (from above) and hybridization mix (50mM Tris-HCl pH 7.5, 7.5mM KCl, 3mM $MgCl_2$) in a total volume of 30μl. The sample was incubated for 30min. at 5°C below the calculated *Td* (for definition see Section 2.4.14) of the oligonucleotide and then put on ice for 15-30min. To this was added 1μl 1M Tris-HCl pH 7.5, 1.5μl 1M KCl, 0.5μl 0.2M $MgCl_2$, 5μl 0.1M DTT, 2.5μl dNTP mix (10mM each), 1μl BSA (5mg/ml) and 7.5μl distilled water. Extension of the primer was initiated by adding 1-2μl (200U/μl) M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase (Bethesda Res. Lab.) and proceeded at 37°C for 60min. The reaction was terminated by boiling in 12.5μl 0.5M NaOH for 3min. and then transferred to ice. After neutralizing with 12.5μl of 0.5M HCl, 12.5μl of 1M Tris-HCl pH 7.4 were added and the nucleic acids were precipitated with 2 volumes of ethanol at -20°C for at least 30min. The pellet obtained after centrifugation (microfuge/15min./4°C) was washed with 66% ethanol. The nucleic acids were re-precipitated and vacuum dried. After being resuspended in 5μl of 2x urea loading buffer (2x: 10M urea, 1xTBE, 0.2% bromophenol blue, 0.2% xylene cyanol) and boiled for 5min., the sample was subjected to denaturing polyacrylamide gel electrophoresis as described in Section 2.4.27.5 except that a pre-run at 40W for 30min. was required to heat up the gel.

2.5.6 Northern Blotting

mRNA transcripts were analysed by Northern hybridization. Total RNA from *P. falciparum* was fractionated under denaturing conditions using an agarose-formaldehyde gel. A 1.2% agarose gel was made up by dissolving 1.8g of agarose (Sigma) in 117ml DEPC-treated water and then cooled to 50°C. To this was added 30ml of 5x MOPS buffer (5x: 41.8g MOPS, 6.8g sodium acetate, 10ml 500mM EDTA; pH to 7 with 5M NaOH in 1 litre final

volume), 3ml formaldehyde and 7.5μl ethidium bromide (10mg/ml). The gel was allowed to set in a fume cupboard and then in cold room for at least 15min.

Total RNA (30μg) was made up to 10.1μl with water. To this was added 4.5μl 5x MOPS, 7.9μl 37% formaldehyde and 22.5μl deionized formamide. The sample was denatured at 55°C for 15min. After a brief chill on ice and the addition of 5μl of formamide/EDTA/dye mix (95% formamide; 20mM EDTA pH 8.0; 0.05% bromophenol blue; 0.05% xylene cyanol), the sample was run at 100V for 2-3hrs in 1x MOPS buffer. Markers used were *E. coli* ribosomal RNA subunits 16S (1.7kb) and 23S (3.5kb) and *P. falciparum* ribosomal RNA subunits of sizes 2.1kb and 3.7kb.

The gel was recovered and photographed under short wave UV light. The gel was rinsed 2 times with 10x SSC for 20min. and transferred to Hybond-N filter in 10x SSC overnight as shown in Fig. 2.5. After transfer the filter was recovered and rinsed 2 times in 2x SSC, air dried and baked at 80°C under vacuum for 2hrs.

Pre-hybridization and hybridization were performed as described in Section 2.4.13.1 using labelled DNA as probe. Unbound labelled DNA fragments were removed by washing the filters in 0.1x SSC, 0.1% SDS at room temperature for 15min. and then 0.2x SSC, 0.1% SDS at 65°C for 30min. before being autoradiographed.

For re-use of the filters, the bound probe could be removed by washing the filters in 5mM Tris pH 8.0, 2mM EDTA at 65°C for 2hrs.

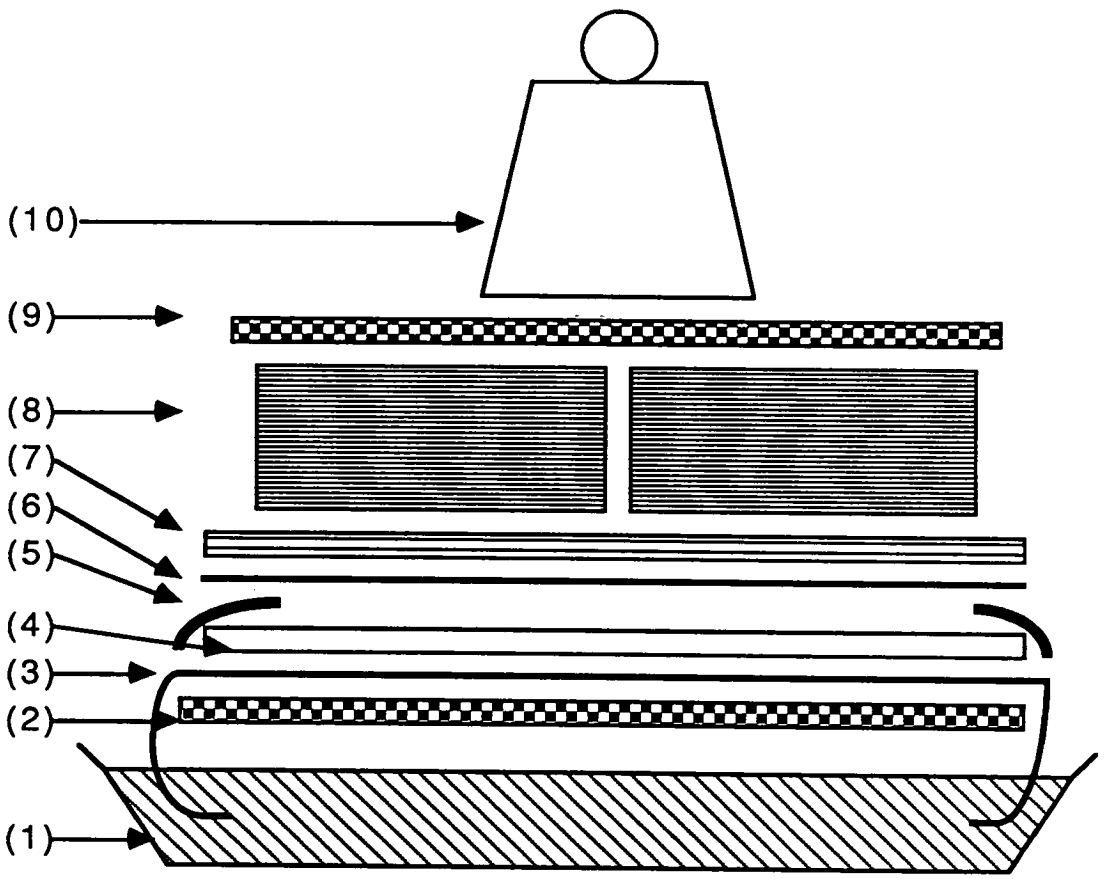


Fig. 2.5 Cross section of a Northern transfer apparatus. (1) tray filled with 10x SSC, (2) glass plate (supported by two sides of the tray), (3) wick of three sheets of Whatman 3MM paper, (4) gel, (5) Parafilm round all sides of gel, (6) Nylon membrane (Hybond-N), (7) three sheets of Whatman 3MM paper, (8) paper towels, (9) glass plate, (10) weight.

2.6 PROTEIN METHODS

2.6.1 Protein Preparation

Total protein was extracted from synchronized cultures of *P. falciparum* according to Inselburg *et al.* (1987) with modifications. A 500ml asynchronized culture containing 40ml infected red blood cells was sorbitol synchronized as in Section 2.2.7. Parasites were then harvested 72hrs after sorbitol synchronization. The number of parasites/ml of red blood cells was determined from the parasitaemia and the haematocrit. These data were used to calculate the final concentration of parasites to be resuspended in buffer. The parasitized red blood cells were washed and resuspended in 40ml of phosphate buffered saline (PBS) and lysed with 0.15% saponin at room temperature for 10min. The pellet, obtained after centrifugation at 3,500rpm for 15min. (4°C) in a Chillspin swingout rotor, was washed 3 times in cold PBS pH7.4. The parasites were resuspended at a concentration of $1x10^9$ parasites/ml TEK (50mM Tris-HCl pH7.4, 1mM EDTA, 0.3M KCl) in the presence of protein inhibitors (10mM benzamidine, 20μg/ml leupeptin, 20μg/ml pepstatin, 50μg/ml aprotinin, 50μg/ml soybean trypsin inhibitor). Parasite protein was extracted by sonication at full power with five 10 seconds bursts with 1min. cooling in between. After spinning down the debris at 15,000rpm in a Sorvall SS-34 rotor for 30min. at 4°C, the supernatant containing parasite protein was collected and stored at -70°C as source of parasite enzyme.

2.6.2 Estimation of Protein Concentration (Bradford's Method)

Bradford's reagent was made up by dissolving 100mg Coomassie Brilliant Blue G-250 (Bio-Rad) in 50ml 95% ethanol which was stirred until dye completely dissolved (Bradford, 1976). Then, 100ml 85% (w/v)

phosphoric acid were added and made up to 1 litre with distilled water.

The test protein (100μl) was mixed with 5ml of Bradford's reagent. Protein standards of 12.5μg-125μg/100μl bovine albumin were used for calibration. The absorbance at wavelength 595nm was read between 2-60min. after mixing against a blank of buffered reagent. A standard curve was plotted from the readings of the protein standards. Protein concentrations of the parasite preparations were determined from the standard curve.

2.6.3 Thymidylate Synthetase (TS) Assay

Expression of the dihydrofolate reductase (DHFR) gene can be measured by quantifying the thymidylate synthetase (TS), since DHFR and TS are translated into a single polypeptide chain. Thus, measurement of the TS will reveal the number of DHFR molecules in the cell.

The assay depends on the use of a substrate analogue F-dUMP tagged with tritium. During the conversion of dUMP to dTMP, an intermediate is formed among the substrate, TS and the co-factor methylenetetrahydrofolate. Substitution of dUMP with F-dUMP will inhibit ion exchange between dUMP and methylenetetrahydrofolate due to the presence of the fluoride ion, thus the reaction will be arrested at this stage. By tagging the F-dUMP with tritium, the products, after PPO (2,5-diphenyloxazole) impregnation, could be detected by fluorography.

2.6.3.1 Preparation of Methylenetetrahydrofolate (CH_2FAH_4): The assay was performed according to Santi *et al.* (1974) and Inselburg *et al.* (1987) with modifications. The co-factor methylenetetrahydrofolate for the conversion of dUMP to dTMP was prepared in a 1ml reaction mixture consisting of 300mM Tris-HCl pH7.2, 20mM DL-tetrahydrofolate (Sigma) and 40 mM formaldehyde. The methylation reaction was carried out at room temperature for 30min. and protected from light.

2.6.3.2 TS Quantification: The assay was done in a 0.4ml assay mixture containing 50mM NMM (N-methylmorpholine) (Sigma), 25mM $MgCl_2$, 1mM EDTA, 75mM 2-mercaptoethanol, 6.5mM formaldehyde, 50μg/ml bovine albumin, 0.17 mM CH_2FAH_4, 95 nM [6-^{3}H] F-dUMP (Moravek Biochemicals, Inc) and 0.3ml parasite protein extract. Control included a reaction mixture without the parasite extracts. Instead, an equivalent volume of TEK buffer was used. The reaction was carried out at room temperature for 1hr, in the dark. After 1hr, 50μl of the assay mixture were mixed with an equal volume of sample buffer and then heat denatured for 5min. before loaded onto a 10% SDS-PAGE gel for further analysis (see below).

2.6.3.3 SDS-PAGE (Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis): Total protein from parasite extracts was fractionated by SDS-PAGE according to Laemmli (1970) and Hames (1981).

The gel mixtures were prepared as listed in Table 2.6. A 10% acrylamide was used in the resolving gel and a 3.75% acrylamide was used in the stacking gel. Sufficient quantity of 10% acrylamide was poured into a 170mm x 150mm x 1.5mm glass-plate, leaving 2-3cm for the stacking gel. Small quantity of distilled water was now gently layered onto the gel surface. After the completion of polymerization, the overlay was poured off. The surface of the separating gel was washed 3 times with stacking buffer. The remaining space between the glass plates was filled with stacking gel mixture. A comb was inserted into the stacking gel to form sample wells in the stacking gel. After the completion of polymerization, the comb was removed and the sample wells were rinsed 3 times with reservoir buffer (0.025M Tris, 0.192M glycine pH 8.3).

The reaction samples were brought to final concentrations of 0.0625M Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol and

Table 2.6 Recipe for gel preparation using SDS-discontinuous buffer system.

Stock solution	Concentrations	
	Final acrylamide concentrations	
	Stacking gel 3.75%	Resolving gel 10%
Acrylamide:bisacrylamide (30:0.8)	2.5ml	20ml
Stacking gel buffer stock* 0.5M Tris-HCl (pH 6.8)	5.0ml	-
Resolving gel buffer stock** 3.0M Tris-HCl (pH 8.8)	-	7.5ml
10% SDS	200μl	600μl
10% ammonium persulphate	100μl	300μl
distilled water	12.2ml	31.6ml
TEMED	15μl	30μl

* Final concentration 0.125M Tris-HCl pH 6.8
** Final concentration 0.375M Tris-HCl pH 8.8

0.002% of bromophenol blue by mixing with an equal volume of sample buffer. Bromophenol blue was included as the tracking dye.

The protein was denatured by boiling for 5min. A sample load of 100μl was used. Molecular weight markers (Prestained SDS Molecular Weight Markers, Sigma) of α_2-macroglobulin (human plasma) 180 kDa, β-galactosidase (*E. coli*) 116 kDa, fructose-6-phosphate kinase (rabbit muscle) 84 kDa, pyruvate kinase (chicken muscle) 58 kDa, fumarase (porcine heart) 48.5 kDa, lactic dehydrogenase (rabbit muscle) 36.5 kDa and triosephosphate isomerase (rabbit muscle) 26.6 kDa were used to calibrate the system.

Electrophoresis in 0.025M Tris, 0.192M glycine pH 8.3, 0.1% SDS was carried out at 100V constant voltage until the tracking dye reached the last centimeter of gel.

The gel was stained in 0.25% Coomassie Brilliant Blue R-250 (Bio-Rad) in water: methanol: glacial acetic acid (5:5:1) at 37°C for 2 hrs. Excess stain was removed by destaining in water: methanol: glacial acetic acid (4.5:4.5:1) at 37°C until a clear background came up. Photographs were taken as records before proceeding to the next step.

2.6.3.4 Detection of the Reaction Products: The tritium labelled reaction products were detected according to Bonner & Laskey (1974). The gel was dehydrated by soaking in about 20x its volume of DMSO for 30min., and then immersed in fresh DMSO for another 30min. These two solutions can be re-used in the same sequence for several times. A third immersion in unused DMSO is recommended if old DMSO solutions are used in the previous two steps. The gel was then immersed in 4 volumes of 22.2% (w/v) of the scintillator 2,5-diphenyloxazole (PPO) in DMSO for 3hrs and then transferred to 20 volumes of water for 1hr. After drying the gel under vacuum, the gel was exposed to an X-ray film at -70°C for fluorography.

CHAPTER THREE
GENETIC BASIS OF PYRIMETHAMINE RESISTANCE

3.1 RESULTS

3.1.1 RFLP Associated with the DHFR-TS Gene of Parent Lines

3.1.2 Segregation of the DHFR-TS Gene with Pyrimethamine Resistance Phenotype in Progeny of a Genetic Cross

3.1.3 No Aberrant Sensitive Alleles were Detected in At Least 100 of the Resistant Progeny Clones

3.1.4 The DHFR-TS Associated RFLP in Other Parasites

3.2 DISCUSSION

Drug chemotherapy has been used extensively to treat malaria patients and it remains one of the most effective ways to combat the disease. However, the global spread of drug resistant parasites has had a great impact on malaria chemotherapy (Spencer, 1985). Resistance to almost all major antimalarial compounds has been reported. Despite this, little is understood about the mechanisms underlying drug resistance in malaria. For example, the precise target for the most widely used drug, chloroquine, is still unknown. Pyrimethamine is an analogue of dihydrofolate and its target is thought to be the enzyme dihydrofolate reductase (DHFR) (Ferone, 1977; Kan & Siddiqui, 1979). It is speculated from sequencing and biochemical data that resistance to pyrimethamine is caused by single base changes in the DHFR gene, resulting in a structurally altered enzyme with reduced affinity for the drug (McCutchan *et al.*, 1984b; Sirawaraporn & Yuthavong, 1984; Dieckmann & Jung, 1986a; Walter, 1986; Chen *et al.*, 1987; Cowman *et al.*, 1988; Peterson *et al.*, 1988; Snewin *et al.*, 1989; Zolg *et al.*, 1989). However, the contribution of other possible mechanisms e.g. over-production of the protein or reduced drug accumulation to pyrimethamine resistance in malaria parasites cannot be excluded. This is open to genetic test. If the sole cause of pyrimethamine resistance is a structural change in the enzyme DHFR, then we should be able to localize the responsible alteration to the gene encoding DHFR.

The first successful cross performed by Professor David Walliker (Walliker *et al.*, 1987) between a pyrimethamine sensitive (pyrS) clone 3D7 and a pyrimethamine resistant (pyrR) clone HB3 was made available to me for the performance of such a genetic test. This, together with the isolation of a cloned dihydrofolate reductase-thymidylate synthetase (DHFR-TS) gene (Snewin *et al.*, 1989) and the feasibility of using restriction-fragment-length-polymorphism (RFLP) as a marker to tag corresponding genes (Section 1.1.4)

form the basis of the present study.

In the study of Walliker *et al.* (1987), the pyrS clone 3D7 was derived from isolate NF54 by limiting dilution and the pyrR clone HB3 was derived from the Honduras isolate H1 by microscopic selection. The method for making crosses has been described in INTRODUCTION Section 1.1.3. Briefly, the gametocytes from the two parent clones were mixed and transmitted through mosquitoes to allow cross-fertilizations to take place. The resulting sporozoites became infective to chimpanzees. Cultures of parasites taken from chimpanzees were established in the laboratory. Genetically pure clones (denoted as X1, X2 etc) were obtained by limiting dilution or micro-manipulation.

The absence of intermediate resistance among the progeny has already suggested the involvement of a single locus in conferring pyrimethamine resistance on the clone HB3 (Walliker *et al.*, 1987). When tested for their sensitivity to pyrimethamine, the progeny gave MIC (minimum-inhibitory-concentration) values characteristic of the two parents of either 10^{-5}M (pyrimethamine resistant) or 10^{-7}M (pyrimethamine sensitive). The MIC is defined as the lowest concentration of the drug that will kill all or nearly all the parasites after exposure to drug containing media for 72hrs (Section 2.2.8).

Analysis of the progeny of the genetic cross for the linkage relationship between the RFLP-tagged DHFR alleles and the resistance phenotype helped us to formulate models about the mechanism underlying resistance to pyrimethamine.

To facilitate the performance of genetic tests on a large number of progeny clones, a simple and rapid protocol for the extraction of DNA from a small quantity of infected blood is an essential pre-requisite. Previously, extraction of DNA from parasites was a time consuming and laborious

process involving weeks of large scale culture with daily medium change. At least 500ml of culture at 8-10% parasitaemia was needed. To circumvent this I devised a mini-prep method (see MATERIALS & METHODS Section 2.4.4.2). Briefly, parasites are collected from 100μl packed red blood cells and subjected to Proteinase K treatment followed by phenol extraction. The nucleic acids are then collected by ethanol precipitation. This method produces an average of 30μg DNA per preparation. The DNA is pure enough for restriction enzyme analysis.

3.1 RESULTS

3.1.1 RFLP Associated with the DHFR-TS Gene of Parent Lines

The lack of available genetic markers for rapid screening of progeny from a genetic cross presents a problem for genetic studies on *Plasmodium*. The emergence of a new class of markers termed restriction-fragment-length-polymorphisms (RFLP) (see INTRODUCTION Section 1.1.4) offers a new approach for linkage analysis. By tagging corresponding genes with a defined RFLP, the alleles of a given gene can be distinguished and thereby the inheritance pattern of the alleles in the progeny of a genetic cross can be analysed. RFLP analysis has been used successfully on several occasions in the study of *Plasmodium* genetics (Wellems *et al.*, 1987; 1990; 1991; Peterson *et al.*, 1988).

The probe used to define RFLP for the DHFR-TS gene was a 5.65kb genomic fragment isolated from the K1 strain by Snewin *et al.* (1989) (see Fig. 3.1). Genomic DNA prepared by the standard method from clones of 3D7 and HB3 was digested with a large number of different restriction enzymes. The resulting patterns in the region of the DHFR-TS gene were

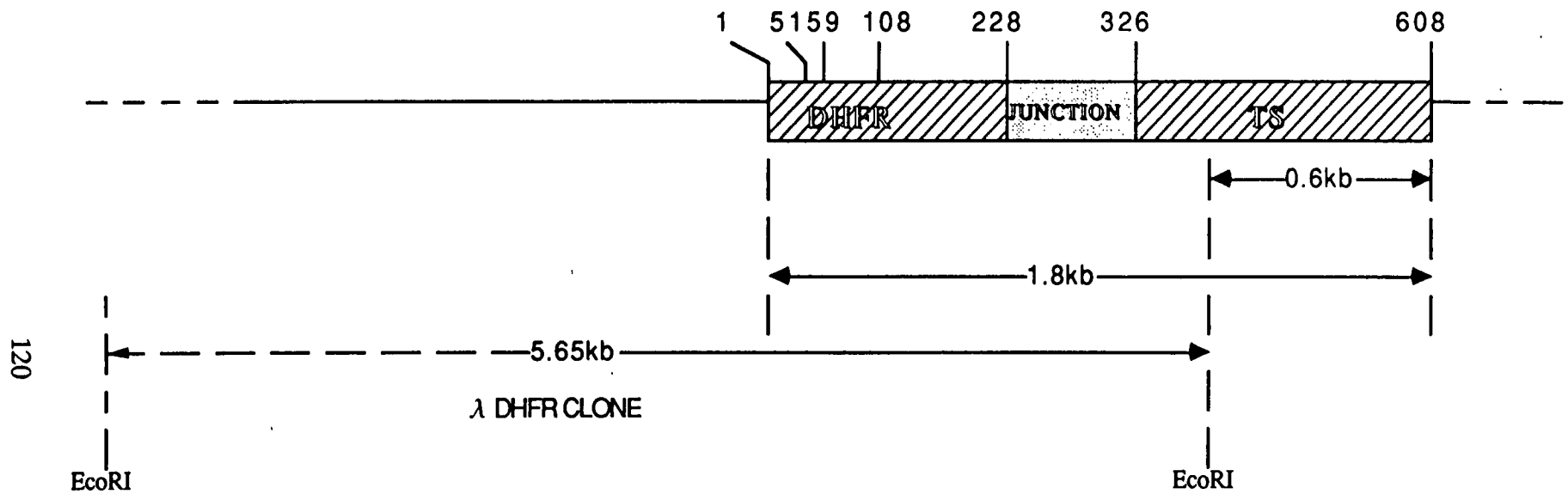


Fig. 3.1 Organization of the gene encoding DHFR-TS isolated from the K1 isolate. A 5.65kb *Eco* RI fragment comprising the 5' flanking region of the DHFR-TS gene, the DHFR domain, the junction domain and the 5' end of the TS domain was cloned in a λ vector (Snewin et al., 1989). Amino acid positions are numbered. Codon 51, 59 and 108 are candidate sites for amino acid changes affecting pyrimethamine sensitivity.

detected by probing Southern blots with the probe described above. The results are shown in Fig. 3.2. An easily recognisable RFLP was observed upon digestion with *Acc*I. In addition to common bands of 1.65kb, 1.1kb and 1.0kb, differing fragment sizes of 2.4kb from 3D7 DNA and 2.15kb from HB3 DNA were found. The difference in apparent sizes between these two fragments from 3D7 and HB3 DNA defines a RFLP for the DHFR-TS which can be used as a marker for the two alleles of the gene.

As mentioned above, the use of a mini-prep method for preparing DNA from *P. falciparum* would greatly facilitate genetic analysis on *Plasmodium*. However, before this mini-prep method could be employed in routine DNA preparation, the quality of DNA prepared by this method must be checked. Genomic DNA from 3D7 and HB3 prepared by the mini-prep method was digested with *Acc*I, blotted and probed. The results are shown in Fig. 3.3. The ethidium bromide stained gel (plate a) revealed complete digestion of the genomic DNA prepared by the mini-prep method. Note that there is a small amount of contaminating RNA shown at the base of the ethidium bromide stained gel. However, this has not affected the quality of the digest (plate b). Restriction patterns identical to that obtained from DNA prepared by the standard method were observed. The pattern observed using 3D7 and HB3 DNA prepared by the conventional large scale method is shown in plate c for comparison. These results illustrate that DNA prepared by the mini-prep method is pure enough for restriction digestion and hence subsequent DNA preparations were obtained by the mini-prep method.

3.1.2 Segregation of the DHFR-TS Gene with Pyrimethamine Resistance Phenotype in Progeny of a Genetic Cross

The genetic determinant for pyrimethamine resistance in HB3 can be

Fig. 3.2 Search for an RFLP associated with the DHFR-TS gene. Southern blots of restriction enzyme digested genomic DNA from 3D7 and HB3, probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate.

Track 1: HB3
Track 2: 3D7

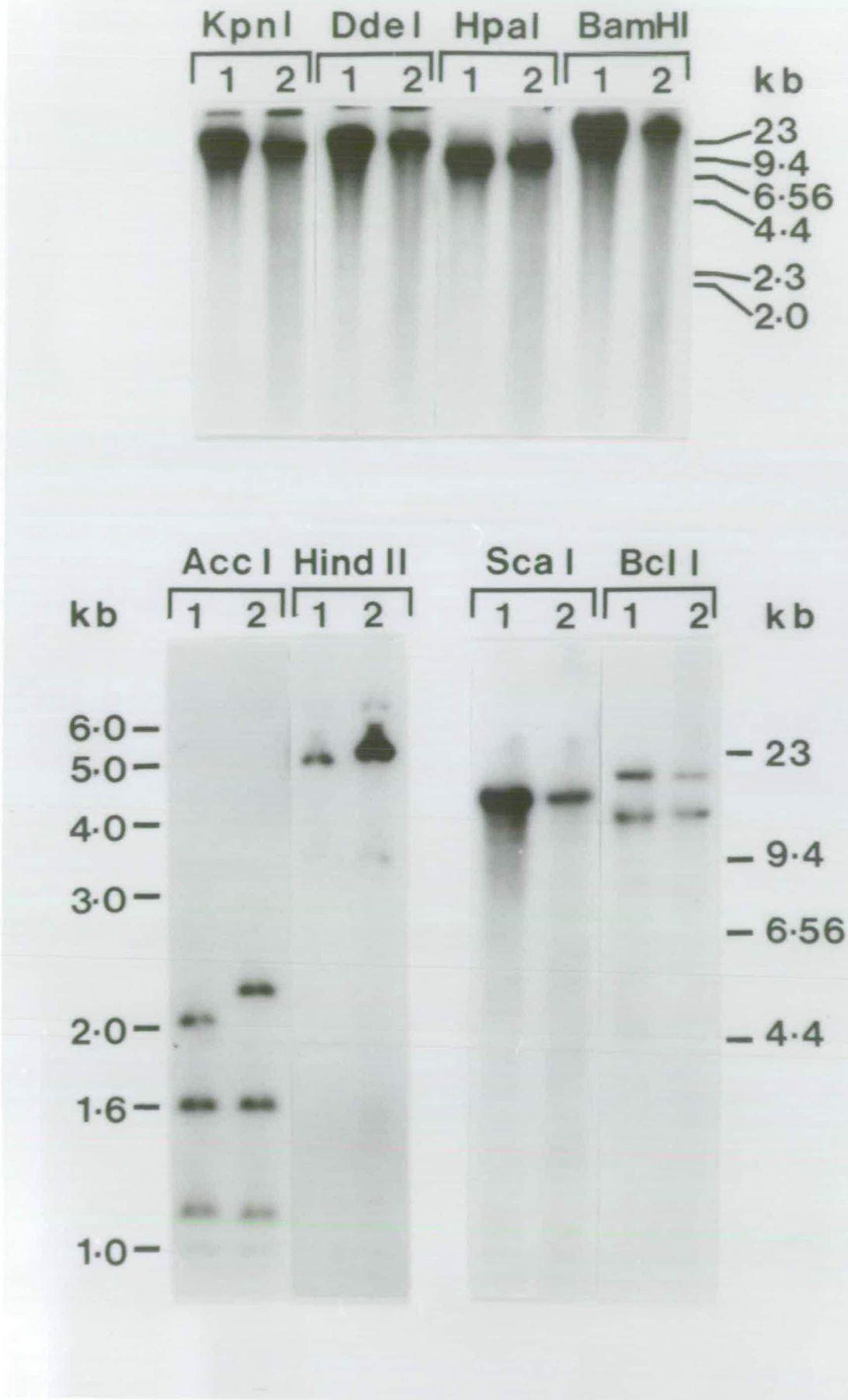

Kpn I
Dde I
Hpa I
BamHI
1 2 1 2 1 2 1 2
kb
23
9·4
6·56
4·4
2·3
2·0
Acc I
Hind II
Sca I
Bcl I
1 2 1 2 1 2 1 2
kb
kb
6·0
5·0
4·0
3·0
2·0
1·6
1·0
23
9·4
6·56
4·4

Fig. 3.3 An AccI RFLP associated with the parasite clones 3D7 and HB3 is detectable using DNA prepared by the mini-prep method. Genomic DNA from 3D7 and HB3 was digested with *Acc*I, fractionated by agarose gel and then blotted for hybridization.

a) Ethidium bromide stained gel of *Acc*I digested genomic DNA of 3D7 and HB3 obtained by mini-prep method (Section 2.4.4.2).

b) Southern blot of gel probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate showing differing sizes of 2.4kb and 2.15kb in the upper band between 3D7 and HB3.

c) Southern blot of *Acc*I digested genomic DNA of 3D7 and HB3 obtained by the conventional large scale method (Section 2.4.4.1), probed with the same gene fragment.

a
HB3
3D7
kb
23
9·4
6·56
4·4
2·3
2·0
0·56
b
HB3
3D7
c
HB3
3D7
kb
4·4
2·3
2·0
0·56

precisely detected using a genetic cross. The cross between a pyrS 3D7 parent clone and a pyrR HB3 parent clone of *P. falciparum* displaying well-defined characteristics enables genetic analysis to be undertaken on the progeny. In many of the progeny the characters of the two parents are reassorted in a Mendelian manner (Walliker *et al.*, 1987). Hence, by mapping the DHFR marker in the cross, we should be able to correlate the inheritance of this DNA marker with the inheritance of resistance to the drug in the cloned progeny.

A total of 29 progeny clones obtained by Walliker *et al.*, (1987) by limiting dilution or by micro-manipulation have been tested for pyrimethamine sensitivity and were made available to me. The recombinant progeny were screened to see if the RFLP segregated with pyrimethamine resistance. Genomic DNA prepared from independent progeny clones was digested with *Acc*I, fractionated by agarose gel electrophoresis, blotted and probed with the 5.65kb DHFR-TS fragment from the K1 isolate. The results are shown in Fig. 3.4 and are summarised in Table 3.1. These results show that the RFLP associated with the DHFR-TS gene co-segregates with the resistance phenotypes. All pyrS progeny had the 2.4kb fragment characteristic of the sensitive parent 3D7 while pyrR progeny had the 2.15kb fragment characteristic of the resistant parent HB3. This implies that the alteration conferring drug-resistance on the clone HB3 is linked to the DHFR-TS gene and is probably located close to the gene or within it.

A control test was performed on clones 3D7B and HB3B which were obtained after passing the parent clones through mosquitoes independently. RFLP patterns similar to the parent 3D7 and HB3 were obtained for 3D7B and HB3B (Fig. 3.4f), showing that the passage through mosquitoes did not affect the RFLP patterns.

Fig. 3.4 RFLP patterns in progeny of a genetic cross.

a-e) Southern blots of *Acc*I digested genomic DNA from progeny, probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate. Note that the progeny clones have only one of the two possible RFLP's. Clone X59 represents a mixed population of sensitive and resistant parasites. The sensitive parent clone 3D7 and resistant parent clone HB3 were included as controls.

f) Southern blot of *Acc*I digested genomic DNA from 3D7B and HB3B, probed with the same gene fragment.

a
HB3
X33
X32
X14
X13
X12
X11
X8
X6
X4
X2
3D7
2·3 kb
2·0 kb
b
HB3
XP9
XP8
XP7
XP5
XP3
XP1
X37
X36
X35
X34
3D7
2·3 kb
2·0 kb
c
HB3
X10
X30
X41
X52
X51
X40
X64
X63
X57
X53
3D7
2·3 kb
2·0 kb

d
HB3
X45
X39
X58
X54
X59
X38
X65
3D7
2·3kb
2·0kb
e
HB3
X67
X66
X56
X50
X48
X47
X44
X43
3D7
2·3kb
2·0kb
f
HB3
HB3B
3D7B
3D7
2·3kb
2·0kb

Table 3.1 Linkage of RFLP with pyrimethamine resistance in the progeny of a cross

	RFLP	PyrR/S	Progeny Phenotype
3D7	1	S	
HB3	2	R	
3D7B	1	S	
HB3B	2	R	
X2	2	R	rec
X4	2	R	rec
X6	2	R	rec
X8	1	S	par
X10	1	S	rec
X11	2	R	rec
X12	2	R	rec
X30	1	S	rec
X33	2	R	rec
X34	2	R	rec
X35	2	R	rec
X36	1	S	par
X37	1	S	par
X41	2	R	rec
X44	1	S	rec
X45	1	S	rec
X47	1	S	rec
X48	1	S	rec
X50	1	S	rec
X51	1	S	rec
X52	1	S	rec
X58	1	S	rec
X63	1	S	rec
XP1	2	R	rec
XP3	2	R	rec
XP5	2	R	rec
XP7	2	R	rec
XP8	2	R	rec
XP9	2	R	rec

Legend to Table 3.1

The segregation of RFLP marker and the pyrimethamine resistance phenotype are shown in progeny of a cross between 3D7 and HB3. The RFLP pattern for 3D7 is denoted as 1 which is observed as a 2.4kb fragment whereas the RFLP pattern for HB3 is denoted as 2 which is observed as a 2.15 kb fragment. The data on pyrimethamine sensitivity is provided by Professor David Walliker. Parasites that are sensitive to 10^{-7}M pyrimethamine or more are denoted as S whereas parasites that are resistant to 10^{-5}M pyrimethamine or less are denoted as R. Progeny are designated either parental (par) from selfed zygotes or recombinant (rec) from crossed zygotes as judged by their reactivities towards different monoclonal antibodies and 2D-PAGE studies (Walliker *et al.*, 1987; D. Walliker, personal communication).

3.1.3 No Aberrant Sensitive Alleles were Detected in At Least 100 of the Resistant Progeny Clones

The number of progeny clones which can be tested is limited by the tedious process of *in vitro* cultivation and DNA preparation. Even with the development of a rapid method to prepare DNA from small scale cultures (see MATERIALS & METHODS Section 2.4.4.2), it is still a time-consuming and laborious process to prepare DNA from a large number of parasites for genetic analysis. The sample size could be increased conveniently if a large number of progeny could be tested without the laborious isolation of individual clones by selecting pyrR parasites from the cross. In order to do this we used the fact that the uncloned progeny represented a mixture of pyrR and pyrS parasites. Treatment with a dosage of pyrimethamine (10^{-6}M) lethal to the sensitive parasites but tolerated by the resistant parasites had previously been used to select a population of parasites resistant to pyrimethamine (denoted as XP) (Walliker *et al.*, 1987).

To detect any aberrant sensitive alleles in XP, DNA was prepared from the uncloned mixture XP and, as a control, DNA was also prepared from the mixture of uncloned unselected progeny (denoted as X). After digestion with *Acc*I and fractionation by agarose gel electrophoresis, the DNA was blotted and probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate. The results are shown in Fig. 3.5. Parasites selected with pyrimethamine (XP) showed the pattern associated with the resistant DHFR-TS allele only, with the characteristic 2.15kb band. Those unselected with pyrimethamine (X) showed a pattern characteristic of both the sensitive and resistant alleles i.e. 2.4kb and 2.15kb.

The limits which a 3D7 type RFLP could be detected in a HB3

Fig. 3.5 RFLP pattern in uncloned progeny selected with pyrimethamine (XP). Southern blot of *Acc*I digested genomic DNA obtained from progeny selected with pyrimethamine (XP), probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate. Controls included *Acc*I digested genomic DNA from 3D7, HB3 and the original uncloned progeny unselected with pyrimethamine (X).

HB3
XP
X
3D7
2·3
2·0
0·56
kb

background was calibrated by mixing known amounts of 3D7 and HB3 DNA in the *Acc*I restriction enzyme digest. The ratio of the two types of DNA varied from 1:1 to $1:10^7$ (Fig. 3.6). The results clearly show that with a ratio of 1:10, the 2.4kb band can be detected in the mixture. Over-exposure of this blot revealed a faint 2.4kb band even when 3D7 DNA was mixed with HB3 DNA in a 1:100 ratio (Fig. 3.7). No such band was ever observed upon probing XP. This indicates that no aberrant sensitive DHFR-TS alleles were detected in at least 100 resistant parasites.

To make sure that no 2.4kb band was masked by the 2.15kb band from the XP digest, *Acc*I digested genomic DNA from X and XP was fractionated and probed with the 5.65kb fragment from the K1 isolate. The blot was initially exposed overnight to reveal the restriction patterns. A piece of filter from the XP track at the position corresponding to the 2.15kb band was cut out and re-exposed. The piece of cut-out filter was exposed alongside as a control. As shown in Fig. 3.8, prolonged exposure of the filter did not reveal any 2.4kb band in the XP track.

3.1.4 The DHFR-TS Associated RFLP in Other Parasites

Parasites isolated from the wild were screened to see whether the occurrence of the *Acc*I RFLP denoted by the 2.15kb/2.4kb fragments was restricted to 3D7 and HB3. Variant parasites tested included a series of Tak9 isolates and clones, the K1 isolate and the cloned line PR70/CB3 from Thailand, a Papua New Guinea strain MAD20 and two *in vitro* selected pyrR mutants derived from T9/94 parent. DNA was prepared from these parasites by the mini-prep method and digested with *Acc*I. After fractionation by agarose gel electrophoresis, the DNA was blotted and probed with the 5.65kb

Fig. 3.6 RFLP analysis on XP (I). Southern blot of *Acc*I digested genomic DNA from the parasite mixtures X and XP, probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate. The sensitivity of the test was calibrated against known amounts of HB3 and 3D7 DNA mixed in different ratios from 1:1 to $1:10^{-7}$. Tracks containing 3D7 and HB3 DNA were included as controls.

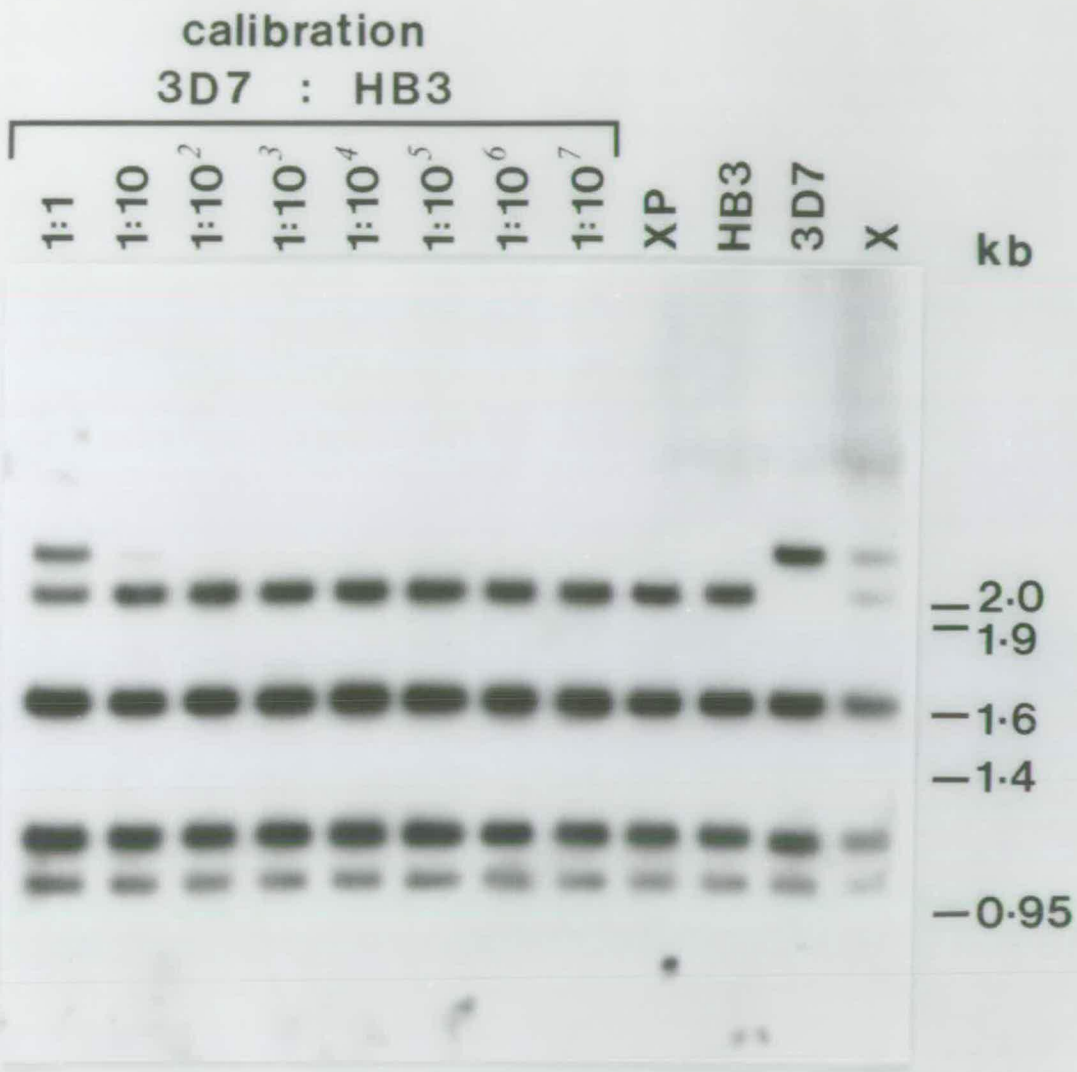
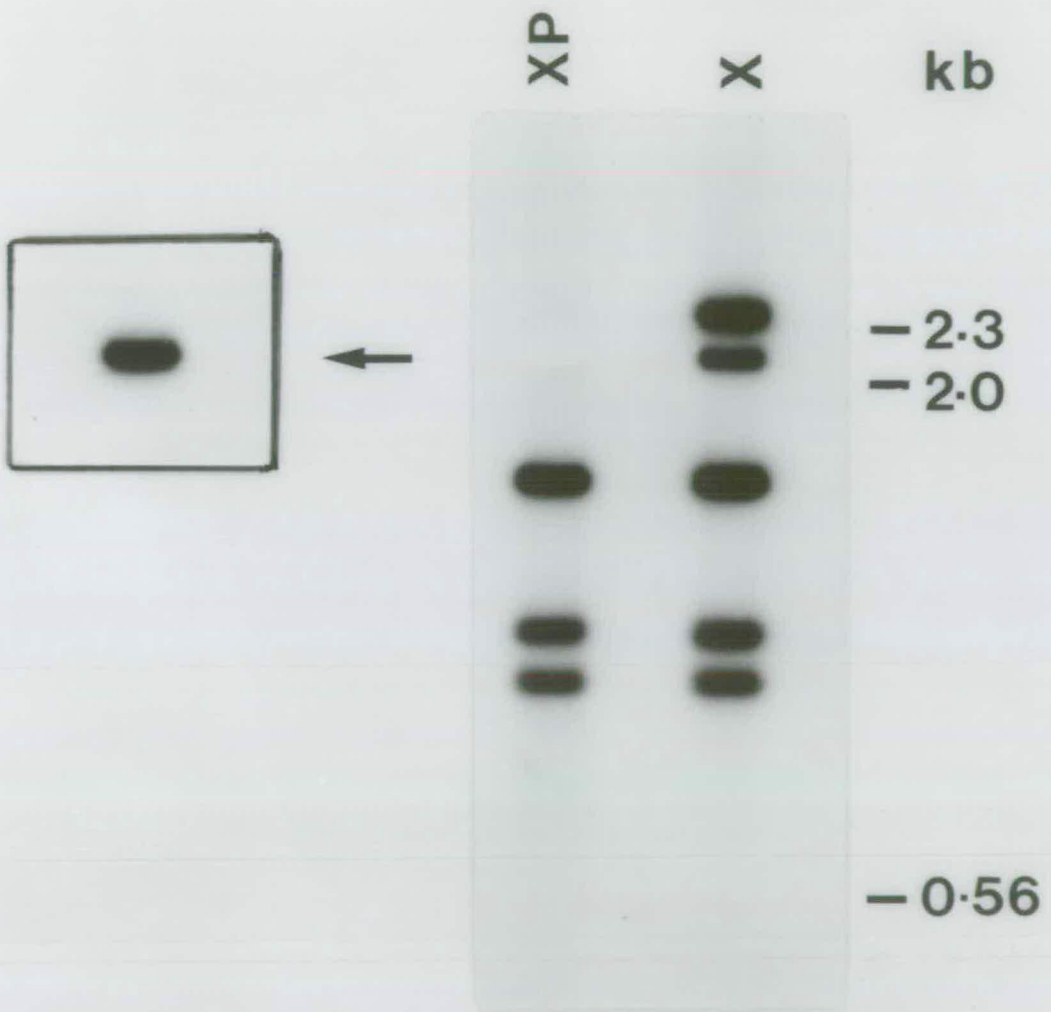

calibration
3D7 : HB3
1:1
1:10
1:10^2
1:10^3
1:10^4
1:10^5
1:10^6
1:10^7
XP
HB3
3D7
X
kb
2·0
1·9
1·6
1·4
0·95

Fig. 3.7 RFLP analysis on XP (II). An over-exposure of the Southern blot in Fig. 3.6 to show the faint band (arrow) detected when 3D7 and HB3 DNA were mixed in a ratio 1:100 (track 5).

Track 1 unselected progeny X
Track 2 selected progeny XP
Track 3-5 3D7 and HB3 DNA mixed in different ratios.


Unselected
Selected
calibration
1:
1
1:
10
1:
100
3D7 DNA
HB3 DNA
(kb)
2·0
1·9
1·6
1·4
0·95
1
2
3
4
5

Fig. 3.8 Absence of the 2.4kb band in XP. An over-exposure of Southern blot of *Acc*I digested genomic DNA from the parasite mixtures X and XP, probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate. The filter was initially exposed overnight to reveal the restriction patterns. A piece of filter at the position corresponding to the 2.15kb band was removed (arrow) and re-exposed.


XP
X
kb
2·3
2·0
0·56

DHFR-TS fragment from the K1 strain. The hybridization patterns were dimorphic in nature (Fig. 3.9). Either the 2.4kb or the 2.15kb dimorphic *Acc*I fragment was present in the parasite strains.

The dimorphic RFLP patterns in these parasites did not seem to have any correlation with their resistance to pyrimethamine, however. For example, the K1 isolate, which is resistant to pyrimethamine, showed a restriction pattern like that of the sensitive clone, 3D7, displaying the characteristic 2.4kb band. From this we conclude that the *Acc*I RFLP associated with DHFR-TS is dimorphic in nature but that this dimorphism in itself does not bear any correlation with pyrimethamine resistance.

3.2 DISCUSSION

Genetic analysis can often provide evidence for the genetic determinants of drug resistance. The success achieved in making crosses of the human malaria parasite, *P. falciparum* (Walliker *et al.*, 1987) enables genetic analysis to be undertaken on this human malaria parasite. The drawback is that the sample size is very often limited. Therefore, in the present study, the number of progeny clones screened was as high as possible. The results of screening 29 progeny show a linkage between the RFLP associated with the DHFR-TS gene and the resistance phenotype. It should be noted that for three of the progeny X8, X36 and X37, using a variety of assays, it has not yet been possible to distinguish them from the sensitive parent 3D7. It may be therefore that these are not true recombinants but could have derived by self-fertilization. Taking this into account, however, the linkage between the

Fig. 3.9 RFLP patterns in different parasite strains and in vitro selected mutants. Southern blot of *Acc*I digested genomic DNA from different parasite strains (K1/MAD20/T9), the cloned lines derived from T9 (T9/94/96/98/101/102/106), the clones 3D7, HB3, PR70/CB3 and the mutants T9/94 (M1-1), T9/94 (S300.300) selected from T9/94. The probe used was derived from the 5.65kb DHFR-TS gene fragment of the K1 isolate.

MAD 20
K1
HB3
T9/106
T9/102
T9/101
T9/98
T9/96
T9
PR70/CB3
T9/94(S300·300)
T9/94(M1-1)
T9/94
3D7
kb
4·4
2·3
2·0
0·56

DHFR-TS gene and pyrimethamine resistance is still confirmed with at least 26 progeny. Moreover, the sample size was raised to 100 when the analysis on parasites selected with pyrimethamine (XP) was calibrated against known mixtures of DNA from 3D7 and HB3. In other RFLP-related studies on *P. falciparum*, the sample sizes have been smaller and still clear predictions have been reached (Wellems *et al.*, 1987; 1990; 1991; Peterson *et al.*, 1988). For example, using 16 progeny, Wellems *et al.*, (1990) are able to disprove the role of the *mdr*-like genes in chloroquine resistance as suggested by Foote *et al.* (1990b). Analysis on segregation of a number of RFLP markers in these 16 progeny has mapped the resistance allele for chloroquine to within 400kb in chromosome 7 (Wellems, 1991).

The present study provides genetic evidence about the target for pyrimethamine and the changes causing pyrimethamine resistance. In the genetic cross pyrimethamine resistance behaved as a single locus (Walliker *et al.*, 1987). As shown in the present study, this locus is linked to the gene for DHFR-TS and is probably located close to the gene or within it. Although the RFLP is used as a genetic marker, the polymorphism itself is not responsible for pyrimethamine resistance i.e. the pyrimethamine resistant K1 isolate displays an RFLP characteristic of the sensitive 3D7 clone.

These results provide genetic confirmation for the conclusion drawn from biochemical and sequencing studies (McCutchan *et al.*, 1984b; Sirawaraporn & Yuthavong, 1984; Dieckmann & Jung, 1986a; Walter, 1986; Chen *et al.*, 1987; Cowman *et al.*, 1988; Peterson *et al.*, 1988; Snewin *et al.*, 1989; Zolg *et al.*, 1989). Their results have suggested that single base changes within the gene for DHFR-TS cause structural changes in the enzyme and thereby reduce its affinity for the drug.

Further proof has come from functional analysis on the products of *in vitro* mutagenized genes expressed in *E. coli* (Sirawaraporn *et al.*, 1990).

Using site-directed *in vitro* mutagenesis the DHFR-TS gene cloned from a resistant parasite was changed to that of a sensitive parasite. The affinity of the expressed protein for pyrimethamine is increased to the same magnitude as that obtained from the wild isolate. Alternatively, the functional importance of the amino acid residues in forming the active site cavity and their interaction with substrate/inhibitor can be studied *in vitro* by X-ray crystallography. Ultimately, the effect of these mutations on phenotypes need to be studied *in vivo* to see whether the mutated gene can confer resistance to a pyrS parasite. This would depend on the development of a transformation system to place altered genes back into the genome of malaria parasites.

The role of single base changes within the DHFR-TS gene in giving rise to pyrimethamine resistance is confirmed from biochemical, sequencing and the present genetic results. It remains to be seen whether other mechanisms also play a part in conferring resistance on malaria parasites. Parasites in nature exhibit a wide range of resistance to pyrimethamine. This varies from a very low level of resistance to as much as 10^6-fold more resistant than the sensitive organism (Thaithong *et al.*, 1983), suggesting the possibility of a variety of mechanisms. Already alterations other than single base changes have been identified. Inselburg's laboratory have generated a class of mutants which it appears over-produce DHFR-TS due to gene amplification (Banyal & Inselburg, 1986; Inselburg *et al.*, 1987; Tanaka *et al.*, 1990a; b). In one wild type isolate measurements of DHFR specific activity from parasite extracts lead Kan & Siddiqui (1979) to conclude that the isolate may have increased levels of enzyme. These results lead us to investigate the role of increased protein levels in pyrimethamine resistance in the next chapter.

CHAPTER FOUR
OVER-PRODUCTION OF DHFR-TS AS A POSSIBLE MECHANISM OF PYRIMETHAMINE RESISTANCE

4.1 BASIS OF THE ASSAY FOR MEASURING THE DHFR-TS CONTENT OF P. FALCIPARUM

4.2 RESULTS

4.2.1 Stability of the Enzyme at 37°C

4.2.2 Synchronization of the Parasites

4.2.3 Calibration of the Conditions Used in TS Assay

4.2.4 The DHFR-TS is Not Over-produced in HB3 Compared to 3D7

4.2.5 The DHFR-TS Gene is Not Amplified in HB3 Compared to 3D7

4.2.6 The DHFR-TS is Over-produced in an in vitro Selected Mutant T9/94 (M1-1) Than in the Parent Clone T9/94

4.2.7 The DHFR-TS Gene is Not Amplified in T9/94(M1-1)

4.2.8 The Level of DHFR-TS is Elevated in a Parasite Clone PR70/CB3 Obtained from a Thai Isolate, PR70 Compared to T9/94

4.2.9 The DHFR-TS Gene is Not Amplified in PR70/CB3

4.3 DISCUSSION

There are several possible basic mechanisms contributing to pyrimethamine resistance in *P. falciparum*:

1. The structure of the target enzyme may be altered so that its affinity for pyrimethamine is reduced.
2. The enzyme may be over-produced to titrate the toxic effect of the drug.
3. The accumulation of the drug inside the parasite may be reduced.
4. The utilization of the metabolic pathway in folate metabolism may be altered to bypass the inhibitory effect of the drug.

The role of single base changes in the dihydrofolate reductase-thymidylate synthetase (DHFR-TS) gene contributing to pyrimethamine resistance by inducing structural alteration in the enzyme has been well established from biochemical, sequencing and genetic results (McCutchan *et al.*, 1984b; Sirawaraporn & Yuthavong, 1984; Walter, 1986; Dieckmann & Jung, 1986a; Chen *et al.*, 1987; Cowman *et al.*, 1988; Peterson *et al.*, 1988; Snewin *et al.*, 1989; Zolg *et al.*, 1989; Sirawarapron *et al.*, 1990 and the present study).

Over-production of the protein as a result of gene amplification and/or increased gene expression has not been documented in naturally occurring *P. falciparum*, however, a mutant isolated *in vitro* (Banyal & Inselburg, 1986) appears to function in this way (Inselburg *et al.*, 1987). Over-production of the gene product is a known mechanism of resistance to another antifolate-methotrexate in *L. major* (Coderre *et al.*, 1983; Beverley *et al.*, 1986) and of cultured mammalian cells (Schimke *et al.*, 1978).

A reduction in the uptake of the antifolate methotrexate has been implicated as one mechanism for drug resistance in the kinetoplastids

(Ellenberger & Beverley, 1987) although this has yet to be found in *P. falciparum*.

The possible use of an alternative pathway in folate metabolism to circumvent the toxic effect of the drug is highlighted by the increasing recognition of the salvage pathway (Krungkrai *et al.*, 1989a; 1990) and the demonstration of a *de novo* methionine synthesis cycle in *P. falciparum* (Krungkrai *et al.*, 1989b). Resistance to sulfadoxine which inhibits the same metabolic pathway as pyrimethamine in *P. falciparum* is thought to be due to shifting from salvage to *de novo* synthesis of *p*-aminobenzoate (Dickemann & Jung, 1986b).

In this chapter the investigation of over-production of DHFR-TS as a possible mechanism of pyrimethamine resistance is described using (a) the cloned line HB3; (b) an *in vitro* selected mutant and (c) a naturally occurring clone PR70/CB3.

4.1 BASIS OF THE ASSAY FOR MEASURING THE DHFR-TS CONTENT OF P. FALCIPARUM

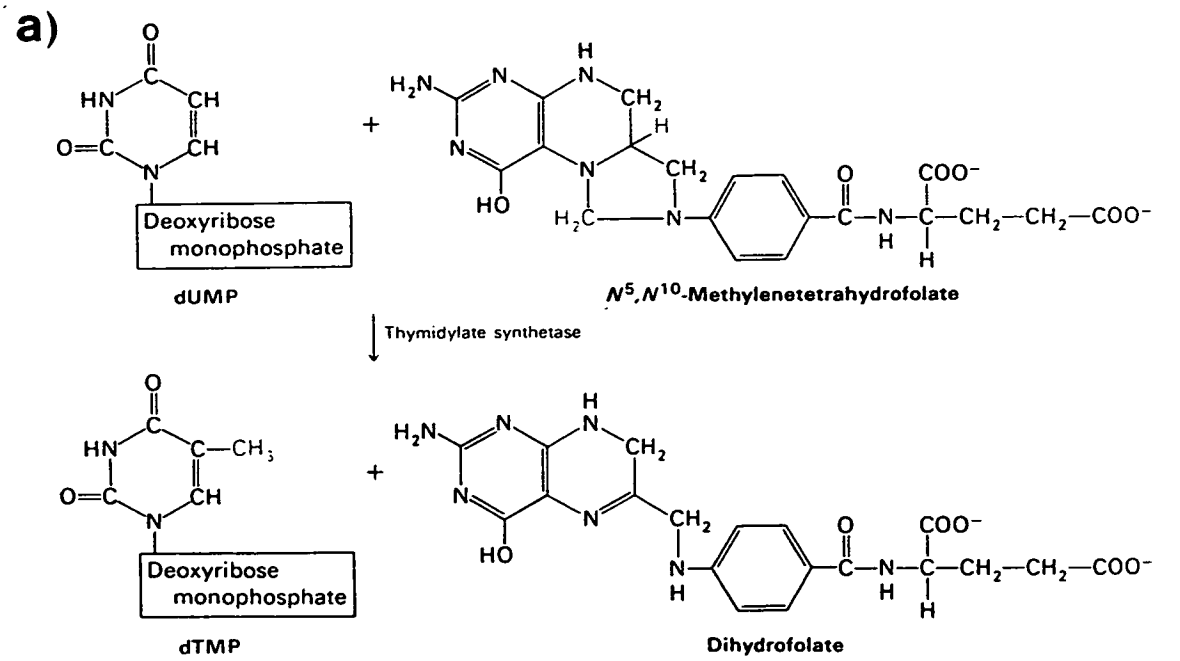
The amount of DHFR-TS enzyme in *P. falciparum* parasites is measured using a thymidylate synthetase assay (TS assay) which quantifies the amount of thymidylate synthetase (TS) in the parasite. Since dihydrofolate reductase (DHFR) and TS are translated into a single polypeptide chain (Bzik *et al.*, 1987; Snewin *et al.*, 1989), expression of the DHFR-TS gene in the two types of parasite can be compared by quantifying the thymidylate synthetase. The basis of the assay is outlined in Fig. 4.1. It relies on the fact that TS is the key enzyme in catalysing the conversion of dUMP to dTMP (Fig. 4.1a). The methylation reaction is mediated by 5, 10-methylenetetrahydrofolate as a co-

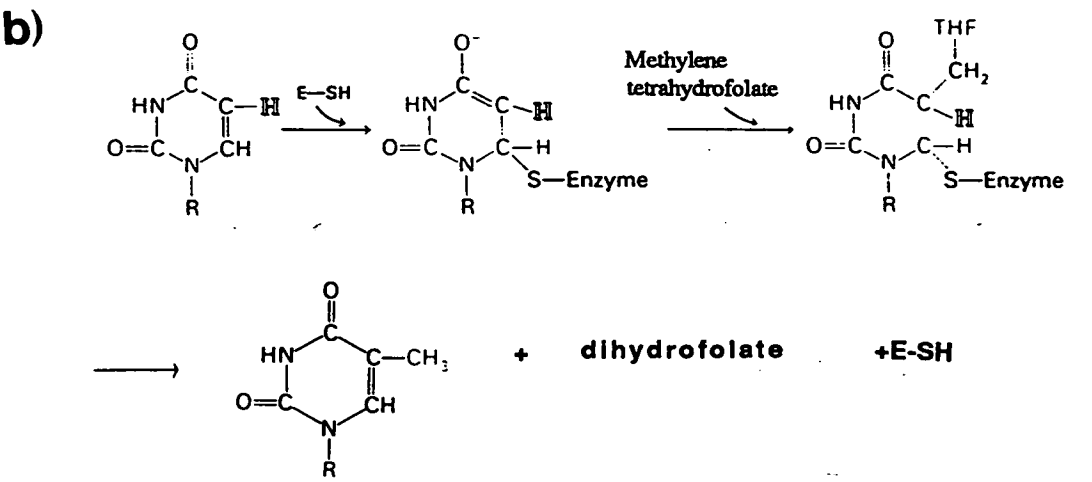
Fig. 4.1 Basis of the TS assay for over-production of DHFR-TS

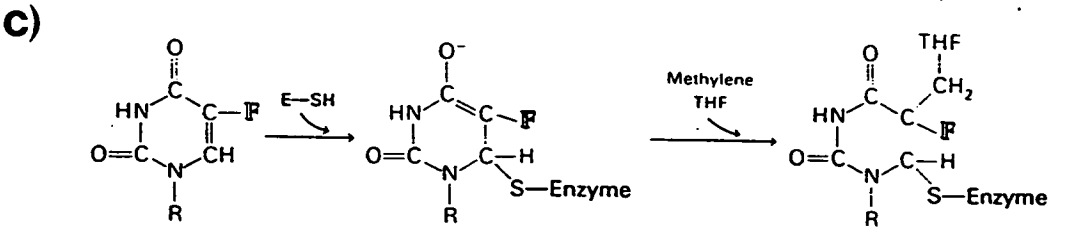
a) The synthesis of dTMP from dUMP. The conversion of dUMP to dTMP is catalysed by the enzyme thymidylate synthetase and mediated by the co-factor 5, 10-methylenetetrahydrofolate. In this reaction, 5, 10-methylenetetrahydrofolate serves as both a one-carbon donor and a reducing agent which is itself converted into dihydrofolate.

b) In a normal reaction, the enzyme will form a covalent complex with the substrate and co-factor 5,10-methylenetetrahydrofolate. After ion exchange among them, the products dTMP and dihydrofolate are formed.

c) When the substrate is replaced by an analogue fluorodeoxyuridylate (F-dUMP), the reaction is irreversibly inhibited at the stage of intermediate formation. The presence of the fluoride ion inhibits ion exchange and hence the conversion of this intermediate into the final products.


a)
Deoxyribose monophosphate
dUMP
N^5,N^{10}-Methylenetetrahydrofolate
Thymidylate synthetase
Deoxyribose monophosphate
dTMP
Dihydrofolate


b)
E—SH
Methylene tetrahydrofolate
THF
S—Enzyme
dihydrofolate
+E-SH


c)
E—SH
Methylene THF
THF
S—Enzyme

factor which serves as both a one-carbon donor and reducing agent. In a normal reaction (Fig. 4.1b), the TS enzyme and the co-factor 5,10-methylenetetrahydrofolate covalently bind to the substrate dUMP to form an intermediate. Further reaction to the final products requires ion exchange between the co-factor, the enzyme and the hydrogen ion of the substrate (shadowed in the figure). When the substrate is replaced with a fluoride analogue F-dUMP, the reaction will be irreversibly inhibited at the stage of covalent complex formation because the fluoride ion cannot be abstracted by the enzyme (Fig. 4.1c).

By tagging this substrate analogue with tritium, binding of the radiolabelled product to thymidylate synthetase can be measured.

To test for TS, I prepared parasites by saponin lysis of the red blood cells (Section 2.6.1). Total protein was then extracted from the parasites by sonication. The lysates were centrifuged and the supernatant which contains DHFR-TS was used in the TS assay as described in MATERIALS & METHODS Section 2.6.3. Briefly, 0.3ml of the protein extract reacted with [6-^{3}H] F-dUMP in a reaction buffer containing the co-factor 5,10-methylenetetrahydrofolate for 1hr. The reaction products were fractionated on SDS-PAGE and the signals detected by fluorography.

4.2 RESULTS

4.2.1 Stability of the Enzyme at 37°C

The instability of the DHFR-TS enzyme from *P. falciparum* has been reported (McCutchan *et al.*, 1984b; Chen & Zolg, 1987). The enzyme lost its activity upon storage (McCutchan *et al.*, 1984b). An experiment was therefore performed to see whether the enzyme stability was affected by some *in vitro* conditions.

The rate of deterioration of the enzyme at 37°C was measured. The total protein was incubated at 37°C for different lengths of time before being subjected to the TS assay. Control reactions without any pre-incubations at 37°C were included. A graph of the relative amount of TS at T_t (as quantified by densitometric scanning of the bands) was plotted against duration of incubation at 37°C.

As shown in Fig. 4.2 and Fig. 4.3, the enzyme content dropped off after the total protein had been incubated at 37°C, resulting in only ≈60% of the original enzyme content after 2hrs of incubation. There was a gradual deterioration of the enzyme with increasing length of incubation at 37°C, indicating that the enzyme is greatly affected by incubation at this temperature. Therefore, great care should be taken in handling the enzymes. Much of the ensuing work depends on comparing DHFR-TS levels between different strains. In the light of these results it was decided that proteins should be prepared simultaneously from the different parasite strains to be compared and that aliquots of parasite extract should be stored under similar conditions.

4.2.2 Synchronization of the Parasites

Based on studies on mammalian systems it is likely that DHFR-TS is cell-cycle regulated and expressed at different rates at different stages of development in *P. falciparum*. Obviously, precise comparison between two parasite strains would require protein obtained from parasites at similar stages of development. This can be achieved by sorbitol treatment to synchronize the parasite growth (see MATERIALS & METHODS Section 2.2.7).

The efficiency of this method was first examined by following the growth of the parasite cloned line T9/94 after sorbitol treatment using Giemsa-stained smears

Fig. 4.2 Deterioration of the DHFR-TS enzyme at 37°C. Fluorograph of the tritium labelled reaction products (arrow) from the TS assay fractionated by 10% SDS-PAGE. Total protein from 3D7 and HB3 were incubated for different length of time at 37°C before being subjected to the TS assay.

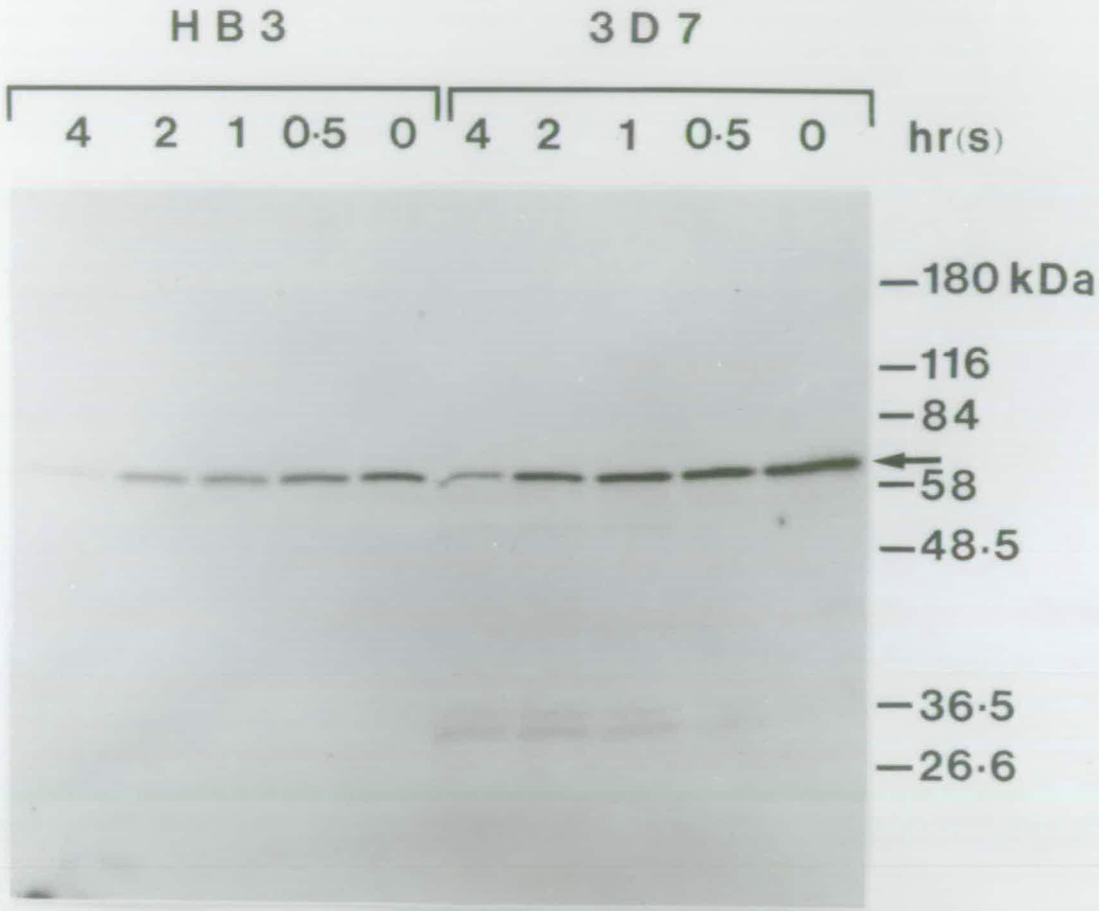
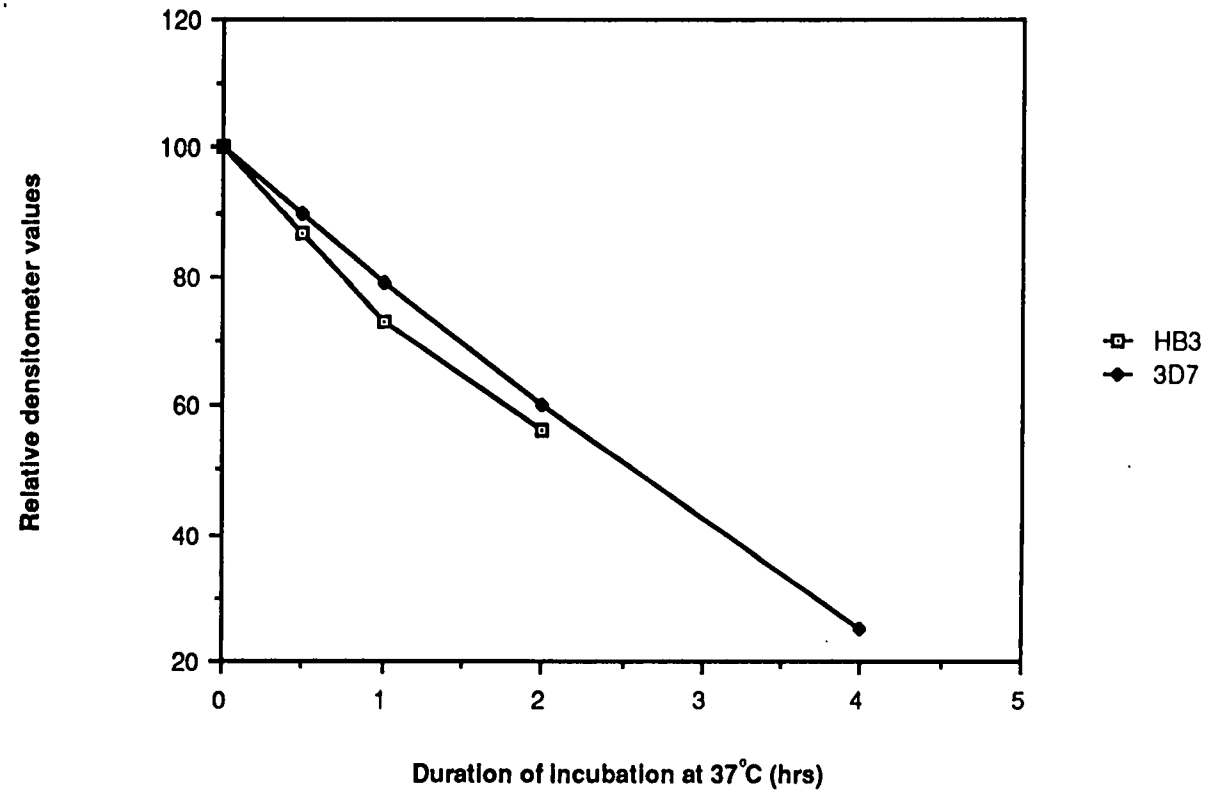

HB3
3D7
4 2 1 0·5 0 4 2 1 0·5 0
hr(s)
—180 kDa
—116
—84
—58
—48·5
—36·5
—26·6

Fig. 4.3 Deterioration of the enzyme content after incubation at 37°C.



Legend to Fig. 4.3
The bands in Fig. 4.2 were scanned by a densitometer. The readings obtained were plotted as percentage of the control band (which was taken as 100) against duration of incubation at 37°C (hrs).

(Table 4.1, Fig. 4.4a). The same culture, which was not subjected to sorbitol treatment was followed as control (Fig. 4.4b). Immediately after sorbitol treatment, the parasitaemia dropped from 6% to 1.8%, due to the toxic effect of sorbitol. The mature stages were killed, leaving 87% of the parasites in the ring forms. In the next 24hr, the ring forms mature to the trophozoites and schizonts. By 48hrs, all parasites were found in the ring stage and the parasitaemia had increased several fold to 5%. The synchrony was maintained up to 72hrs when more than 95% of the parasites were in the trophozoite and schizont stages. After another round of re-invasion and replication (t=120hrs), however, the culture lost its synchrony and a mixed population with all three stages of parasites were again obvious. From these results the optimal conditions for harvesting were judged to be at t=72hrs. At that time the parasites were predominantly in the late trophozoite and early schizont stages when the DNA synthesis is exponential (Newbold *et al.*, 1982; Inselburg & Banyal, 1984; de Rojas & Wesserman, 1985) and the parasitaemia was relatively high (5%).

4.2.3 Calibration of the Conditions Used in TS Assay

Comparative studies require equal amounts of total protein from each of the parasite clones investigated to be used in the TS assay. This was ensured in several ways.

1. All parasites were harvested 72hrs after sorbitol synchronization at similar stages of development (see above). Before protein was extracted, the parasites were resuspended to the same density as calculated from the parasitaemia and haematocrit.
2. Total protein content obtained from the parasite extract was measured with Bradford's reagent (Bradford, 1976). Similar

Table 4.1 Parasite* distribution after sorbitol treatment

Time after sorbitol treatment (hrs)	Sorbitol treated				Control without sorbitol treatment			
	Percentage(%)				Percentage (%)			
	R	T	S	Parasi-taemia	R	T	S	Parasi-taemia
0	87	13	0	1.8	64	14	19	2.1
3	72	26	2	1.5	74	0	26	1.9
24	10	20	70	1.5	84	16	0	1.9
48	100	0	0	5.0	42	8	50	3.6
72	4.3	65	30	5.1	53	18	30	5.7
96	94	2	4	8.5	33	8	59	8.3
120	24	31	45	8.7	57	5	38	9.6

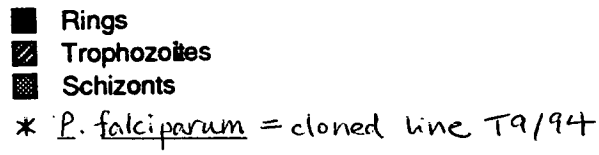
Key: R= rings
T= trophozoites
S= schizonts
* Parasite = cloned line T9/94

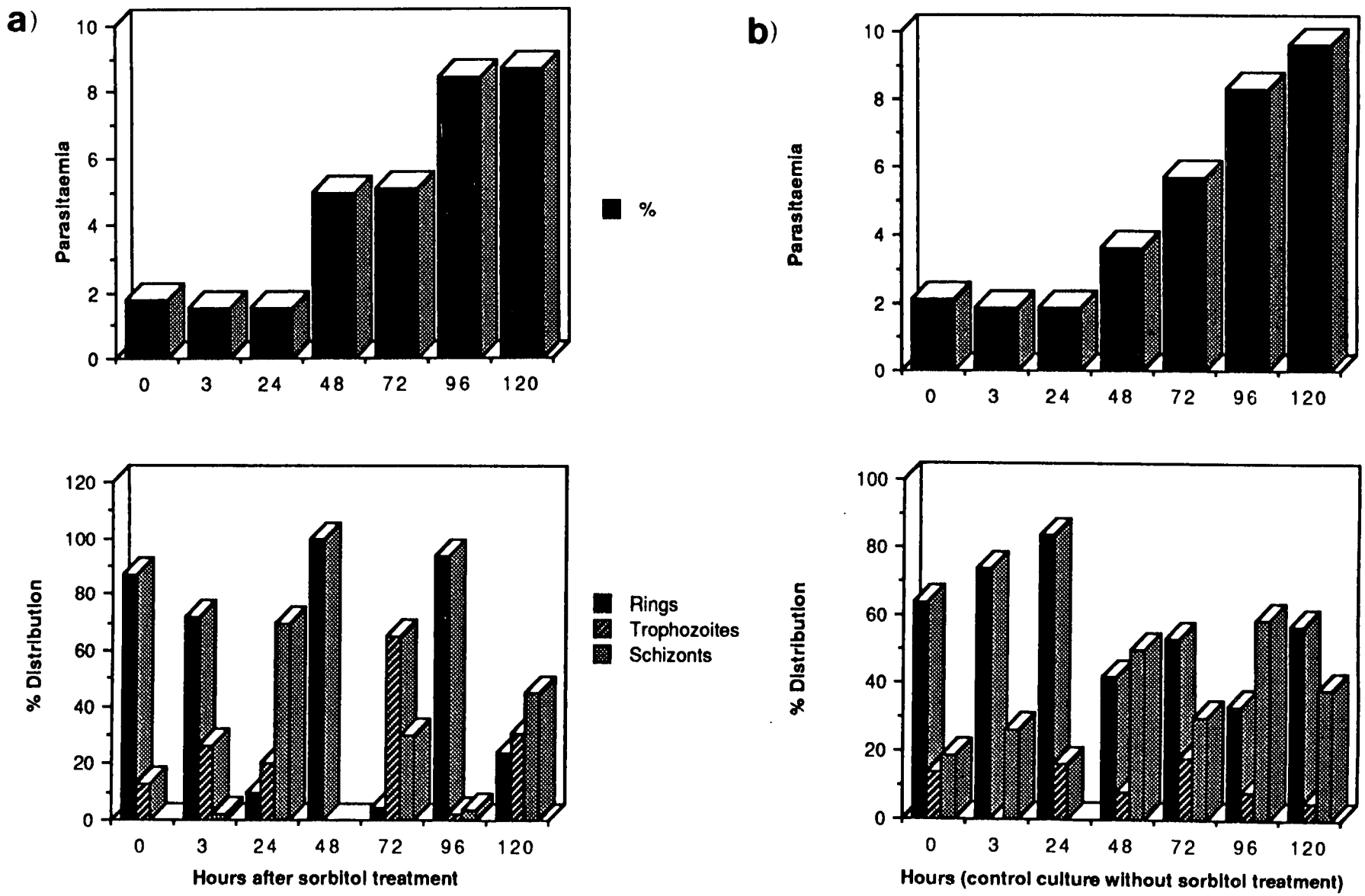
Fig. 4.4 Relative percent distribution of P. falciparum* stages during three cycles of synchronized growth in culture.

a) culture treated with 5% sorbitol for 5min.

b) control culture without sorbitol treatment.

Time is given in hours after sorbitol treatment.




a)
Parasitaemia
10
8
6
4
2
0
0 3 24 48 72 96 120
%
% Distribution
120
100
80
60
40
20
0
0 3 24 48 72 96 120
Hours after sorbitol treatment
Rings
Trophozoites
Schizonts
b)
Parasitaemia
10
8
6
4
2
0
0 3 24 48 72 96 120
% Distribution
100
80
60
40
20
0
0 3 24 48 72 96 120
Hours (control culture without sorbitol treatment)

amounts of protein, as adjusted from the measurements, were then used in the reaction.

3. The reaction products were fractionated by 10% SDS-PAGE gel which was then stained. The staining pattern of the fractionated polypeptides invariably indicated that similar amounts of protein had been applied to the gel.

It is important when quantifying the enzyme, other factors should not be limiting. The use of 1μl and 10μl of the substrate [6-^{3}H] F-dUMP was therefore compared in the quantification of TS in initial experiments (see below). As no differences in the intensity of the bands were detected, this indicated that 1μl of the substrate is not limiting and therefore this quantity of [6-^{3}H] F-dUMP was used in subsequent experiments.

4.2.4 The DHFR-TS is Not Over-produced in HB3 Compared to 3D7

At the time of harvest, the parasites of 3D7 and HB3 were at similar stages of synchronized development, with about 80% of the parasites in the schizont stage (Table 4.2). After resuspending the parasites to a similar density and extracting the total protein by sonication, equal amounts of protein, as measured by Bradford's reagents, were used for the assay. The results of the assay are shown in Fig. 4.5 and the densitometric values are listed in Table 4.3. Either 1μl (tracks 3 & 4) or 10μl (tracks 1 & 2) of [6-^{3}H] F-dUMP were used in the assay. Similar staining patterns of the fractionated polypeptides from 3D7 (tracks 2 & 4) and HB3 (tracks 1 & 3) on SDS-PAGE gel indicated that similar amounts of protein were loaded onto the gel. The [6-^{3}H] F-dUMP labelled proteins of 3D7 and HB3 had a relative molecular mass of 70kDa, as resolved on SDS-PAGE gel, confirming that there were no gross aberrant structural changes in the proteins which would otherwise affect its mobility on polyacrylamide gel. The molecular mass of the DHFR-TS

Table 4.2 The percentage distribution of parasites at different stages of development from HB3 and 3D7.

	HB3				3D7			
	Percentage (%)				Percentage (%)			
	R	T	S	G	R	T	S	G
	10	2	83	5	12	2	79	7
Parasitaemia	6				8.6			

R= rings; T= trophozoites; S= schizonts; G=gametocytes

Fig. 4.5 Measurement of the quantity of thymidylate synthetase in 3D7 and HB3. Total protein from 3D7 (tracks 2 & 4) and HB3 (tracks 1 & 3) was quantified by the TS assay using either 1μl (tracks 3 & 4) or 10μl (tracks 1 & 2) of [6-^{3}H] F-dUMP in the reaction. The reaction products were fractionated by 10% SDS-PAGE gel before subjected to processing for fluorography.

a) Coomassie stained SDS-PAGE gel of the fractionated polypeptides.

b) Fluorographs of the gel. The tritium labelled reaction products are indicated (arrow).

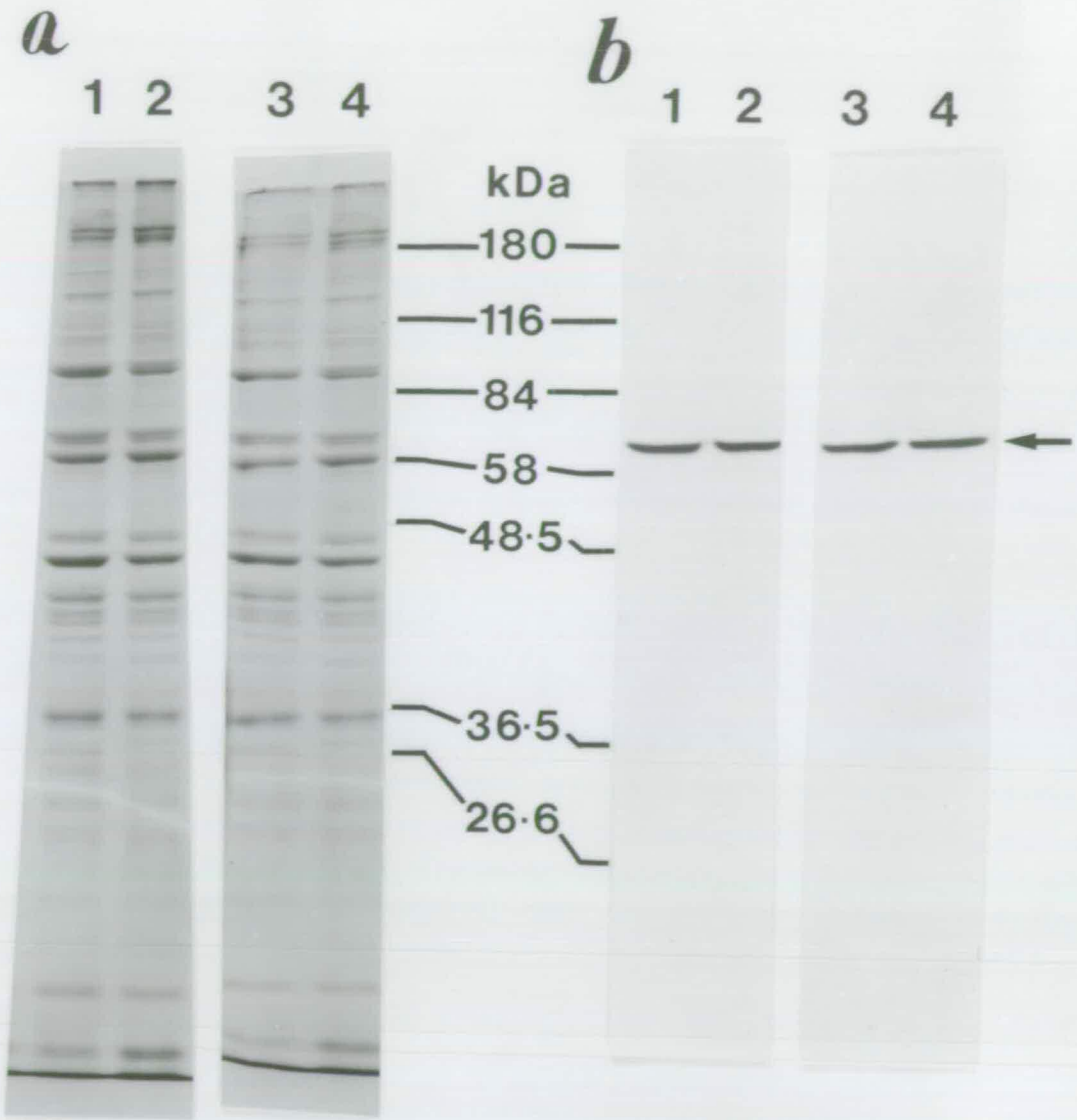

a
1 2 3 4
kDa
180
116
84
58
48·5
36·5
26·6
b
1 2 3 4

Table 4.3 Densitometric values of the reaction products of the thymidylate synthetase assay from HB3 and 3D7.

Quantity of [6-^{3}H] F-dUMP used in the assay	Densitometric values		Ratio $\frac{\text{HB3}}{\text{3D7}}$
	HB3	3D7	
10μl	10578	10277	1.0
1μl	10317	10172	1.0

protein has been estimated by gel chromatography and SDS-PAGE to be ~70kDa (Chen & Zolg, 1987). Similarly, the DHFR-TS gene sequence predicts a protein with MW of 72kDa (Bzik *et al.*, 1987; Snewin *et al.*, 1989).

Densitometric scanning of the 3D7 and HB3 bands gave similar values showing that the DHFR-TS was produced at a similar level in the two parasites. Similar readings were obtained with 1μl or 10μl of [6-^{3}H] F-dUMP in the assay. Control reactions replacing the parasite extracts with equal volume of buffer were performed in parallel. The absence of any bands confirmed the parasitic origin of the radiolabelled products in previous experiments (data not shown).

The results shown here were reproducible and I therefore conclude that the protein is produced at a similar level in the two parasite clones.

4.2.5 The DHFR-TS Gene is Not Amplified in HB3 Compared to 3D7

Since the enzyme is not over-produced in HB3, we would not expect to see any amplification of the gene in HB3. This was confirmed using dot-blot analysis on genomic DNA obtained from the two clones HB3 and 3D7.

The concentrations of DNA were determined spectrophotometrically (see MATERIALS & METHODS Section 2.4.3). Measured amounts of DNA from 3D7 and HB3 at serial dilutions (from 125/123ng to 8ng) were spotted onto Hybond-N filters which were then probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate. To confirm that equal amounts of DNA were spotted onto the filters from the two clones, control filters containing serial dilutions of DNA (900ng, 450ng, 225ng and 113ng) were probed with a rRNA gene fragment whose content is conserved among various strains (Langsley *et al.*, 1983).

The results are shown in Fig. 4.6 and the densitometric values are listed in Table 4.4. Hybridization of different dilutions of the two DNA

Fig. 4.6 Dot-blot analysis of DNA from parasite clones 3D7 and HB3. Dilutions of DNA (as shown) were spotted onto Hybond-N filters, denatured and probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate or the rRNA gene fragment prib2.


125 63 31 16 8 ng total DNA
3D7
HB3
DHFR
probe
123 61 31 15 8 ng total DNA
900 450 225 113 ng total DNA
3D7
HB3
rRNA gene
probe
900 450 225 113 ng total DNA

Table 4.4 Dot-blot analysis of DNA from HB3 and 3D7.

a) DHFR probe

DNA concentrations (ng)	Densitometric values		Ratio $\frac{\text{HB3}}{\text{3D7}}$
	HB3	3D7	
123/125	26804	29156	0.91
61/63	15046	15665	0.95
31	10068	9864	1.04
15/16	3778	3754	1.00
8	n.m.	n.m.	-
	Average		1.00

n.m.= no measurements

b) rRNA gene probe

DNA concentrations (ng)	Densitometric values		Ratio $\frac{\text{HB3}}{\text{3D7}}$
	HB3	3D7	
900	18575	16663	1.10
450	12980	11470	1.10
225	7408	7309	1.00
113	4584	4042	1.10
	Average		1.08

samples with equal intensity to the DHFR-TS gene probe, as determined by densitometric scanning of the dots, suggested that the 3D7 and HB3 parasites have the same number of copies of the gene. The control filters probed with the rRNA gene fragment gave similar densitometric values for the two DNA samples at the four different dilutions of DNA, confirming that equal amounts of DNA were spotted onto the filter.

We can therefore conclude that the two parasites possess the same number of copies of the DHFR-TS gene which is expressed at a similar level in the two parasites. The change associated with the DHFR-TS gene which affects the level of resistance to pyrimethamine in HB3 is most likely the single base change converting a serine to an asparagine at codon 108, resulting in a structural altered enzyme with reduced affinity for pyrimethamine (Cowman *et al.*, 1988; Peterson *et al.*, 1988).

4.2.6 The DHFR-TS is Over-produced in an in vitro Selected Mutant T9/94 (M1-1) Than in the Parent Clone T9/94

An array of pyrimethamine resistant (pyrR) mutants has already been obtained in the laboratory of Dr Sodsri Thaithong, Chulalongkorn University, Thailand. Briefly, $1.7x10^7$ parasites of the parent clone T9/94 synchronized to the early schizont stage were treated with 1.0μg/ml of the mutagen N-methyl-N-nitro-N-nitrosoguanidine (MNNG) in complete RPMI medium when the parasites were actively replicating. After 12hrs, a second treatment with the same concentration of mutagen was given. Parasites surviving after 12hrs of incubation in the mutagen were allowed to go through asexual cycles of development without the mutagen for 20 days to express the mutant phenotypes. Parasites resistant to pyrimethamine were selected by exposing $5x10^7$ parasites to $5x10^{-8}$M pyrimethamine for five days. The selection procedure was repeated with increasing concentrations of pyrimethamine (10^-

^{7}M and $3x10^{-7}$M) on surviving parasites expressing resistance to pyrimethamine. Individual pyrR clones were then isolated. One of them, T9/94 (M1-1), had a MIC of $5x10^{-6}$M and is 100 times more resistant than the parent clone T9/94. This clone was selected for analysis in this study.

The level of DHFR-TS expressed in the pyrR mutant T9/94 (M1-1), as compared to its sensitive parent T9/94, was first quantified by the TS assay to see whether there is any over-production of the enzyme in the mutant. Similar measures as described in Secion 4.2.3 were undertaken to ensure that equal amounts of protein were put into the reactions. At the time of harvest, the parasites of T9/94 and T9/94(M1-1) were at similar stages of synchronized development, with more than 86% of the parasites in the young schizont stage (Table 4.5). Protein extracts prepared from these synchronized cultures were used in equal amounts, as determined by the Bradford's method, in comparative studies. The reaction products [6-^{3}H] F-dUMP labelled proteins were electrophoresed on SDS-PAGE gel and visualised by PPO impregnation followed by fluorography.

The results are shown in Fig. 4.7. The Coomassie staining pattern of the fractionated polypeptides from T9/94 (tracks 2 & 4) and T9/94 (M1-1) (tracks 1 & 3) revealed that similar amounts of protein were applied to the gel. Both the T9/94 and T9/94 (M1-1) DHFR-TS enzymes had a relative molecular mass of 70kDa suggesting that there were no gross structural changes in the mutant protein. Densitometric scanning of the bands detected a two-fold increase in the level of DHFR-TS in the mutant (Table 4.6). Similar readings were obtained with either 1μl (tracks 3 & 4) or 10μl (tracks 1 & 2) of [6-^{3}H] F-dUMP in the assay. Control reactions using equivalent volume of buffer instead of parasite extracts did not reveal any radiolabelled bands (data not shown).

The assay was repeated several times starting from fresh cultures of

Table 4.5 The percentage distribution of parasites at different stages of development from T9/94 (M1-1) and T9/94.

	T9/94			T9/94 (M1-1)		
	Percentage (%)			Percentage (%)		
	R	T	S	R	T	S
	8	6	86	6	7	87
Parasitaemia	6.9			6.8		

R= rings; T= trophozoites; S= schizonts

Fig. 4.7 Measurement of the quantity of thymidylate synthetase in T9/94 and T9/94 (M1-1). Total protein from T9/94 (tracks 2 & 4) and T9/94 (M1-1) (tracks 1 & 3) was quantified by the TS assay using either 1μl (tracks 3 & 4) or 10μl (tracks 1 & 2) of [6-^{3}H] F-dUMP in the reaction. The reaction products were fractionated by 10% SDS-PAGE gel before subjected to processing for fluorography.

a) Coomassie stained SDS-PAGE gel of the fractionated polypeptides.

b) Fluorographs of the gel. The tritium labelled reaction products are indicated (arrow).

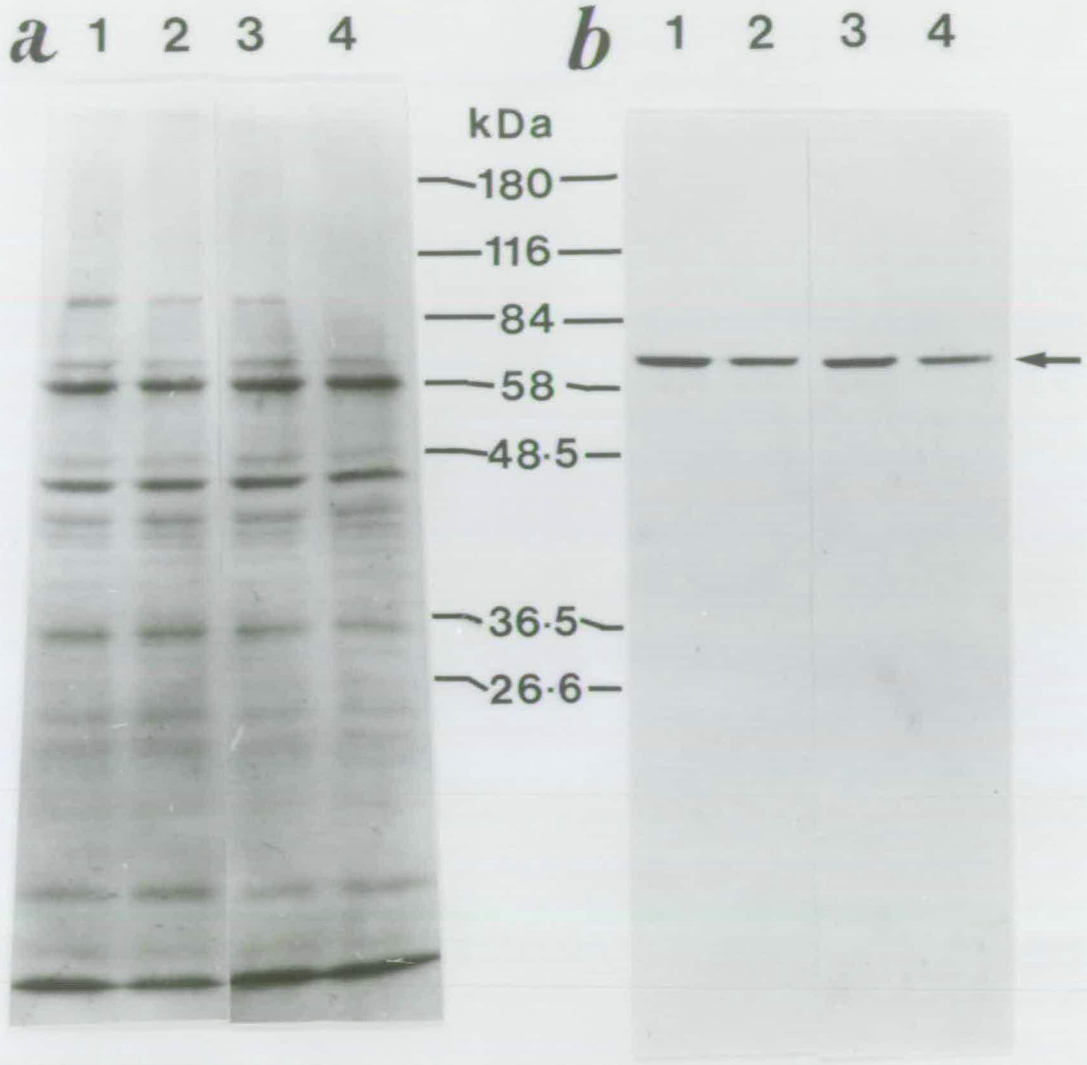

a
1
2
3
4
kDa
180
116
84
58
48·5
36·5
26·6
b
1
2
3
4

Table 4.6 Densitometric values of the reaction products of the thymidylate synthetase assay from T9/94 (M1-1) and T9/94.

Quantity of [6-^{3}H] F-dUMP used in the assay	Densitometric values		Ratio $\frac{\text{T9/94 (M1-1)}}{\text{T9/94}}$
	T9/94	T9/94 (M1-1)	
10μl	2150	4026	1.90
1μl	1695	3682	2.20

parasite and these results were fully reproducible giving increased protein values ranging from 1.80 to 2.20.

4.2.7 The DHFR-TS Gene is Not Amplified in T9/94 (M1-1)

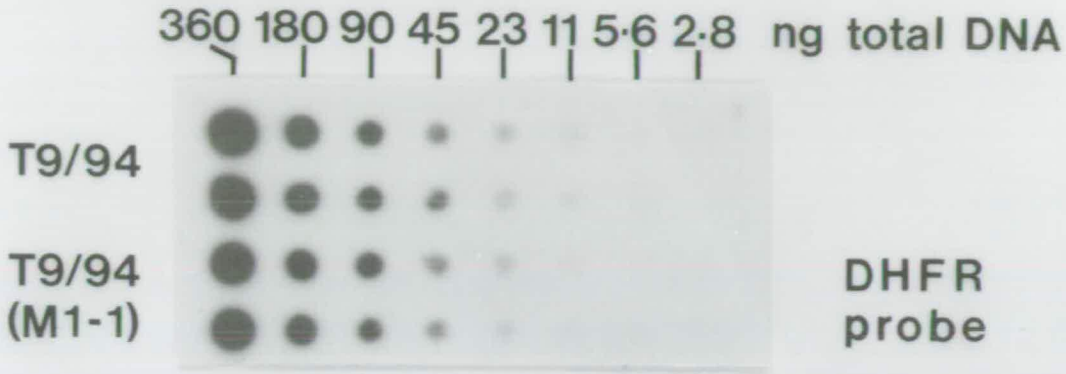
Having established that there is a two-fold increase in the level of the protein in the pyrimethamine resistant mutant from T9/94, it is necessary to find out the mutation responsible for the change in the enzyme level.

An obvious cause could be amplification of the gene. Attempts were made to detect any variation in the number of copies of the gene in the mutant by dot-blot analysis. Equal amounts of the two DNA samples, as determined spectrophotometrically (Section 2.4.3), were spotted at serial dilutions from 360ng to 2.8ng onto Hybond-N filters. These filters were then probed with the 5.65kb DHFR-TS fragment from the K1 isolate to quantify the number of copies of the gene. As a control, serial dilutions of 500ng, 125ng, 50ng and 12.5ng DNA were spotted onto the filters which were then probed with the rRNA gene fragment.

The results are shown in Fig. 4.8 and the densitometric values are listed in Table 4.7. Hybridization of different dilutions of the two DNA samples with equal intensity to the DHFR-TS gene probe, as determined by densitometric scanning of the dots, suggested that the number of copies of the gene is unchanged in the mutant. The control filters probed with the rRNA gene fragment gave similar densitometric values for the two DNA samples at the four different dilutions of DNA, confirming that equal amounts of DNA were spotted onto the filters.

These results suggest that amplification of the DHFR-TS gene had not occurred in the mutant T9/94 (M1-1) to account for the over-production of the protein in this mutant. In addition, it is worth noting that the parent clone T9/94 and the mutant T9/94 (M1-1) were included in an RFLP

Fig. 4.8 Dot-blot analysis of DNA from parasite clones T9/94 and T9/94 (M1-1). Dilutions of DNA (as shown) were spotted onto Hybond-N filters, denatured and probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate or a rDNA gene fragment.


360 180 90 45 23 11 5·6 2·8 ng total DNA
T9/94
T9/94
(M1-1)
DHFR
probe

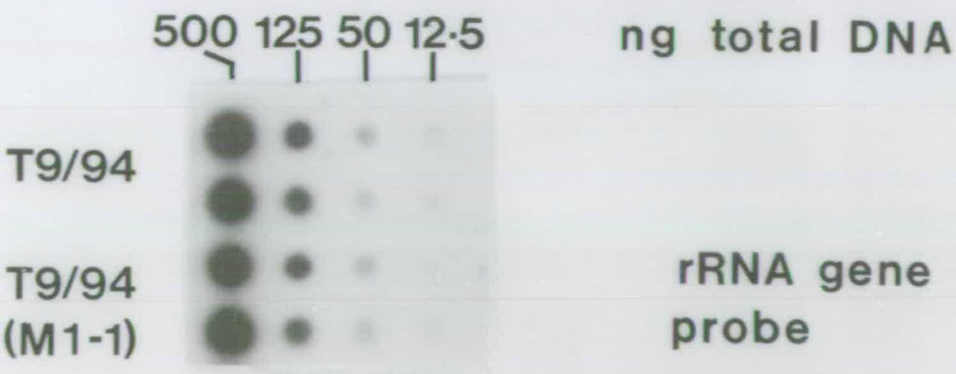

500 125 50 12·5 ng total DNA
T9/94
T9/94
(M1-1)
rRNA gene
probe

Table 4.7 Dot-blot analysis of DNA from T9/94 (M1-1) and T9/94.

a) DHFR probe

DNA concentrations (ng)	Densitometric values		Ratio $\frac{\text{T9/94 (M1-1)}}{\text{T9/94}}$
	T9/94	T9/94 (M1-1)	
180	41922	41446	1.0
90	22793	23595	1.0
45	13056	11209	0.9
22.5	4582	3624	0.8
	Average		0.9

b) rRNA gene probe

DNA concentrations (ng)	Densitometric values		Ratio $\frac{\text{T9/94 (M1-1)}}{\text{T9/94}}$
	T9/94	T9/94 (M1-1)	
500	77907	69300	0.9
125	13999	11401	0.8
50	1920	1708	0.9
	Average		0.9

analysis in CHAPTER 3 (Fig. 3.9). No difference was observed between the two patterns suggesting that there is no gross rearrangement across 5.65kb within the immediate vicinity of the gene that may affect gene expression. Therefore, an alteration resulting in over-production of the protein at the transcriptional level would seem to be likely.

4.2.8 The Level of DHFR-TS is Elevated in a Parasite Clone PR70/CB3 Obtained from a Thai Isolate, PR70 Compared to T9/94

Identification of an *in vitro* selected mutant over-producing the protein suggests this as a possible mechanism for pyrimethamine resistance. It is therefore interesting to see whether over-production of the enzyme occurs in the wild isolates. A preliminary study was carried out with a cloned line of PR70, PR70/CB3. This cloned line is 400 times more resistant to pyrimethamine than the pyrS Thai isolate T9/94.

The level of TS was compared in T9/94 and PR70/CB3 by the TS assay using equal amounts of synchronous parasites. Both parasite cultures were harvested at the time of synchronized development, with more than 80% of the parasites in schizont stage (Table 4.8). Similar amounts of protein, as measured by Bradford's reagent, were put into the reactions. The results are depicted in Fig. 4.9. The Coomassie staining patterns of the fractionated polypeptides confirmed that equal amounts of protein were used in the reactions. The fluorographs showed that both enzymes had an identical relative molecular mass of 70kDa, suggesting that there were no gross aberrant changes in the proteins. The amount of TS, however, was quantitatively greater in PR70/CB3. Densitometric scanning of the bands showed that the level of the protein was at least 1.3-fold higher in PR70/CB3 than in T9/94. Control reactions using an equivalent volume of buffer in place of parasite extracts did not reveal any radiolabelled bands (data not

Table 4.8 The percentage distribution of parasites at different stages of development from PR70/CB3 and T9/94.

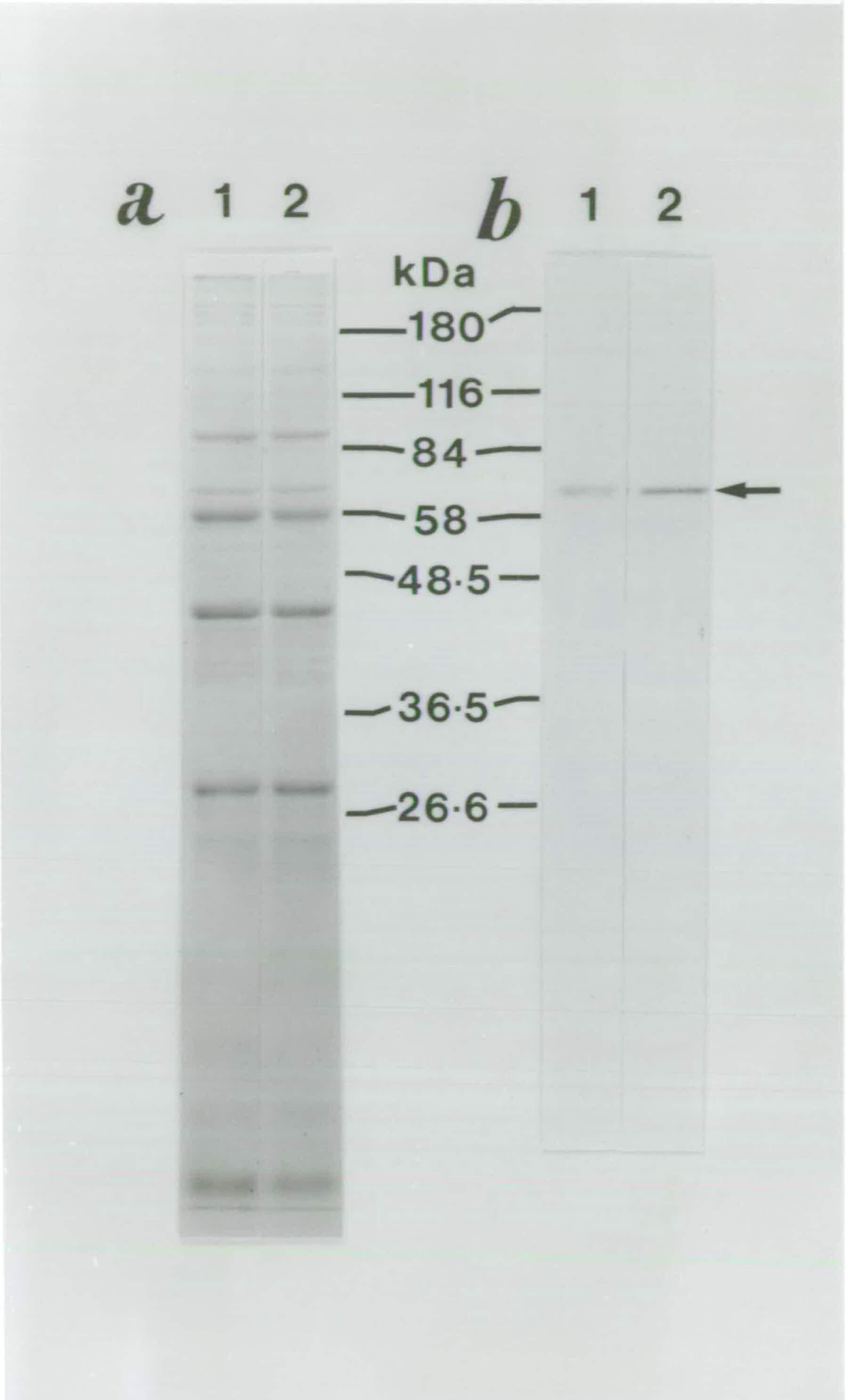
	PR70/CB3				T9/94			
	Percentage (%)				Percentage (%)			
	R	T	S	G	R	T	S	G
	5	9	82	4	9	6	85	0
Parasitaemia	5.2				6.9			

R= rings; T= trophozoites; S= schizonts; G=gametocytes

Fig. 4.9 Measurement of the quantity of thymidylate synthetase in PR70/CB3 and T9/94. Total protein from PR70/CB3 (track 2) and T9/94 (track 1) was quantified by the TS assay using 1μl of [6-^{3}H] F-dUMP in the reaction. The reaction products were fractionated by 10% SDS-PAGE gel before subjected to processing for fluorography.

a) Coomassie stained SDS-PAGE gel of the fractionated polypeptides.

b) Fluorographs of the gel. The tritium labelled reaction products are indicated (arrow).


a
1
2
b
1
2
kDa
180
116
84
58
48·5
36·5
26·6

shown). It would therefore seem possible that PR70/CB3 produces the enzyme at a higher level than T9/94.

4.2.9 The DHFR-TS Gene is Not Amplified in PR70/CB3

The increased level of the protein in PR70/CB3 as compared to T9/94 might be due to changes such as gene amplification. However, dot-blot analysis excluded this possibility as the same number of copies of gene was detected in PR70/CB3 and T9/94.

The results are shown in Fig. 4.10 and the densitometric values are listed in Table 4.9. Serial dilutions of equal amounts of the two DNA samples, as determined spectrophotometrically, were spotted onto Hybond-N filters. The filters were probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate and the rRNA fragment respectively. As revealed from the hybridization intensity to the rRNA probe, about 1.7 times more DNA from PR70/CB3 was spotted onto the filters. Similarly, hybridization of the PR70/CB3 DNA to the DHFR-TS gene probe was about 1.7 times more intense than T9/94 DNA. These results suggest that the number of copies of the gene in PR70/CB3 is the same as that in T9/94.

This experiment confirms the importance of the controls. If a control was not included, the above results would be wrongly interpreted as the presence of an increased number of copies of the gene in PR70/CB3 compared to that of T9/94.

In addition, there were no gross changes within the immediate vicinity of the gene to account for the increased level of the enzyme in PR70/CB3 since the *Acc*I restriction patterns of the genomic DNA from both clones were identical upon probing with the 5.65kb DHFR-TS fragment from the K1 isolate (see CHAPTER 3 Fig. 3.9).

Fig. 4.10 Dot-blot analysis of DNA from parasite clones PR70/CB3 and T9/94. Dilutions of DNA (as shown) were spotted onto Hybond-N filters, denatured and probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate or the rRNA gene fragment prib2.

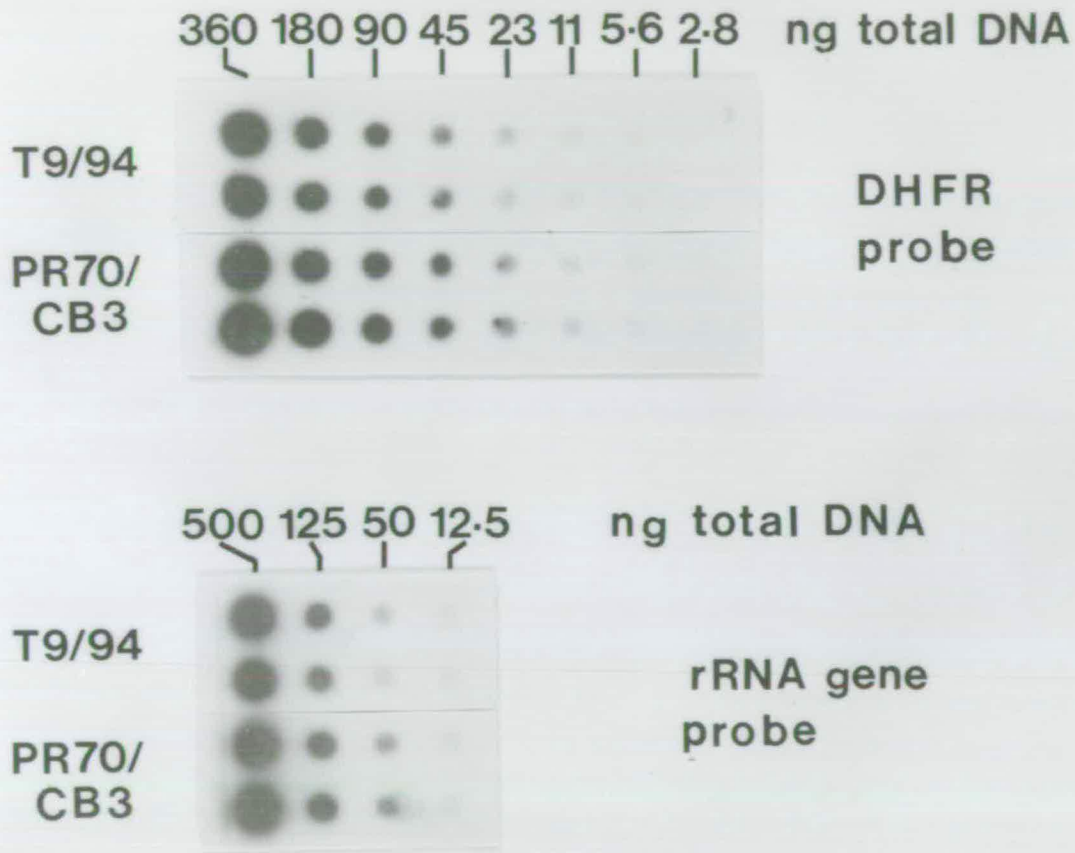

360 180 90 45 23 11 5·6 2·8 ng total DNA
T9/94
PR70/
CB3
DHFR
probe
500 125 50 12·5 ng total DNA
T9/94
PR70/
CB3
rRNA gene
probe

Table 4.9 Dot-blot analysis of DNA from PR70/CB3 and T9/94.

a) DHFR probe

DNA concentrations (ng)	Densitometric values		Ratio PR70/CB3 / T9/94
	PR70/CB3	T9/94	
180	72370	41921	1.70
90	36988	22792	1.62
45	21674	13056	1.70
22.5	9512	5281	1.80
	Average		1.70

b) rRNA probe

DNA concentrations (ng)	Densitometric values		Ratio PR70/CB3 / T9/94
	PR70/CB3	T9/94	
125	27218	16559	1.65
50	11076	6221	1.78
12.5	2452	1475	1.70
	Average		1.71

4.3 DISCUSSION

Resistance to pyrimethamine can be due to one or more of the following mechanisms. Firstly, the structure of the enzyme may be altered due to single base changes within the coding region. Operation of this mechanism in *P. falciparum* is well confirmed from many sources. Secondly, the amount of the enzyme may be increased. This mechanism is found in *in vitro* selected mutants and may occur in wild isolates. Thirdly, the accumulation of the drug inside the parasites may be reduced. Finally, the parasites may utilize an alternative folate metabolic pathway to bypass the toxic effect of the drug. These last two mechanisms have yet to be found in *P. falciparum*.

In the clone HB3, a single base substitution within the coding region of the gene is detected as the only change so far identified which can account for its resistance to pyrimethamine although Zolg's results (Chen *et al.*, 1987; Zolg *et al.*, 1989) have pointed out the possible participation of other mechanisms contributing to pyrimethamine resistance in HB3. In their studies, they found a disproportionality in the concentration of pyrimethamine needed to inhibit the growth of the parasite and the concentration of pyrimethamine required to inhibit the activity of the enzyme. The present study has excluded over-production of the protein as one of these possible changes.

The existence of other possible mechanisms is illustrated by an *in vitro* selected mutant T9/94 (M1-1) which over-produces the protein two-fold compared to the T9/94 parent from which it is derived. In this mutant, the gene has not been amplified. The sensitivity of the tests in detecting over-production of the enzyme or gene amplification is evident from the reproducibility of these results and similar analyses undertaken by Inselburg and co-workers (1987). Using the same techniques as in the present study,

Inselburg's group was able to detect a mutant which over-produces the protein ten-fold and contains about three copies of the DHFR-TS gene. Evidently, the present results identify a mutant which over-produces the protein but not because of amplification of the gene. The region encoding DHFR of the DHFR-TS gene of T9/94 (M1-1) has been sequenced and showed no difference from that of the parent clone T9/94 (G. Beale, personal communication). A change at the transcriptional level would seem to be likely.

The fact that this mutant derives from a genetically pure parent clone makes more precise analysis of the mutation(s) causing pyrimethamine resistance possible. However, during the process of transcription of DNA to RNA and translation of RNA to protein, a number of events may have occurred resulting in an over-production of the final product. It is therefore necessary to carry out further tests to define more precisely the number and the type of mutation(s) resulting in an over-production of the enzyme in T9/94 (M1-1). Northern blot analysis and pulsed-field gel electrophoresis would be required to detect any changes in the level of mRNA transcribed and the structure of the chromosome in the mutant DNA.

The readiness to select mutants which over-produce the protein in two independent selection procedures, namely those of Inselburg and ourselves, suggests that these mutations may already exist and constitute a major portion of the natural gene pool. Perhaps over-production of the protein may have a more common occurrence in nature than has so far been thought. It is important that more clones obtained from pyrR field isolates be investigated and their DHFR-TS quantified. The results may reveal a high incidence of over-production of the protein among those pyrR parasite strains. Preliminary data in the present study has already suggested an elevated level of the enzyme in the pyrimethamine resistant cloned line PR70/CB3

compared to a pyrimethamine sensitive clone T9/94. Previous studies of Kan & Siddiqui (1979) also suggested an elevated level of the protein, as measured by the specific activity of the enzyme, in the Uganda isolate Palo-alto.

The T9/94 (M1-1) represents a putative expression mutant which derived from a genetically pure clone. This mutant offers a well-defined model for comparative studies to map the changes giving rise to increased production of the protein. To further identify the changes causing variation in gene expression, we need to look at transcriptional control. An initial step will be to map the transcriptional start sites. However, previous results in CHAPTER 3 show that a RFLP dimorphism exists in the region which could contain the transcriptional start sites. Therefore, we must first investigate the nature of the RFLP before proceeding with mapping the transcriptional start sites.

CHAPTER FIVE
THE NATURE OF THE RFLP ASSOCIATED WITH THE DHFR-TS GENE

5.1 RESULTS

5.2 DISCUSSION

The mutations giving rise to restriction-fragment-length-polymorphisms (RFLPs) of human genes are most often point mutation(s) resulting in creation or destruction of restriction enzyme sites such as occurs in the case of the β-globin gene (Kan *et al.*, 1980; Orkin *et al.*, 1982). It is also possible, however, that large insertion/deletion of DNA sequences is involved as in the case of α-globin genes (Higgs *et al.*, 1981). RFLP markers have proved to be useful and effective in prenatal diagnosis of human genetic diseases such as sickle cell anaemia and β-thalassaemia (Kan & Dozy, 1978a; b; Philips III *et al.*, 1980; Harper *et al.*, 1984; Oberle *et al.*, 1985).

RFLPs have been found associated with the dihydrofolate reductase-thymidylate synthetase (DHFR-TS) gene of *P. falciparum* (Peterson *et al.*, 1988 and see CHAPTER 3) and also the *pfmdr1* gene (Foote *et al.*, 1990b; Wellems *et al.*, 1990; 1991), the histidine-rich-protein-II (HRP-II) and HRP-III genes (Wellems *et al.*, 1987). Despite the utilization of RFLP markers in genetic studies of *P. falciparum*, the nature of a given RFLP has yet to be defined in this organism.

In this chapter I will describe results on the DHFR-TS gene which exclude the possibility of a simple creation/destruction of a restriction enzyme site. They predict the possible involvement of an insertion or deletion as large as 250bp within 1.35-1.6kb 5' of the initiating methionine codon. To further characterize the RFLP the non-coding regions of both the 3D7 and HB3 DHFR-TS genes were cloned and sequenced. Furthermore, sequencing the 5' untranslated regions upstream of the DHFR-TS gene was a pre-requisite for attempts to characterize the transcriptional unit of this gene (see CHAPTER 6) in the two dimorphic alleles described by the *Acc*I RFLP.

5.1 RESULTS

5.1.1 A Deletion Appears Responsible for the RFLP

A composite restriction map for the *P. falciparum* DHFR-TS genomic clone (Fig. 5.1) was constructed based on information obtained from restriction mapping of parasite strains K1 and Honduras-1 (Bzik *et al.*, 1987; Snewin *et al.*, 1989) and sequence data from a number of parasite strains (Cowman *et al.*, 1988; Peterson *et al.*, 1988). On the map, an *Acc*I site was located immediately 3' to the gene while another unique *Acc*I site was found within the coding region of the gene. These two sites delimited a fragment of 1.1kb in length which was common to 3D7 and HB3. The *Acc*I site within the coding region of the gene is conserved in both 3D7 and HB3 (Cowman *et al.*, 1988; Peterson *et al.*, 1988).

Since the *Acc*I restriction pattern gave four bands, two further *Acc*I sites were likely to be positioned upstream of the gene. The RFLP pattern is consistent with deletions. Sequence data on these genes did not reveal a large deletion of sequence within the coding region (Cowman *et al.*, 1988; Peterson *et al.*, 1988; Snewin *et al.*, 1989), the putative deletion covered by the probe is therefore likely to occur in the 5' flanking region. If so, cleavage with *Xba*I and *Eco*RI should also give dimorphic patterns. To do this, genomic DNA from 3D7 and HB3 was digested with either *Eco*RI or *Xba*I. After fractionation by agarose gel electrophoresis, the gel was blotted and the restriction patterns revealed by hybridization with the 5.65kb DHFR-TS fragment from the K1 isolate. The results are illustrated in Fig. 5.2. The dimorphic pattern was not unique to *Acc*I digestion, instead, consistent RFLP patterns were visualized upon enzymatic digestion with *Xba*I and *Eco*RI.

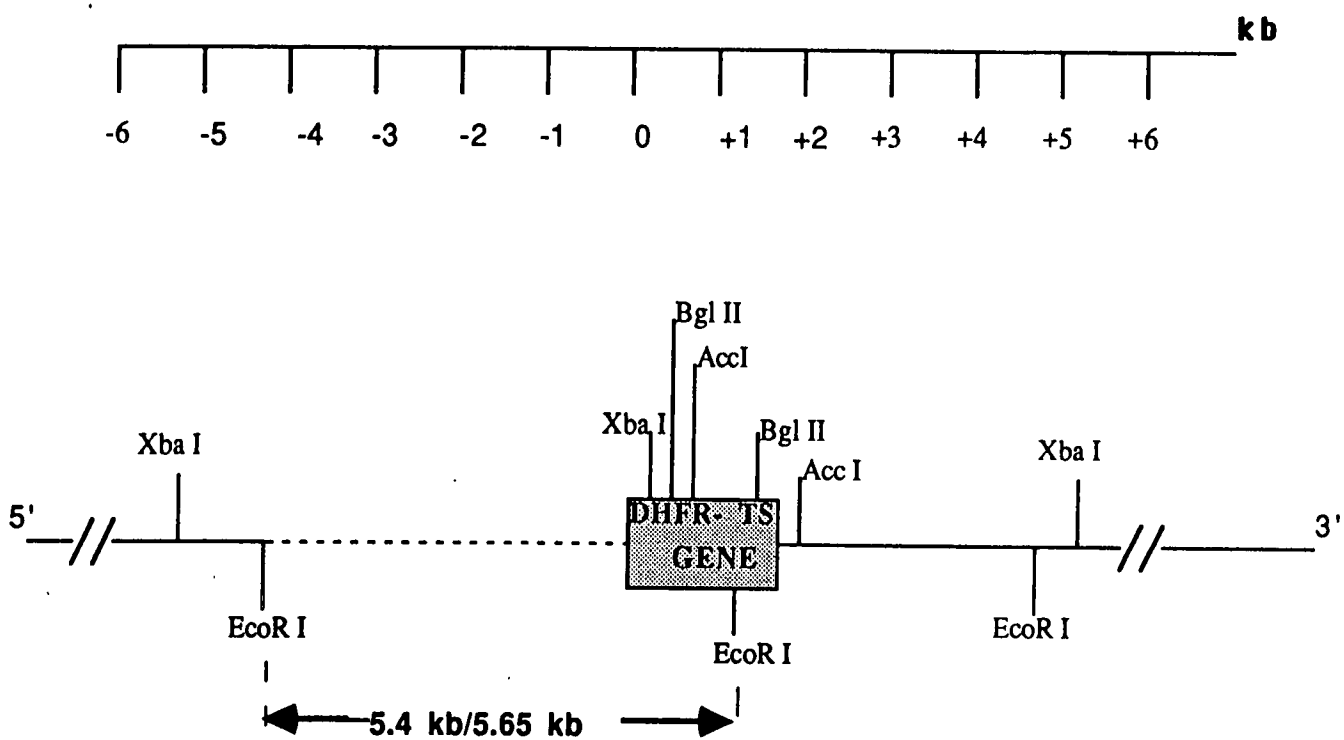
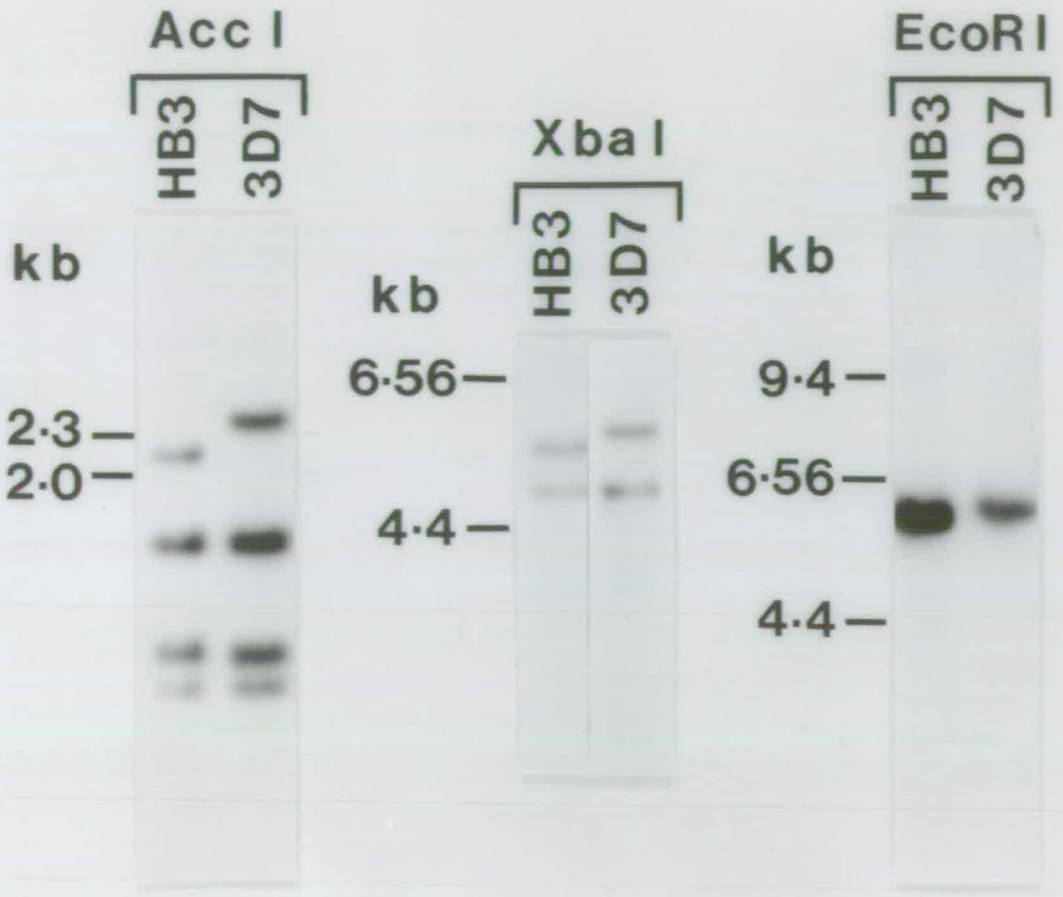


Fig. 5.1 The restriction map of the *P. falciparum* DHFR-TS gene. The map is composed from restriction mapping data of parasite strains K1 and Honduras-I and sequencing data from a number of parasite strains. (Bzik et al.,1987; Cowman et al., 1988; Peterson et al., 1988; Snewin et al., 1989). The dotted line represents the upstream region of the gene which contains 2 *Acc* I sites and a putative deletion in the HB3 clone. The 5.4/5.65kb *Eco* RI insert used as a probe for these studies is indicated.

Fig. 5.2 Dimorphic restriction patterns of 3D7 and HB3 genomic DNA. Autoradiographs of enzyme restricted genomic DNA from 3D7 and HB3 DNA, probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate.


Acc I
HB3
3D7
kb
2·3
2·0
Xba I
HB3
3D7
kb
6·56
4·4
EcoR I
HB3
3D7
kb
9·4
6·56
4·4

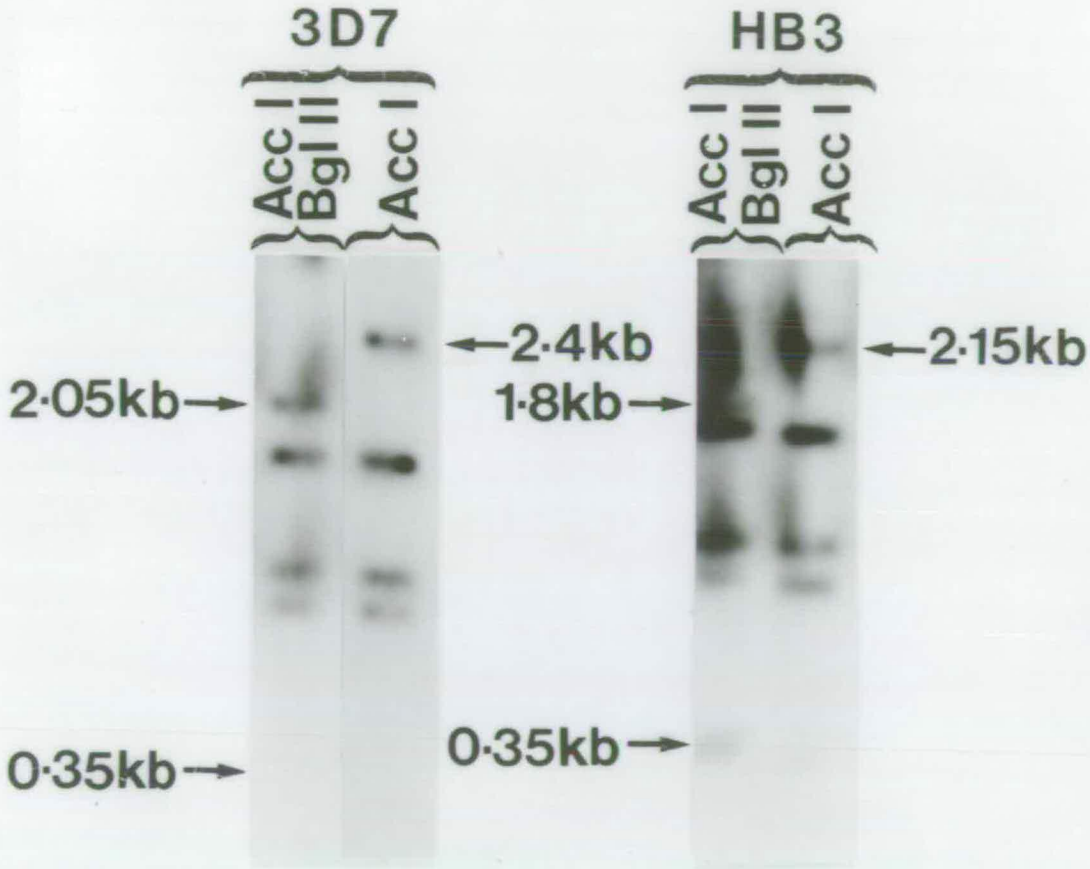
Cleavage with *Xba*I generated two fragments from each DNA. Size dimorphism was detected in the upper band around 5.5kb. Cleavage with *Eco*RI released one band from each DNA. A difference in size between the two fragments was again obvious.

These results suggest that the RFLP as observed in the 3D7 and HB3 genomic DNA is associated with a deletion of 250bp in HB3 DNA sequence and this deletion occurs 5' to the gene.

5.1.2 Mapping of the RFLP

From these results, we suspect that there is a deletion 5' to the gene and that two *Acc*I sites are located 5' to the gene in the region covered by the probe. To define the position of the first *Acc*I site 5' to the coding region, the *Bgl*II site at 5' end of the coding region (see Fig. 5.1) can be utilized since the *Acc*I fragment immediately 5' to the gene will be cut by *Bgl*II. Genomic DNA from 3D7 and HB3 was cleaved with restriction enzymes *Acc*I and *Bgl*II, fractionated by agarose gel electrophoresis, blotted on to a Nylon membrane and then probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate. The results are shown in Fig. 5.3. The dimorphic *Acc*I fragments (2.15/2.4kb) were cut by *Bgl*II into smaller fragments. Therefore, the putative deletion must occur within this fragment and map to within 1.35kb/1.6kb upstream from the methionine initiation codon. The relative position of the putative deletion to the gene is shown in the revised figure of the restriction map (Fig. 5.4).

Fig. 5.3 Identification of the first AccI fragment 5' to the gene. Autoradiographs of *Acc*I or *Acc*I/*Bgl*II restricted genomic DNA from 3D7 and HB3 DNA, probed with the 5.65kb DHFR-TS gene fragment from the K1 isolate.


3D7
HB3
Acc I
Bgl II
Acc I
Acc I
Bgl II
Acc I
2·4kb
2·05kb
1·8kb
2·15kb
0·35kb
0·35kb

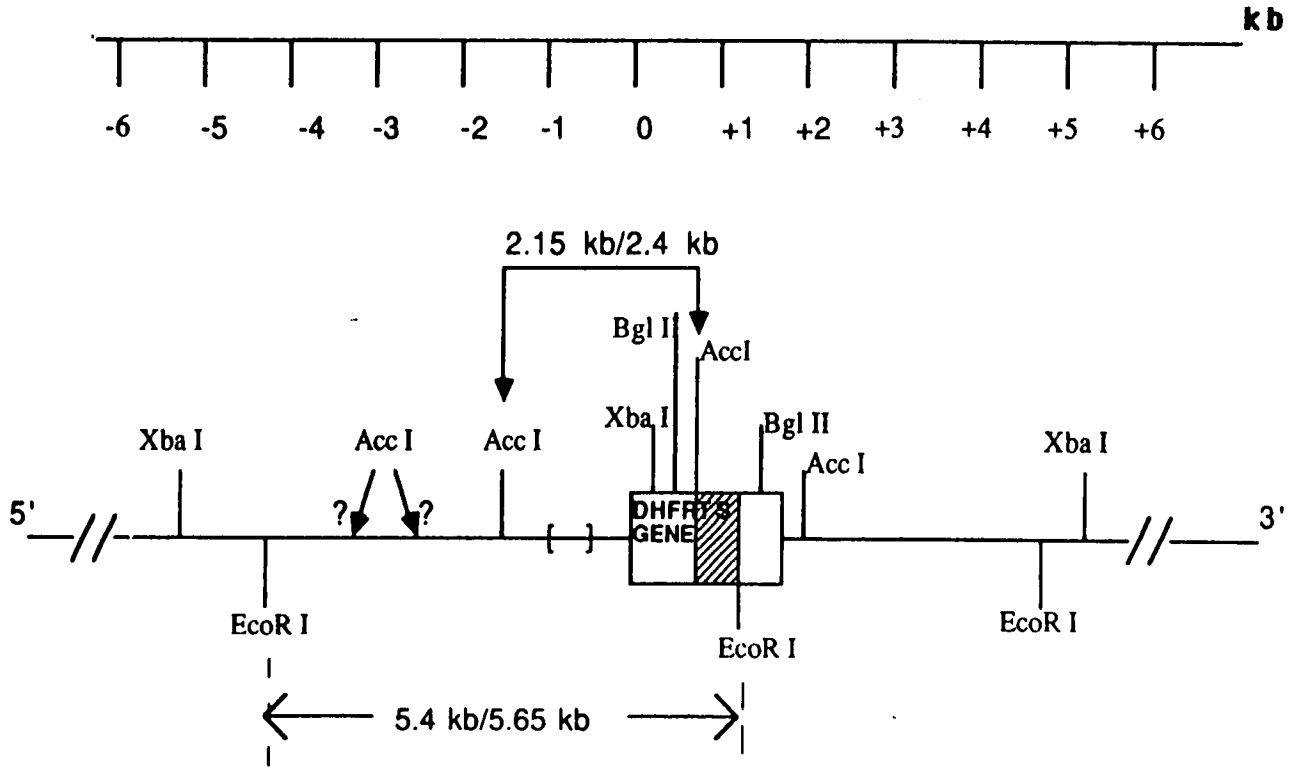


Fig. 5.4 A revised restriction map of the *P. falciparum* DHFR-TS gene. An additional *Acc* I site was located 1.35 kb/1.6kb upstream of the gene. This region also contains a deletion in the HB3 clone (see bracket). The arrows and question marks indicate the possible positions for another *Acc* I site. A 0.6 kb *Acc* I - *Eco* R I fragment used as a probe in many of these studies is indicated. (▨)

5.1.3. Deletion Occurs in a Gene Fragment from the K1 Isolate during λ Replication in E. coli

Further evidence from studying the K1 genomic *Eco*RI fragment used as a probe for most of this study also suggests that deletion may occur in the non-coding region.

Preparations of the K1 cloned insert invariably produced a doublet despite efforts to purify the bacteriophage by selecting isolated plaques. This is illustrated in Fig. 5.5 (plate a). Further digestion of the purified *Eco*RI insert with *Acc*I yielded a pattern identical to that of 3D7 and HB3 (plate b). The *Acc*I fragment also occurred as a doublet of 2.15kb/2.4kb. By analogy to the dimorphic *Acc*I fragments in 3D7 and HB3, the 2.15kb/2.4kb doublet was also cleaved by *Bgl*II into three smaller fragments of 2.05kb, 1.8kb and 0.35kb.

It is thought that the 2.15kb/2.4kb doublet is derived from the same region of DNA. This was shown to be true as cross-hybridization was observed between these two fragments. Purified *Eco*RI insert was digested with *Acc*I, fractionated by agarose gel electrophoresis, blotted and probed with either the 2.4kb or 2.15kb fragment purified independently from the doublet. The results (Fig. 5.6) reveal that both fragments were lit up by either probe. No cross-hybridization with other *Acc*I fragments were observed.

It appears that a deletion within the cloned K1 fragment has occurred during λ replication in *E. coli*. This is analogous to the dimorphic *Acc*I fragments which apparently arose from deletion of 250bp of sequence from the 5' flanking region of HB3 DNA. Subsequent sections in this chapter attempt to identify this dimorphism by cloning and sequencing the 5' non-

Fig. 5.5 Studies on the DHFR-TS insert from the isolate K1 cloned into λ.

a) Ethidium bromide stained gel of DNA cut with *Eco*RI to release two λ arms and the insert which occurred as a doublet (arrow).

b Ethidium bromide stained gel of the *Eco*RI inserts cut with either *Acc*I alone or with *Acc*I and *Bgl*II.

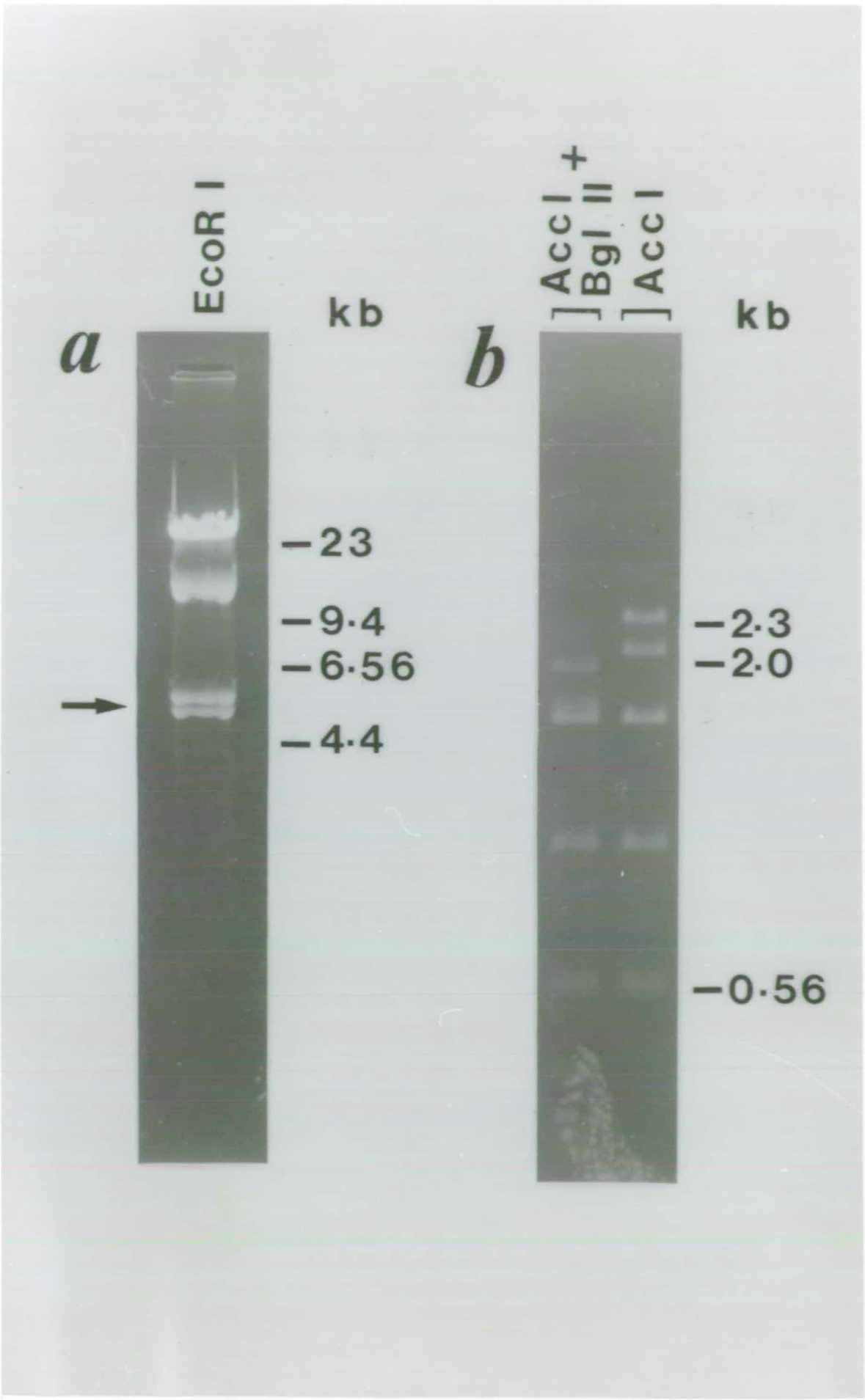
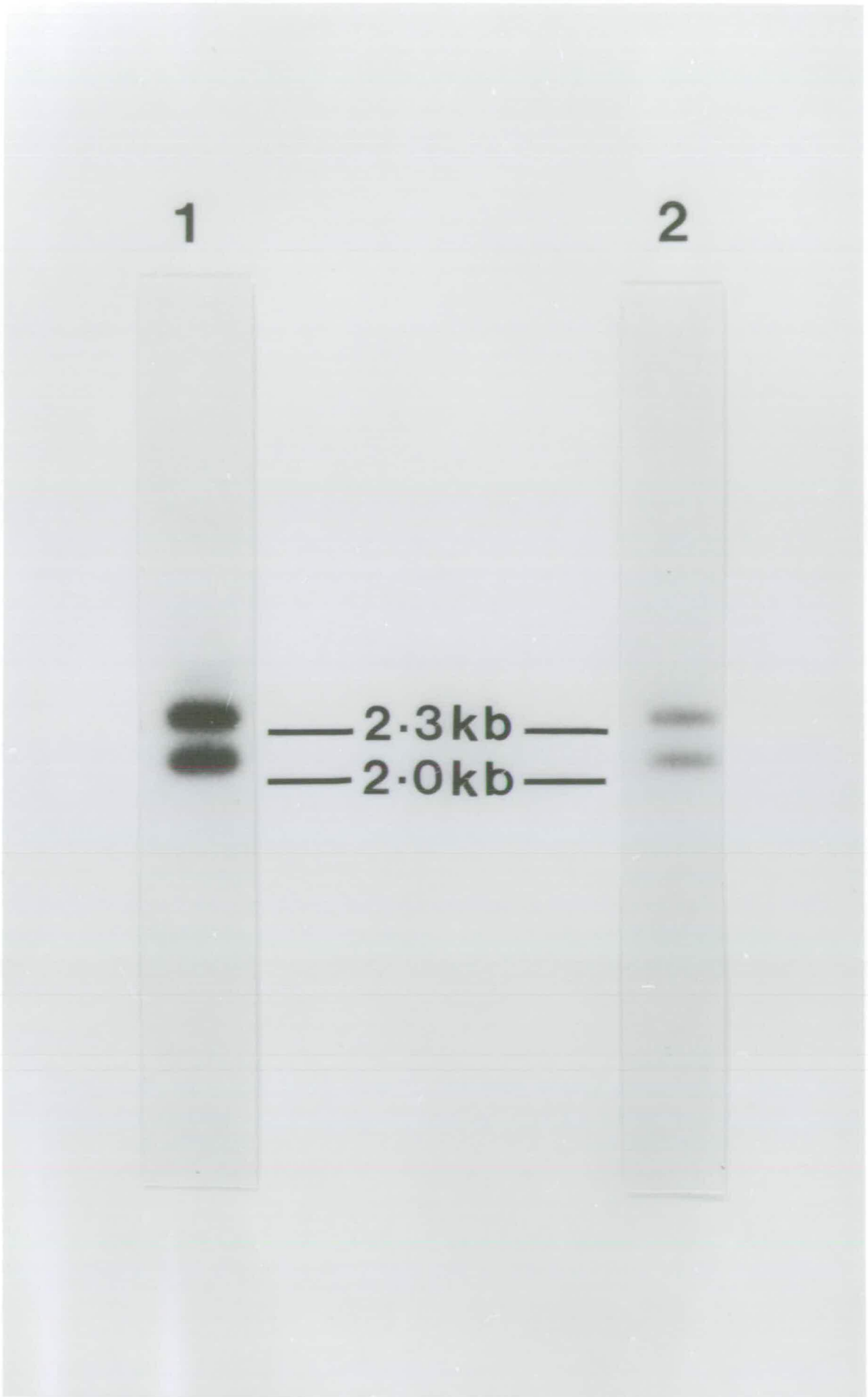

a
EcoR I
kb
23
9·4
6·56
4·4
b
] Acc I + Bgl II
] Acc I
kb
2·3
2·0
0·56

Fig. 5.6 Homology between the 2.4kb and 2.15kb fragments of the doublet. Autoradiographs of gels containing *Acc*I subfragments of the *Eco*RI insert from the K1 isolate, probed with the purified 2.4kb (track 1) or 2.15kb (track 2) fragments.


1
2
2·3kb
2·0kb

coding region of the DHFR-TS gene from both the 3D7 and HB3 clones.

5.1.4 Cloning of DHFR-TS Gene from HB3

The DHFR-TS gene has been successfully cloned from the K1 strain as an *Eco*RI fragment in the bacteriophage vector λNM1149 (Snewin *et al.*, 1989). Therefore, conventional cloning procedures were employed to clone the gene from HB3 into the same vector.

The cloned insert of K1 gave rise to a doublet and 3D7, which generated the same sized *Eco*RI fragment as K1, may also generate a doublet once cloned into a λ vector, yielding fragments of uncertain origin. On the other hand, HB3, which yielded a smaller *Eco*RI fragment, was expected to be able to maintain the insert in a stable form in λ. Therefore, an initial attempt was to clone the gene from HB3. To achieve this, I constructed a library of *Eco*RI fragments from the genomic DNA of HB3 in the λ vector NM1149 (Section 2.4.23). The library was screened for clones containing the DHFR-TS gene with a homologous 0.6kb *Acc*I-*Eco*RI gene fragment isolated from the λ K1 clone (see Fig. 5.4). Plaques giving positive signals were picked, enriched and purified (Section 2.4.24). The purification of one of these clones is illustrated in Fig. 5.7. Positive plaques were detected at a rate of 1 in 3,200 recombinant phages.

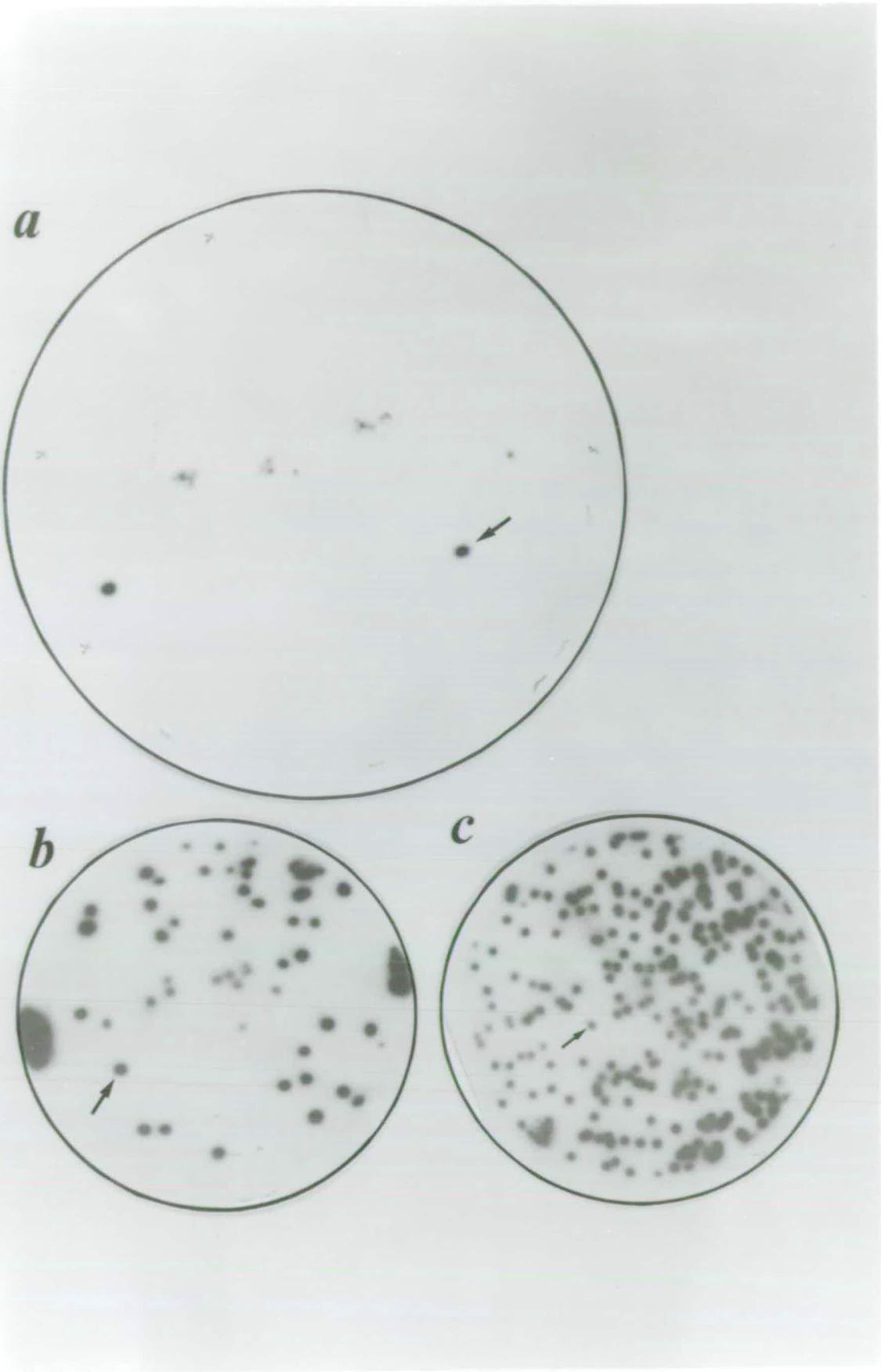
5.1.4.1. Characterization of the DHFR-TS Genomic Clones: To further characterize the inserts from a number of putative positive clones, bacteriophage λ DNA was prepared from 11 of them by the mini-prep method (Section 2.4.5.2). To check the sizes of the inserts, the phage DNA was digested with *Eco*RI to release the inserts from the λ arms. The sizes of the restricted DNA were then determined by agarose gel electrophoresis.

Fig. 5.7 Screening of HB3 EcoRI genomic library for recombinant clones containing DHFR-TS gene fragment.

a) Primary screening: Autoradiograph of plaque lift from the HB3 *Eco*RI genomic library, probed with the 0.6kb *Acc*I-*Eco*RI gene fragment from the K1 isolate. Agar containing positive plaque (arrow) was removed, diluted and the phages re-plated at an appropriate dilution to give well isolated plaques for re-screening.

b) Secondary screening: Autoradiograph of plaque lift after re-plating, probed with the same probe. During the secondary screening, positive plaques were segregated from negative plaques. One of them (arrow) was picked and re-plated at an appropriate dilution for tertiary screening.

c) Tertiary screening: Autoradiograph of plaque lift from second re-plating, probed with the same probe. Results showed that at this stage the phage were effectively plaque pure.


a
b
c

The results are depicted in Fig. 5.8 (plate a). In contrast to the cloned K1 insert which occurred as a 5.4kb/5.65kb doublet, only one insert of 5.4kb was released from each of these clones. This demonstrates the stability of the insert in λ.

To confirm that the DHFR-TS gene has been cloned, the gel was blotted and probed with the 0.6kb *Acc*I-*Eco*RI gene fragment from the K1 isolate. The results (Fig. 5.8, plate b) show that all of these 5.4kb inserts hybridized with the probe, which illustrates a high positive rate in clones screened from the library.

Two clones 423 and 92a3 were selected for further analysis. A large amount of DNA from these clones was prepared by the maxi-prep method (Section 2.4.5.1) and the *Eco*RI insert purified by electroelution from agarose gels (see MATERIALS & METHODS Section 2.4.10.1). These inserts were then analysed by restriction enzyme mapping to confirm the nature of the cloned insert by comparing with that of the cloned insert from the K1 isolate.

The results are illustrated in Fig. 5.9. Upon digestion with *Eco*RI, a 5.4kb insert was released from the HB3 clones. Further digestion of the purified *Eco*RI insert by either *Acc*I alone or *Acc*I and *Bgl*II yielded a pattern similar to that of K1. The DNA was then blotted and probed with the 0.6kb *Acc*I-*Eco*RI fragment from the K1 isolate. These results confirmed the isolation of the DHFR-TS gene of HB3 and allowed a restriction enzyme map of the gene to be produced (Fig. 5.10). The clone 423 was selected for all further work.

Fig. 5.8 Characterization of the DHFR-TS genomic clones.

a) Ethidium bromide stained gel of *Eco*RI digested DNA from 11 positive recombinant clones (tracks 1-11) of HB3.

b) Southern blot of gel probed with the 0.6kb *Acc*I-*Eco*RI fragment from the K1 isolate.

Tracks 1-11: recombinant clones from the *Eco*RI genomic library of HB3

Track 1: 92b2
Track 2: 92b1
Track 3: 92a2
Track 4: 92a1
Track 5: 911
Track 6: 712
Track 7: 711
Track 8: 422
Track 9: 421
Track 10: 112
Track 11: 111

Track 12: recombinant clone of the K1 isolate

Track 13: DNA markers *Hin*dIII digested λcI_{857} DNA

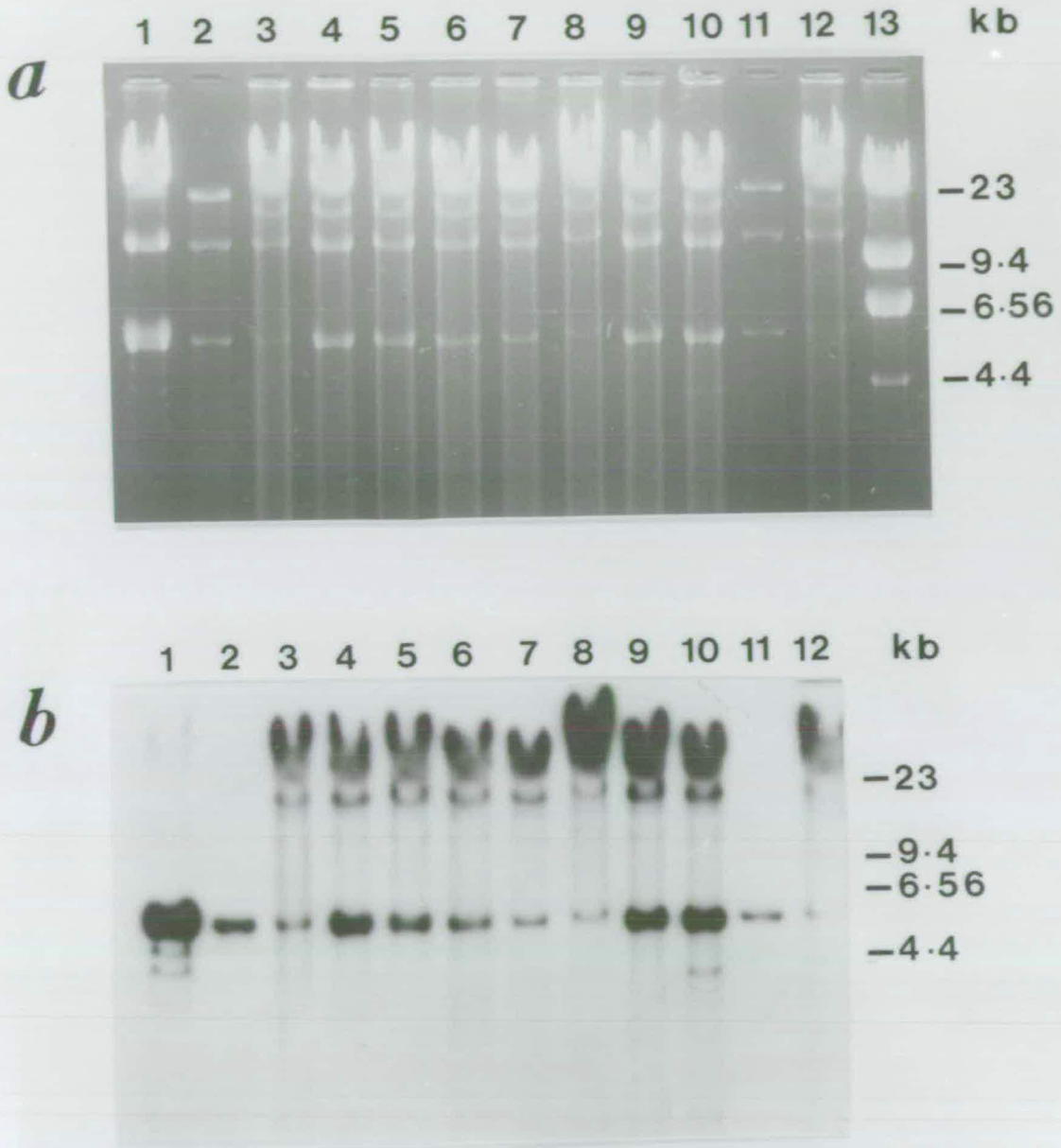

a
1 2 3 4 5 6 7 8 9 10 11 12 13 kb
23
9·4
6·56
4·4
b
1 2 3 4 5 6 7 8 9 10 11 12 kb
23
9·4
6·56
4·4

Fig. 5.9 Characterization of the DHFR-TS genomic clones 423 and 92a3.

a) Ethidium bromide stained gel of enzyme digested DNA from the HB3 clones 423, 92a3 and the λ K1 clone.

b) Southern blot of gel probed with the 0.6kb *Acc*I-*Eco*RI fragment from the K1 isolate.

Tracks 1-3: Purified *Eco*RI fragment from the recombinant clones

Track 1: 92a3
Track 2: K1
Track 3: 423

Tracks 4-5: Insert DNA from clone 92a3 digested with *Acc*I and *Bgl*II (track 4) or *Acc*I (track 5)

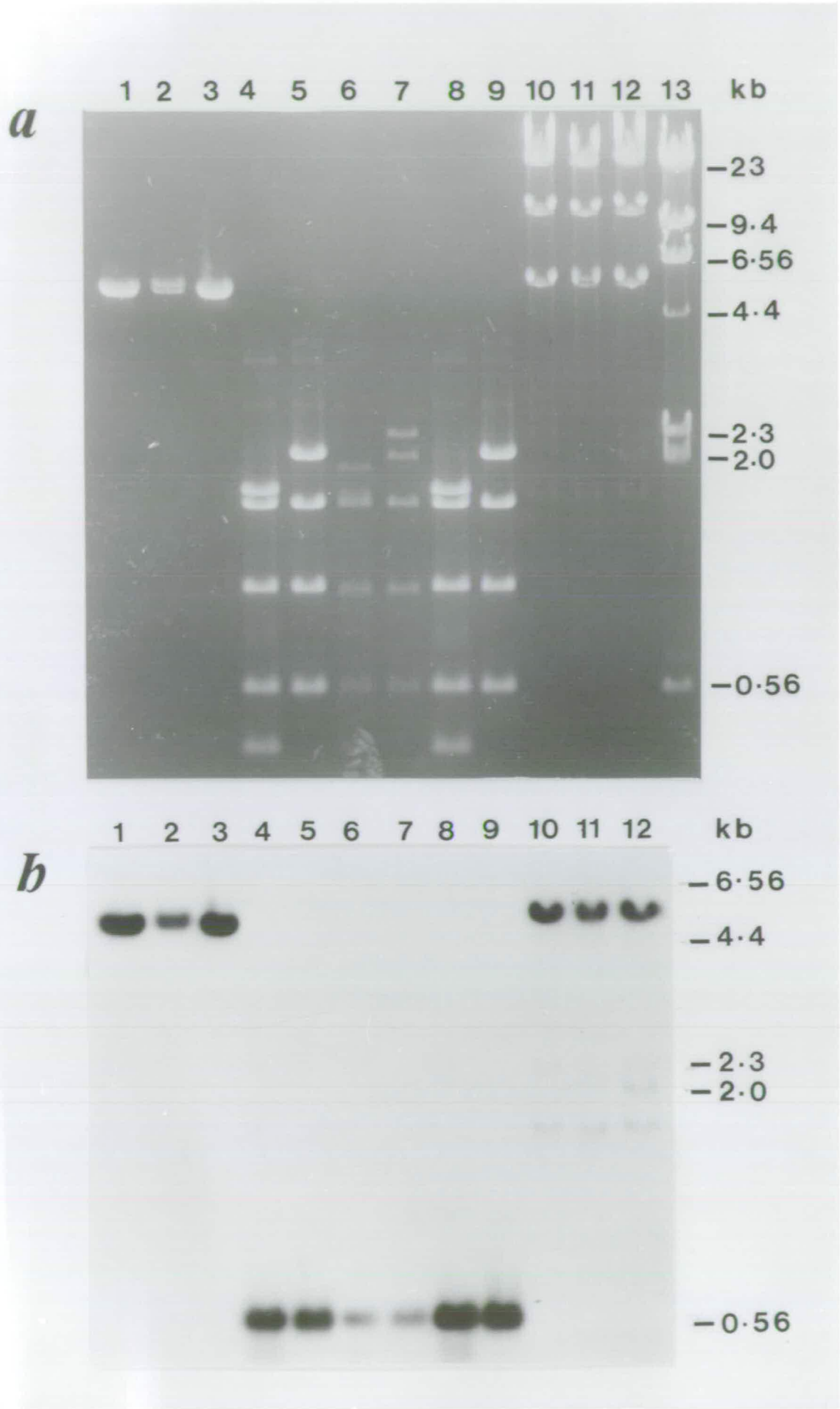
Tracks 6-7: Insert DNA from λ K1 clone digested with *Acc*I and *Bgl*II (track 6) or *Acc*I (track 7)

Tracks 8-9: Insert DNA from clone 423 digested with *Acc*I and *Bgl*II (track 8) or *Acc*I (track 9)

Tracks 10-12: DNA from recombinant clones digested with *Eco*RI

Track 10: 92a3
Track 11: K1
Track 12: 423

Track 13: DNA markers *Hin*dIII digested λcI_{857} DNA


a
1 2 3 4 5 6 7 8 9 10 11 12 13 kb
23
9·4
6·56
4·4
2·3
2·0
0·56
b
1 2 3 4 5 6 7 8 9 10 11 12 kb
6·56
4·4
2·3
2·0
0·56

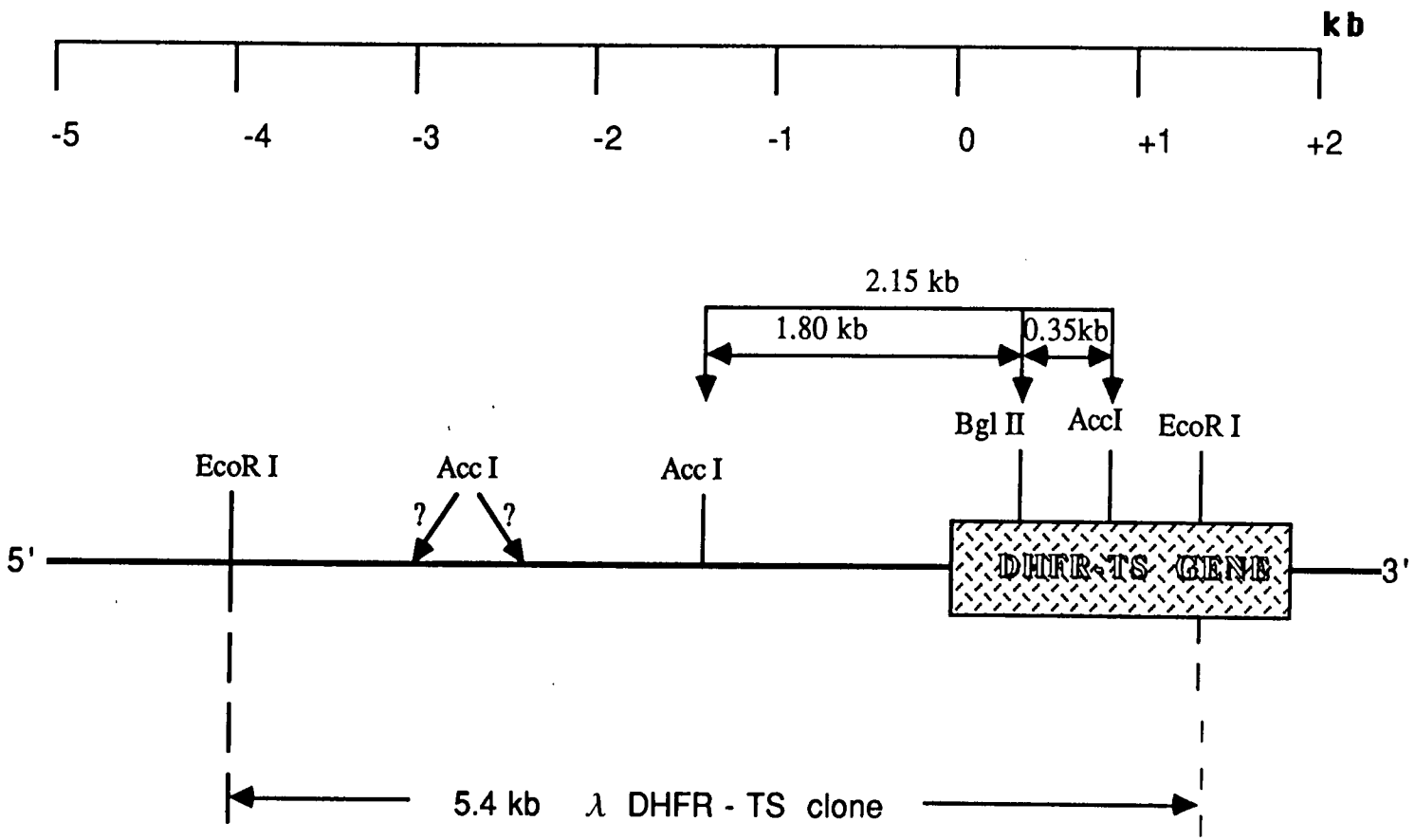


Fig. 5.10 The restriction enzyme map of the *P. falciparum* HB3 DHFR-TS genomic clone

5.1.5 Sequencing Studies on 5' Non-coding Region of DHFR-TS Gene from HB3

Having obtained the cloned DHFR-TS insert from HB3, I was able to initiate sequencing studies. The first aim was to sequence the 2.15kb *Acc*I fragment which is thought to contain the putative deletion of 250bp. To do this, it was necessary to subclone the fragment into a suitable sequencing vector. Attempts were made to subclone the entire *Acc*I fragment into M13 but the insert was unstable. Therefore, another cloning strategy was tried which required initial mapping of cloning sites within the fragment. Preliminary analysis suggested that the cloned insert possessed many target sites for restriction enzyme *Alu*I. Those within and neighbouring the 2.15kb dimorphic *Acc*I fragment were mapped and are diagrammatically represented in Fig. 5.11. These sites were utilized in the subsequent cloning strategy.

The cloning and sequencing strategies are illustrated in Fig. 5.12. Basically, an *Alu*I-*Alu*I or *Alu*I-*Bgl*II fragment was subcloned into M13 vectors (Section 2.4.25). Positive plaques were identified by plaque hybridization with the appropriate probe (Section 2.4.20). An example of this is shown in Fig. 5.13. Plaques were isolated and grown to generate single-stranded sequencing templates (Section 2.4.27.1). Sequencing was performed by the standard methods of dideoxy sequencing (Section 2.4.27.2), using both M13 universal 17bp and internal oligonucleotide primers. Sequence to 1453bp upstream of the gene was obtained. However, a region in the 817bp *Alu*I-*Alu*I fragment which was bounded on both sides by a stretch of T cannot be sequenced by the M13 method. This might be due to formation of secondary structure within this region or instability of the cloned insert. Other workers have encountered similar problems in sequencing *P. falciparum* DNA in M13 (A.

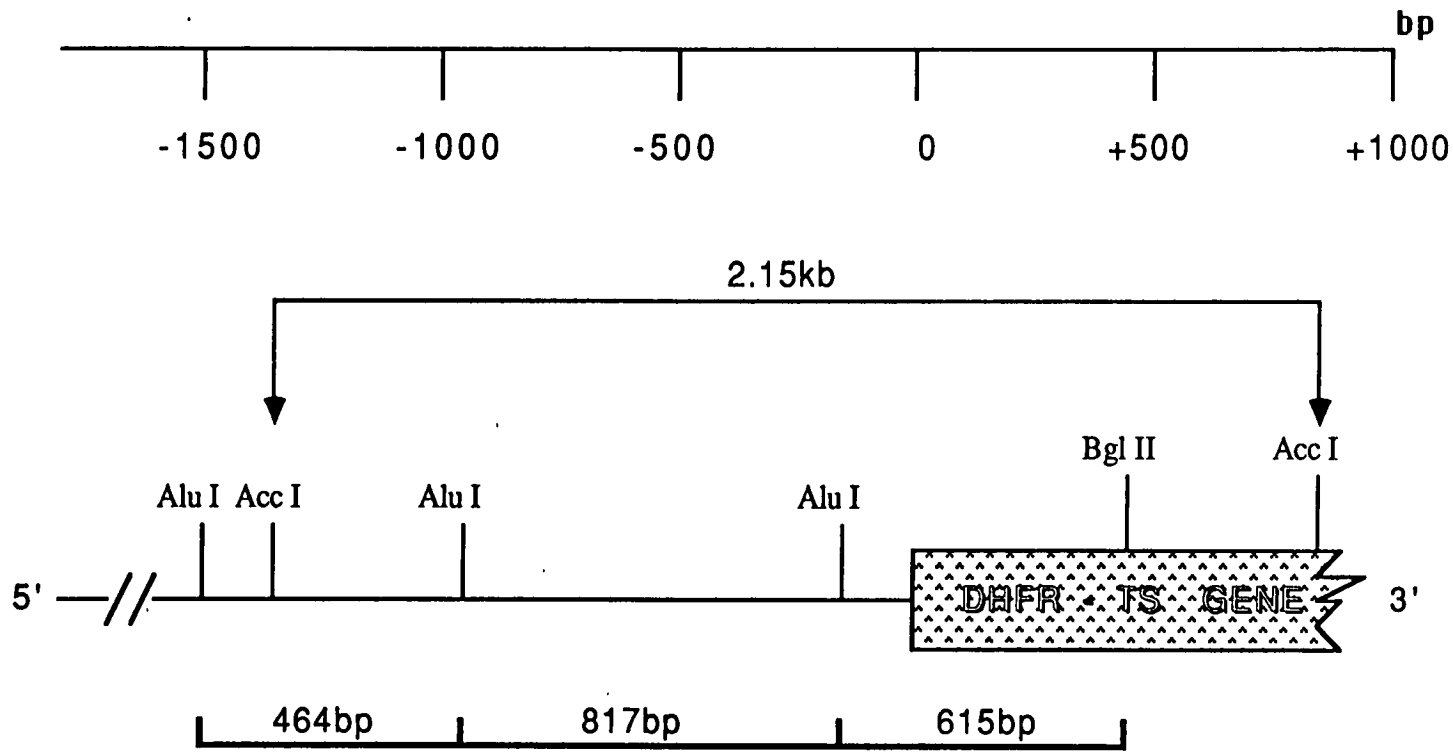


Fig. 5.11 Location of the *Alu* I site within or neighbouring the 2.15 kb *Acc* I dimorphic fragment of the HB3 DHFR-TS genomic clone.

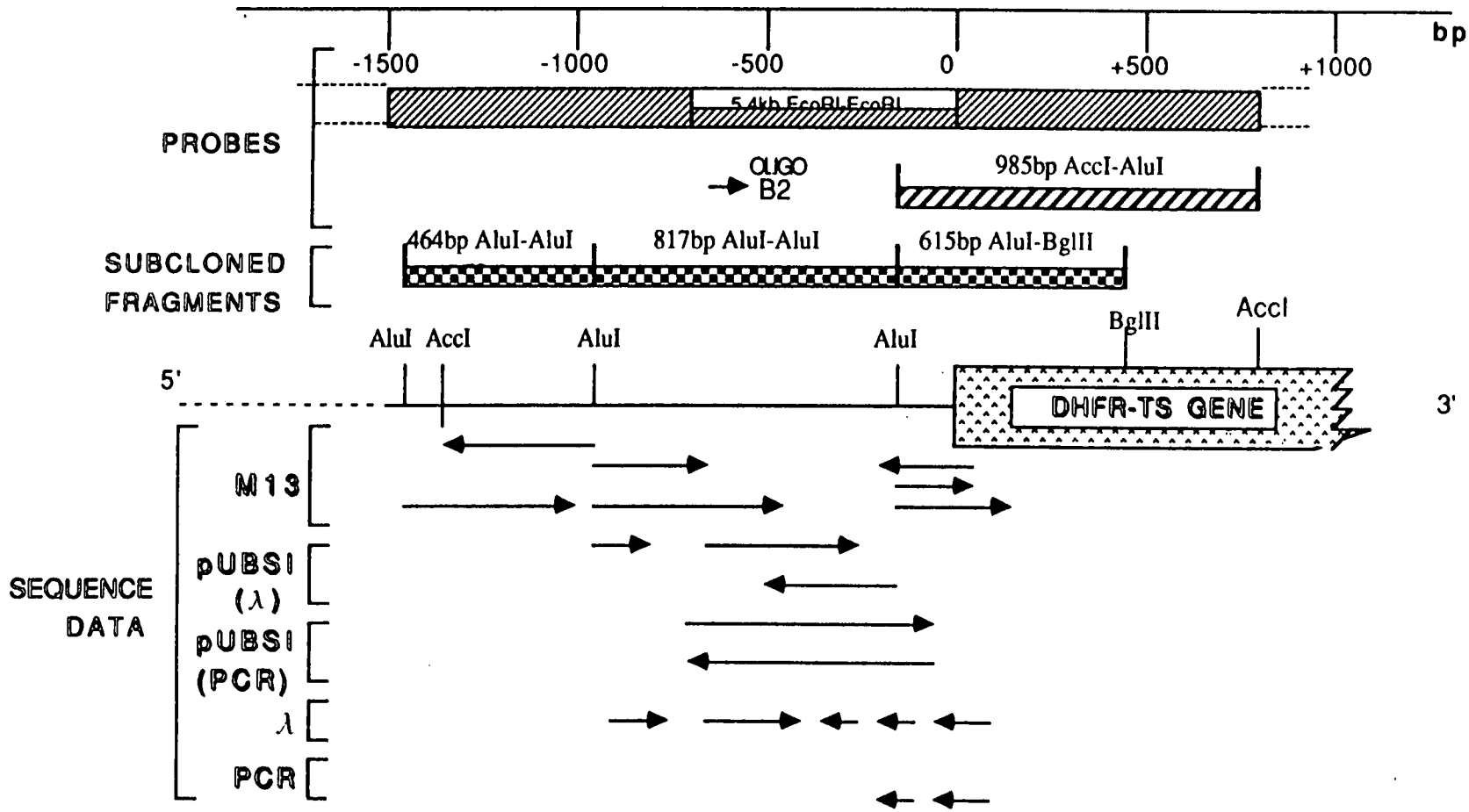
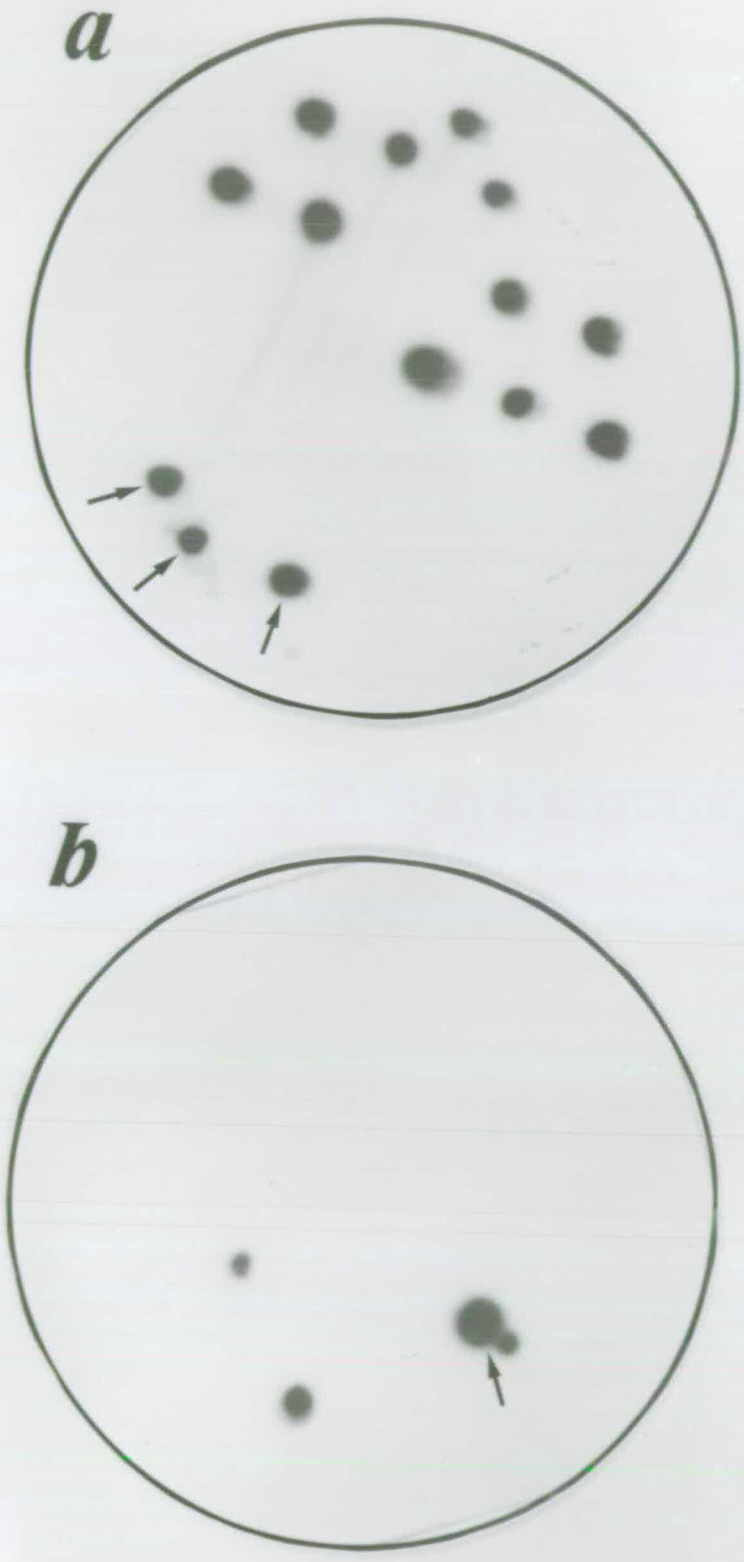


Fig. 5.12 Subcloning and sequencing strategies of 5' non-coding region of DHFR-TS gene from *P. falciparum* clone HB3. Sequence data were obtained from M13 subclones and pUBSI subclones of insert subfragment; pUBSI subclones of PCR fragments; purified λ DNA and directly from PCR fragments.

Fig. 5.13 Isolation of M13 subclones containing 0.6kb AluI-BglII subfragment of the DHFR-TS insert. Autoradiographs of plaque lifts from a) M13mp18 and b) M13mp19 recombinant clones probed with the 985bp *Acc*I-*Alu*I gene fragment from the cloned line HB3. Arrows indicate positive controls.


a
b

Khan; V.A. Snewin, personal communications).

The apparent stability of cloned inserts in the plasmid vector pUBS1 (Goman *et al.*, 1991) prompted an attempt to obtain the rest of the sequence by subcloning the 817bp *Alu*I-*Alu*I fragment into the *Sma*I site of this vector (see MATERIALS & METHODS Section 2.4.26) for double-stranded sequencing. The insert could be removed by utilizing the flanking *Xba*I and *Hin*dIII sites as illustrated in Fig. 5.14. The remaining sequence was obtained from these clones by the modified methods of dideoxy sequencing (Section 2.4.27.3), using both the M13 17bp universal primer and internal oligonucleotide primers. The complete sequence of 1453bp 5' non-coding region of the DHFR-TS gene is compiled in Fig. 5.15. Part of the sequence was also confirmed by three other double-stranded sequencing methods. These included direct sequencing from the λ clone 423, direct sequencing of polymerase chain reaction (PCR) products and sequencing of pUBS1 subclones of PCR fragments.

5.1.5.1 Features of the 5' Flanking Sequence of the HB3 DHFR-TS Gene: No extensive sequencing of *P. falciparum* non-coding regions has been done probably because of the difficulties in sequencing through AT-rich regions. As a result of this work the *P. falciparum* DHFR-TS gene is one of the few genes which has its non-coding 5' region sequenced as far as 1453bp from the translation start site.

The 1453bp 5' flanking sequence showed an elevated frequency of A and T. An average of 82% AT was found in this region compared with 75% AT in the coding region. The occurrence of AT had gone up to 94% in the immediate vicinity of the gene and was as high as 87.5% up to 1kb from the ATG codon. While the coding region showed an asymmetry with the purines

Fig. 5.14 Characterization of pUBS1 subclones containing the 817bp AluI-AluI subfragment of the DHFR-TS insert.

a) Ethidium bromide stained gel of *Xba*I-*Hin*dIII digested DNA from pUBS1 subclones.

b) Southern blot of gel probed with a synthetic oligonucleotide B2.

Tracks 1 & 10: (Control) pUBS1 vector DNA cut with *Xba*I and *Hin*dIII

Tracks 2-5 & 8-9: Plasmid DNA from pUBS1 subclones cut with *Xba*I and *Hin*dIII

Track 6: DNA markers 123bp ladder

Track 7: (Control) Purified 2.15kb *Acc*I fragment cut with *Alu*I

Track 11: (Control) pUBS1 vector DNA

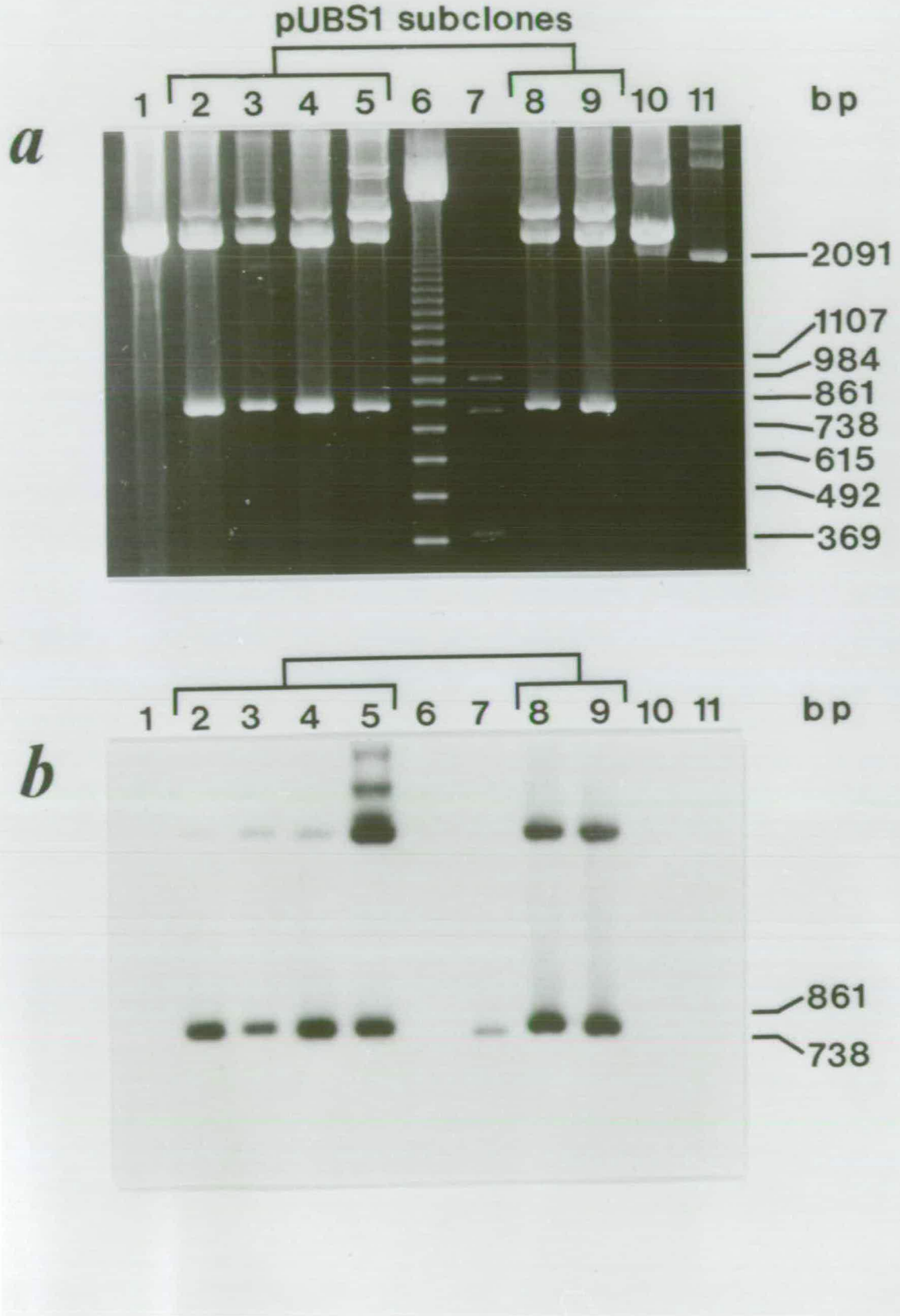

pUBS1 subclones
1 2 3 4 5 6 7 8 9 10 11
bp
a
2091
1107
984
861
738
615
492
369
1 2 3 4 5 6 7 8 9 10 11
bp
b
861
738

Fig. 5.15 The nucleotide sequence of the 5' non-coding region of the DHFR-TS gene from HB3. Part of the sequence for the coding region is also shown. In places where AT tracts and A or T tracts occurred, slight variation in the number of AT, As or Ts was obtained by different sequencing methods.

Key: underlined = poly (dT) or poly (dA) and CAT
double underlined = poly (AT)
boxed = ATTT or TAAA repeats
wavy underlined = potential Z-DNA forming sequences

-1453 CTTGTCCATATGACTGCGAGAATTAACCGTGGTGAGGCGA -1414

-1413 TCCCTGAACCAGTAAAACAACTTCCTGTCATGGGCGGTAG -1374

-1373 ACATAAAATGAAAACAAGAAAAATATATATACAAAGTATA -1334

-1333 TATACATATACTTATTATTATTTTTATTATTTTAATTTTT -1294

-1293 TTATTGATGTTCCTTTTCAAATAATTCCAAAGTAATATAC -1254

-1253 ACAACGTTACAGTTGTTGAATAAATATAGGACCAAATAGA -1214

-1213 TATATGCCATATAATTTATAAAACCATCACATGTTTAAAG -1174

-1173 TAAATTTTTCTTCATTTCAAATATTCATGTTGTTCTCTTT -1134

-1133 CCTATTTCTTTATAAGTTACTCTTATATATGTCTTTTTAT -1094

-1093 TTTCTATTTTAATTTTCTTTTTTTTATAAGTGAGTAAATA -1054

-1053 TGAATGTGCATGCTTAATGGAAAAAAAGAGAGAAAAAAAA -1014

-1013 GAAAAGCCTTTATATTGAAACTAGCTCAACAAAAATTAAA -974

-973 TAAATAAATATATATATATTATATATATGTATAATTCTTT -934

-933 CAGTGCACATATATATATATATATATATATATATATATAT -894

-893 ATATATATATATAATATTCTCCGTTTTATTTTATTTTATT -854

-853 TTTTTTTTTTTTAAATATTATATTTTAAAGATATCAATCT -814

-813 TTTATACAATCATATATATGTCTATGGGAATCTTATAATA -774

-773 TGTAAAAATAAGAAACAAAAGGATATATAAATAATAATAT -734

-733 ACCTTAAAAAAATTATTATAGAGAAGGATAAATTTATTTT -694

-693 TATTATAAGTTCTATTCACATATCCATATGATATAGGTAT -654

-653 AAAAAAATAATTATTATTTATTTATTTATTTATTTATTTA -614

-613 TTTATTTTATTTTTCTTATATGCACTTTCCTTGTAAATGT -574

-573 CATTCTTTATATTAAGAAGAACATATTATACATATATAAA -534

-533 AATATTATATCTCATATAATTTTTGTACAGTTTTTTATTG -494

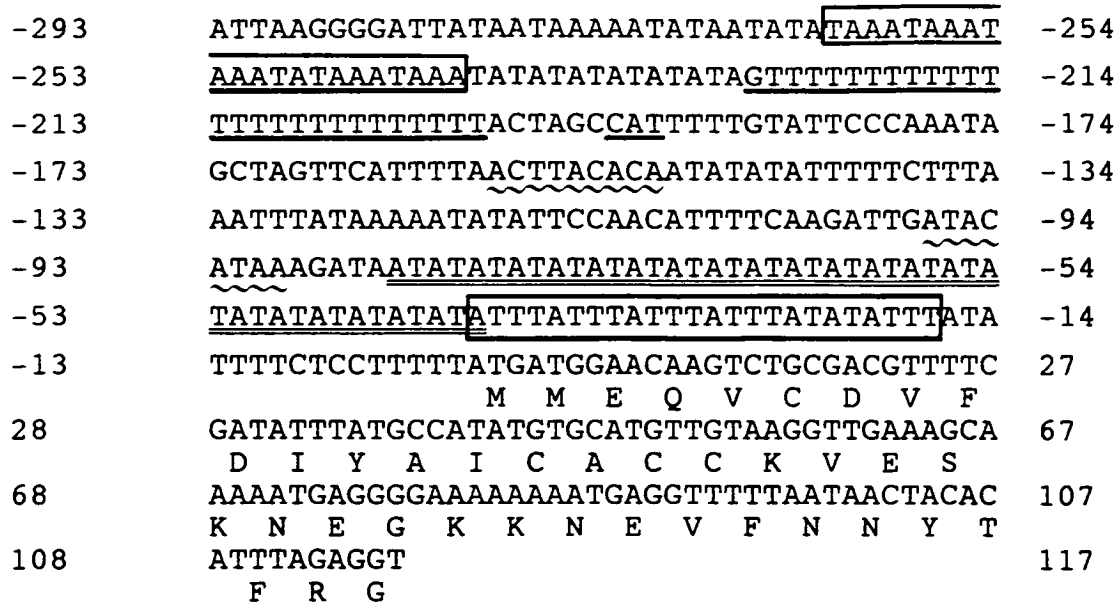
-493 GTTTCTTAATTGTTTTTTTTTTTTTTTTTTTTTCTCATTC -454

-453 CATTTATATAAGTATGATTAGATTTGTTTAAGAAAAAAAA -414

-413 AAAAAAAAAAATTATCATCATAAGAAATATTTTACACATT -374

-373 TTGAAATATAATTAAGATATTTATTTTATATATATTTAAA -334

-333 TGAATAGATACATTTATTTATGAATCTGTTTAAAAGTTAT -294


-293 ATTAAGGGGATTATAATAAAAATATAATATATAAATAAAT -254
-253 AAATATAAATAAATATATATATATATAGTTTTTTTTTTTT -214
-213 TTTTTTTTTTTTTTACTAGCCATTTTTGTATTCCCAAATA -174
-173 GCTAGTTCATTTTAACTTACACAATATATATTTTTCTTTA -134
-133 AATTTATAAAAATATATTCCAACATTTTCAAGATTGATAC -94
-93 ATAAAGATAATATATATATATATATATATATATATATATA -54
-53 TATATATATATATATTTATTTATTTATTTATATATTTATA -14
-13 TTTTCTCCTTTTTATGATGGAACAAGTCTGCGACGTTTTC 27
M M E Q V C D V F
28 GATATTTATGCCATATGTGCATGTTGTAAGGTTGAAAGCA 67
D I Y A I C A C C K V E S
68 AAAATGAGGGGAAAAAAAATGAGGTTTTTAATAACTACAC 107
K N E G K K N E V F N N Y T
108 ATTTAGAGGT 117
F R G

in excess by 14% in the coding strand, the non-coding region did not reveal any significant bias. Only a 5% pyrimidine excess of the total bases was found in the non-coding region. This is in accord with the results obtained by Hyde & Sims (1987) who observed a marked excess of purines over pyrimidines in the mRNA-synonymous strand of coding regions. The purine excess ranges from 14-32% of the total bases. By contrast, there is no consistent bias seen in the non-coding region of 25 genes analysed.

Two long oligo (dT) stretches of 26 and 21 bases and one long oligo (dA) stretch of 19 bases were found around -210, -470 and -410bp respectively. These sequences were preceded by a G base and followed by CAT. A shorter oligo (dT) stretch of 14 bases was located around -850bp.

A particularly long tract of AT repeats occurred twice in the sequence, one between -39 and -84bp and the other between -882 and -925bp. The numbers of the repeated AT bases were 22 for the distal tract and 23 for the proximal tract.

Another characteristic of the sequence was the common occurrence of the ATTT repeats. Most of the ATTT repeats were found together with other AT_n domains. The sequence ATTT repeated seven times was found from -611 to -638bp preceded by $(ATT)_2$ and followed by AT_4 and then AT_5. It is also found repeated five times at -17 to -40. Five inverted repeats TAAA alternating with other TA_n domains occurred around -241 to -261bp.

Putative Z-DNA forming sequences were found along the 5' non-coding region of *P. falciparum* DHFR-TS gene. They consist of short stretches of alternating purine-pyrimidine base pairs which are reported to have the potential to form Z-DNA when no more than one base pair is out of purine-pyrimidine alternations (Nordheim & Rich, 1983a; b). Alternatively, a Z-DNA forming sequence is favoured in nucleotide sequences such as poly d(GC), poly d(CA), poly d(GT), d(CGCG), d(CGCGCG),

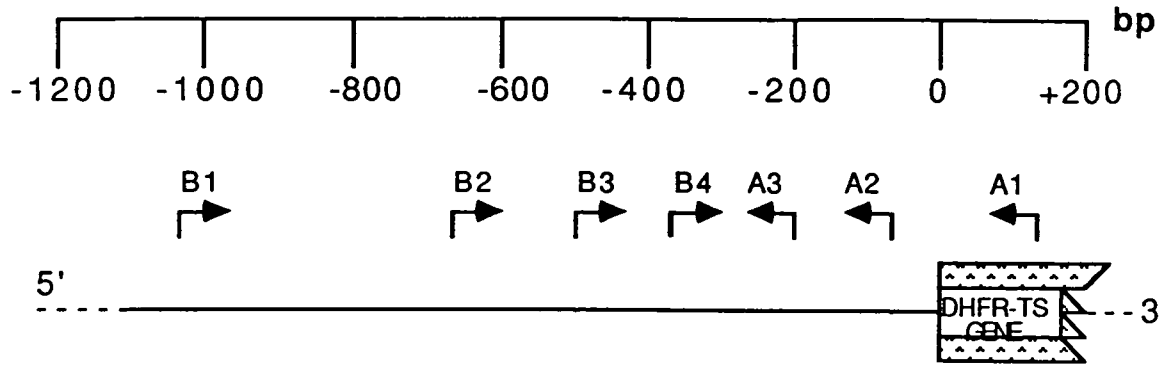
$d(CA/TG)_n$ which has not been found in the non-coding region of *P. falciparum* DHFR-TS gene.

5.1.6 Cloning of 5' Non-coding Region of DHFR-TS Gene from 3D7

After the sequence of HB3 DNA was completed, oligonucleotide primers were used to amplify corresponding segments from 3D7 DNA by PCR. The sequences and the relative positions of the synthetic oligonucleotides in the gene map are illustrated in Fig. 5.16. The amplified fragments were then subcloned into a plasmid vector for sequencing. The oligonucleotides, namely A2 and B2 corresponded to nucleotides -93 to -116 and -677 to -654 of the HB3 sequence given in Fig. 5.15. Using these oligonucleotides, a PCR fragment of 585bp was expected from HB3 DNA and a fragment of ≈835bp was expected from 3D7 DNA.

As shown in Fig. 5.17, fragments amplified from 3D7 and HB3 DNA using oligonucleotide primers A2 and B2 gave the expected sizes (plate a). This implied that the source of the dimorphism was present in this region i.e. the putative deletion was contained within this fragment. The identity of the fragments was confirmed by probing a Southern blot of this gel with an oligonucleotide A3 occurring in between A2 and B2 at position -285 to -308 (plate b).

After purification from an agarose gel, these two fragments were subcloned into pUBS1 plasmid (Section 2.4.26). Positive clones were identified by screening with the internal oligonucleotide A3 (Section 2.4.21). The sizes of the inserts from these clones were checked by agarose gel



synthetic oligonucleotide

A1 (60 to 37)	5'-AAC CTT ACA ACA TGC ACA TAT GGC-3'
A2 (-93 to -116)	5'-TGT ATC AAT CTT GAA AAT GTT GGA-3'
A3 (-285 to -308)	5'-CCC CTT AAT ATA ACT TTT AAA CAG-3'
B1 (-1008 to -984)	5'-GCC TTT ATA TTG AAA CTA GCT CAA C-3'
B2 (-677 to -654)	5'-CAC ATA TCC ATA TGA TAT AGG TAT-3'
B3 (-509 to -486)	5'-GTA CAG TTT TTT ATT GGT TTC TTA-3'
B4 (-398 to -375)	5'-CAT CAT AAG AAA TAT TTT ACA CAT-3'

Fig. 5.16 The sequences of the primers used in polymerase chain reactions (PCR) and the relative positions of the synthetic oligonucleotides in the gene map. The positions of the oligonucleotides (in brackets) are numbered according to the HB3 sequence in Fig. 5.15.

Fig. 5.17 Dimorphic fragments amplified from 3D7 and HB3 DNA using primers A2 and B2 in PCR.

a) Ethidium bromide stained gel of PCR amplified fragments from 3D7 and HB3 DNA.

b) Southern blot of gel probed with the internal oligonucleotide A3.

a
HB3
3D7
Markers
bp
1230
1107
984
861
738
615
492
369
246
123
b
HB3
3D7

electrophoresis to confirm that no apparent deletion had occurred during the cloning process. The results are shown in Fig. 5.18. Fragments of the correct sizes 580bp and 830bp respectively were maintained in HB3 and 3D7 subclones (plate a). The specificity of these fragments was proved by their cross-hybridization with internal oligonucleotide A3 (plate b).

5.1.7 Sequencing Studies on 5' Non-coding Region of DHFR-TS Gene from 3D7

The sequencing strategy is shown in Fig. 5.19. The complete sequence of the subcloned fragment was obtained by the modified methods of dideoxy sequencing (Section 2.4.27.3), using the M13 17bp universal primer, the M13 16bp reverse primer and internal oligonucleotide primers. Part of the sequence within this region was confirmed by direct sequencing from PCR products. Sequences on the 5' and 3' sides of the fragment were obtained by direct sequencing from another PCR product obtained using the oligonucleotides A1 and B1 corresponding to positions -137 to -160 and -1008 to -984 respectively of the HB3 sequence (Fig. 5.15).

5.1.7.1 Sequence Comparison of the 5' Flanking Regions of 3D7 and HB3 DHFR-TS Genes: The sequence of the 5' non-coding region of the DHFR-TS gene for 3D7 and its comparison with the sequence for the HB3 cloned line is shown in Fig. 5.20. There was no 250bp insertion of sequence in 3D7 DNA as predicted from the apparent size of 3D7 DNA in agarose gel. Instead, only minor variations in the two sequences were detected. The presence of an insertion might be mimicked by the presence of 3D7 sequence which favoured the formation of secondary structures e.g. bent DNA and retarded the passage of the fragment through the agarose but before this could be evaluated the difference in the two sequences are illustrated by

Fig. 5.18 Characterization of pUBS1 subclones of PCR fragments.

a) Ethidium bromide stained gel of *Xba*I-*Hin*dIII digested DNA from pUBS1 subclones containing PCR fragments from either 3D7 or HB3 DNA.

b) Southern blot of gel probed wtih a synthetic oligonucleotide A3.

Tracks 1-3: DNA from pUBS1 subclones containing PCR fragment from 3D7 cut with *Xba*I and *Hin*dIII

Track 1: D9
Track 2: D8
Track 3: D3a

Track 4: (Control) PCR fragment from 3D7

Track 5: DNA markers 1kb ladder

Track 6: (Control) PCR fragment from HB3

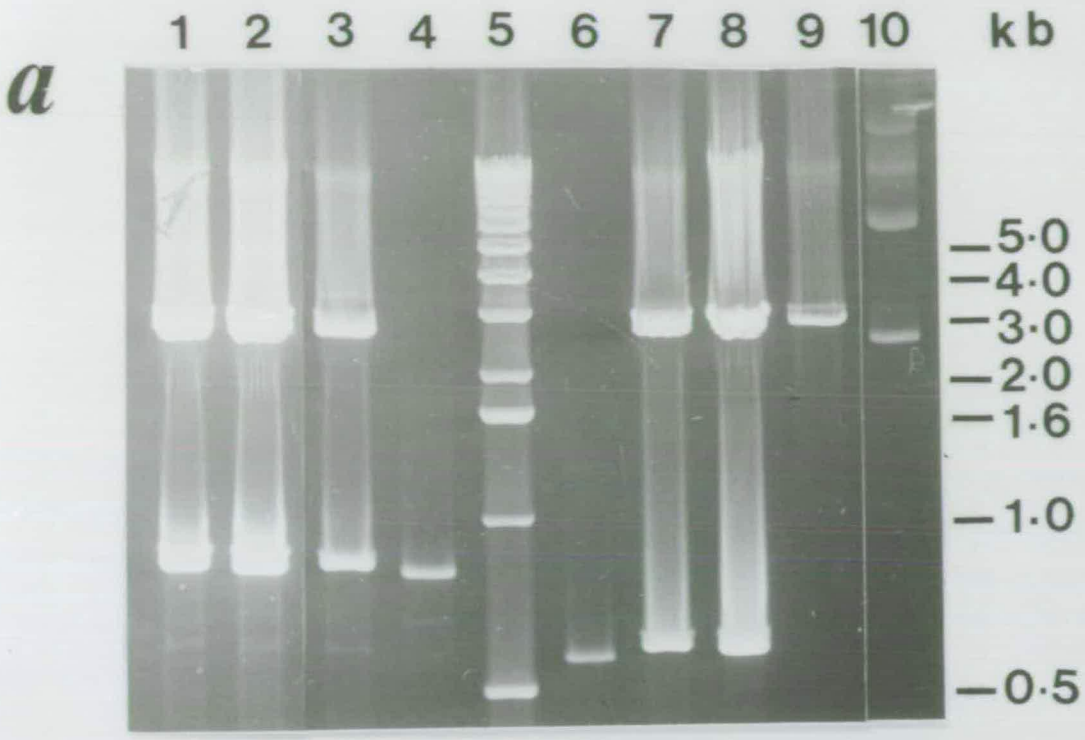
Tracks 7-8: DNA from pUBS1 subclones containing PCR fragment from HB3 cut with *Xba*I and *Hin*dIII

Track 7: H4
Track 8: H5

Track 9: (Control) vector DNA cut with *Xba*I and *Hin*dIII

Track 10: (Control) vector DNA

Note that the slightly increase in size of the released inserts is due to cleavage with enzymes flanking the *Sma*I cloning site, which are ≈20bp either sides of the *Sma*I site.


a
1 2 3 4 5 6 7 8 9 10 kb
5·0
4·0
3·0
2·0
1·6
1·0
0·5


b
1 2 3 4 5 6 7 8 9 10 kb
1·0
0·5

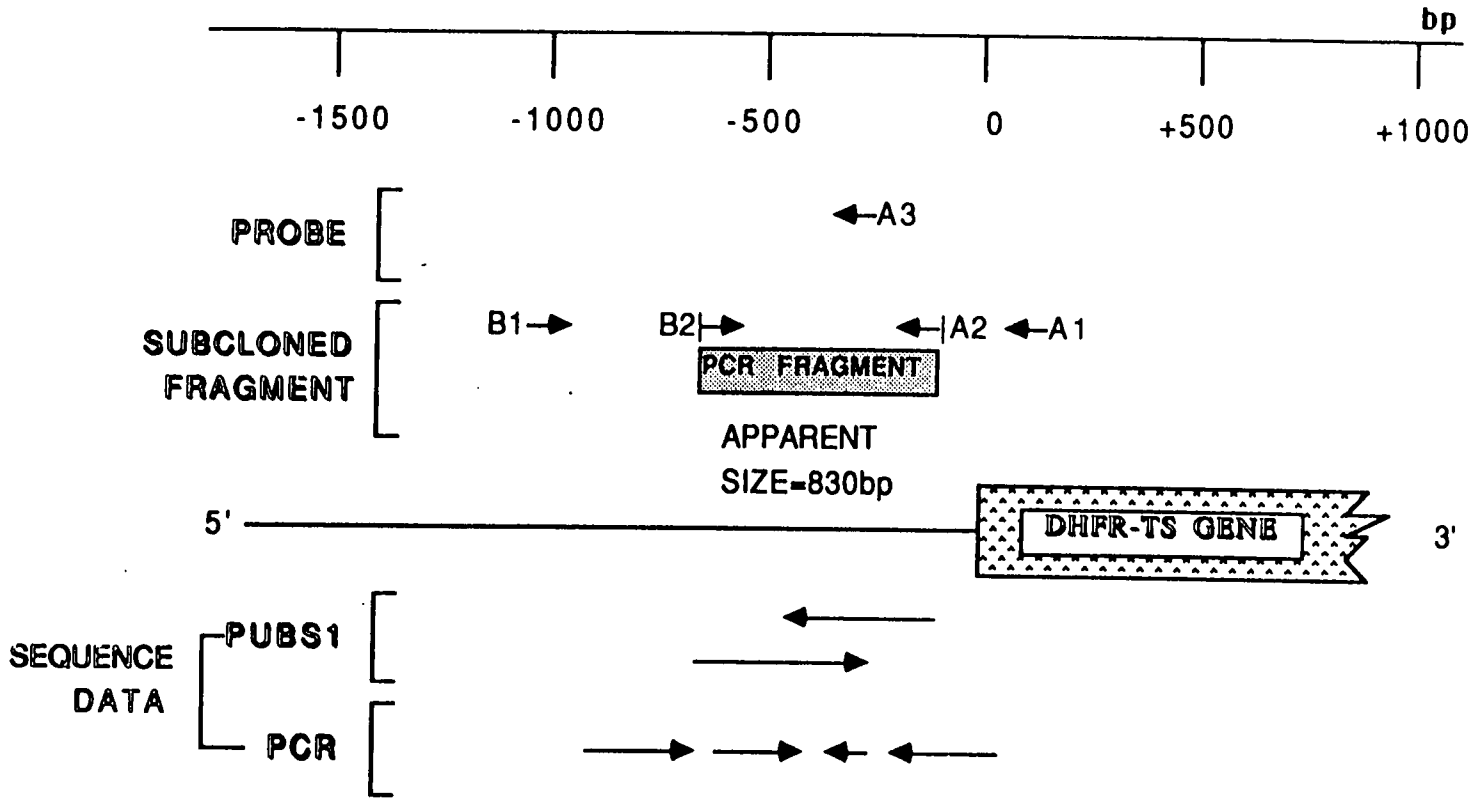


Fig. 5.19 PCR and sequencing strategies of the 5' non-coding region of the DHFR-TS gene from *P. falciparum* clone 3D7. Sequence data were obtained from either pUBS1 subclones of PCR fragments or PCR fragments as indicated.

Fig. 5.20 Alignment of 3D7 and HB3 sequences from the 5' non-coding regions of the DHFR-TS gene. The sequence of 3D7 is shown on top. The differences in the HB3 sequence are shown as either insertion of sequences or deletion of sequences (dotted line) in the bottom line.

```
3D7  TTAAATAAATAAATATATATATATTTTATATATATATGTA      -901
HB3                              ....              -943

3D7  TAATTCTTTCAGTGCAC..............ATATATATA      -875
HB3                    ATATATATATATAT              -903

3D7  TATATATATATATATATATATAATATTCTCCGTTTTATTT      -835
HB3                                               -863

3D7  TATTTTATTTTTTTTTTTTTTAAATATTATATTTTAAAGA      -795
HB3                                               -823

3D7  TATCAATCTTTTATACAATCATATATATGTCTATGGGAAT      -755
HB3                                               -783

3D7  CTTATAATATGTAAAAATAAGAAACAAAAGGATATATAAA      -715
HB3                                               -743

3D7  TAATAATATACCTTAAAAAAATTATTATAGAGAAGGATAA      -675
HB3                                               -703

3D7  ATTTATTTTTATTATAAGTTCTATTCACATATCCATATGA      -635
HB3                                               -663

3D7  TATAGGTATAAAAAAATAATTATT............ATTT      -607
HB3                               ATTTATTTATTT    -623

3D7  ATTTATTTATTTATTTTATTTTTCTTATATGCACTTTCCT      -567
HB3                                               -583

3D7  TGTAAATGTCATTCTTTATATTAAGAAGAACATATTATAC      -527
HB3                                               -543

3D7  ATATATAAAAATATTATATCTCATATAATTTTTGTACAGT      -487
HB3                                               -503

3D7  TTTTTATTGGTTTCTTAATTGTTTTTTTTTTTTTTTTTT       -447
HB3                                               -463

3D7  TTCTCATTCCATTTATATAAGTATGATTAGATTTGTTTAA      -407
HB3                                               -423

3D7  GAAAAAAAAAAAAAAAAAAATTATCATCATAAGAAATATT     -367
HB3                                               -383
```

```
3D7  TTACACATTTTGAAATATAATTAAGATATTTATTTTATAT      -327
HB3                                              -343

3D7  ATATTTAAATGAATAGATACATTTATTTATGAATCTGTTT      -287
HB3                                              -303

3D7  AAAAGTTATATTAAGGGGATTATAATAAAAATATAATATA     -247
HB3                                              -263

3D7  TAAATAAATAAATATAAATAAATATATATATATATATATA     -207
HB3                          ....                -227

3D7  GTTTTTTTTTTTTTTTTTTTTTTTTTTACTAGCCATTTTT    -167
HB3                                              -187

3D7  GTATTCCCAAATAGCTAGTTCATTTTAACTTACACAATAT     -127
HB3                                              -147

3D7  ATATTTTTCTTTAAATTTATAAAAATATATTCCAACATTT     -87
HB3                                              -107

3D7  TCAAGATTGATACATAAAGATAATATATATATATATATAT     -47
HB3                                              -67

3D7  ATATATATATA.....................TTTATTTAT    -27
HB3              TATATATATATATATATTTA            -27

3D7  TTATATATTTATATTTTCTCCTTTTTATGATGGAACAAGT     14
HB3                                              14
```

the sequencing gels shown in Fig. 5.21. Nucleotide positions given refer to those of 3D7 sequence shown in Fig. 5.20. There are six differences involving either insertion or deletion of A and T bases.

Two distinctive differences involving the number of AT repeats in two AT tracts at positions -36 to -63 and -854 to -883 are shown in plate a and b. The 3D7 sequence had eight less TA in the proximal AT tract (positions -36 to -63) (plate a) and seven less AT in the distal AT tract (position -854 to -883) (plate b). Plate a also shows another difference in the number of TTTA repeats (position -24 to -35) which occurred immediately preceding the proximal AT tract. A deletion of TTTA in 3D7 sequence (arrow) reduced the number of TTTA repeats from four to three. Plate b also shows an insertion of TTAT (positions -912 to -915) in the 3D7 sequence which added one AT to a $(AT)_4$ repeats.

Plate c shows a difference in the number of ATTT repeats (positions -595 to -610). There are four ATTT repeats in 3D7 sequence whereas seven ATTT repeats are present in HB3 sequence. Plate d shows another difference in the number of TA repeats (positions -207 to -214) in the two sequences. Nine TA repeats were found in 3D7 sequence while only seven were present in HB3 sequence.

5.1.7.2 Comments on Various Methods Used in Sequencing: In the course of sequencing the 5' non-coding region of the DHFR-TS gene, difficulties were encountered mainly in sequencing through AT ladders and poly (dA) and poly (dT) tracts. Many of the methods used in sequencing the 5' non-coding region of the *P. falciparum* DHFR-TS gene could not read through sequences after stretches of A or T. This was a particular problem in the case of M13 single-stranded sequencing and direct sequencing from

Fig. 5.21 Differences in the two sequences of the 5' non-coding regions from 3D7 and HB3.

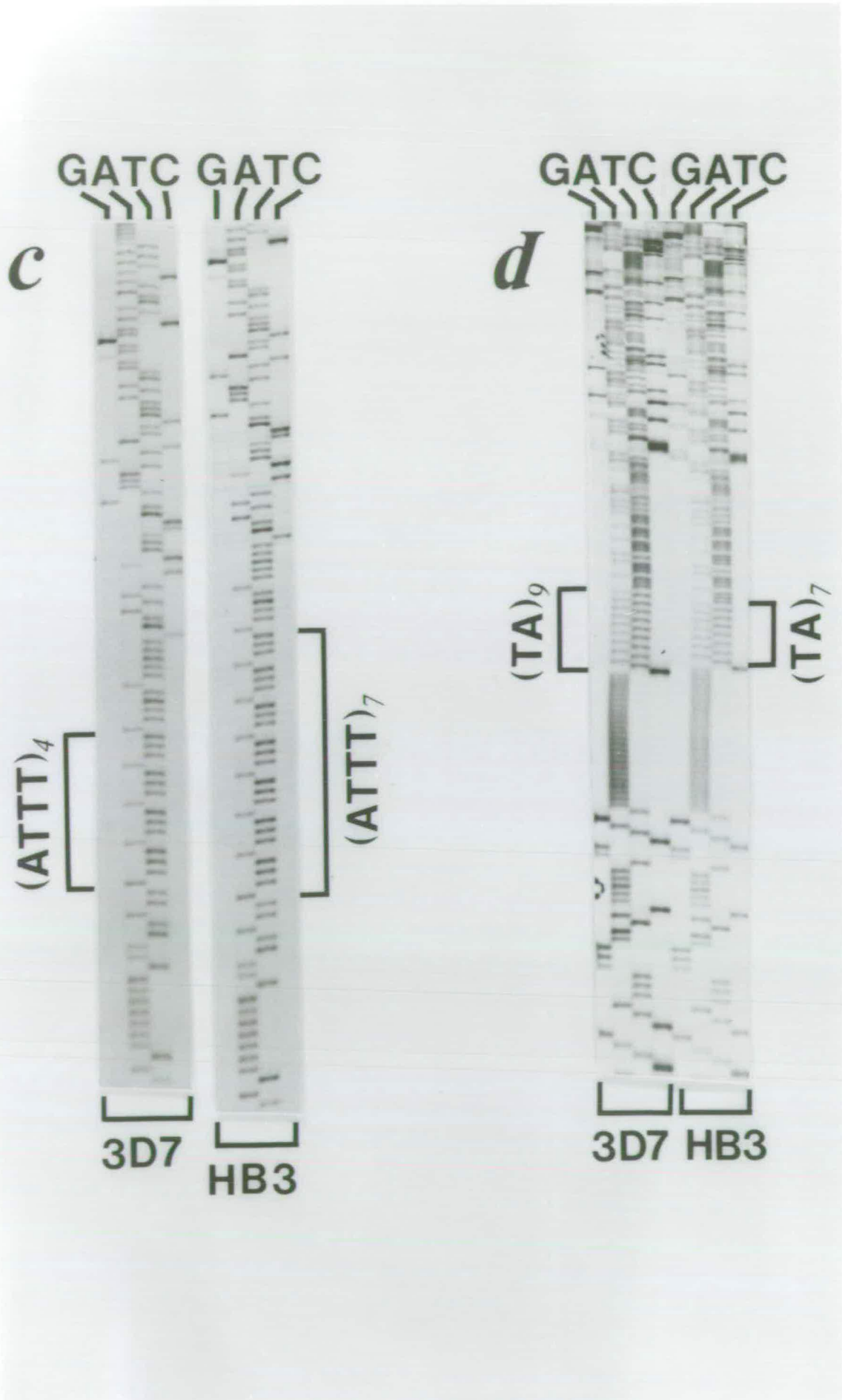
a) Autoradiographs of sequencing gels showing a difference in the number of AT repeats in an AT ladder positioned -36 to -63 of 3D7 sequence. It also shows a deletion of TTTA (arrow) from a repeat (-24 to -35) of 3D7 sequence.

b) Autoradiographs of sequencing gels showing a difference in the number of AT repeats in an AT ladder positioned -854 to -883 of 3D7 sequence. It also shows an addition of TTAT (-912 to -915) in the 3D7 sequence.

c) Autoradiographs of sequencing gels showing a difference in the number of ATTT repeats positioned -595 to -610 of 3D7 sequence.

d) Autoradiographs of sequencing gels showing a difference in the number of TA repeats positioned -207 to -214 of 3D7 sequence.

a
GATC GATC
TA3(TA)8
3D7 HB3
b
GATC GATC
(AT)15
(AT)22
TTAT
3D7 HB3


GATC GATC
c
(ATTT)4
(ATTT)7
3D7
HB3
GATC GATC
d
(TA)9
(TA)7
3D7 HB3

λ or PCR products. The reason for this is unknown but is presumably due to formation of secondary structures or instability of inserts. The plasmid vector pUBS1 proved to be a suitable sequencing vector for *Plasmodium* genes since there were no difficulties in sequencing through these stretches after the DNA fragments were subcloned into pUBS1. It gave a long continuous, clear and unambiguous sequence. This was found when sequencing both the 3D7 and HB3 DHFR-TS non-coding regions.

5.1.8 Evaluation of the Secondary Structure as the Cause of the RFLP

The failure to detect a 250bp difference in the DNA sequence to account for the RFLP implies that there must be differences in the DNA sequences affecting the mobility of the DNA fragments on agarose gel which are not deletions. Since only minor differences in sequence were detected, it is tempting to speculate that the anomalous mobility of the DNA fragments is due to formation of secondary DNA structure induced by unusual DNA sequence e.g. bent DNA. This was tested as described in the following experiments.

5.1.8.1 Dissociation of RFLP by PCR: The occurrence of sequence variation in different parts along the 5' non-coding regions leads to the speculation that some kind of sequence interaction is involved in giving rise to the secondary structure. An experiment was designed to test this possibility.

PCR was used to amplify fragments from different 5' and 3' sites of the gene fragment encompassing the RFLP region. The PCR strategy is illustrated in Fig. 5.22 and the results summarised in Fig. 5.22 and Table 5.1.

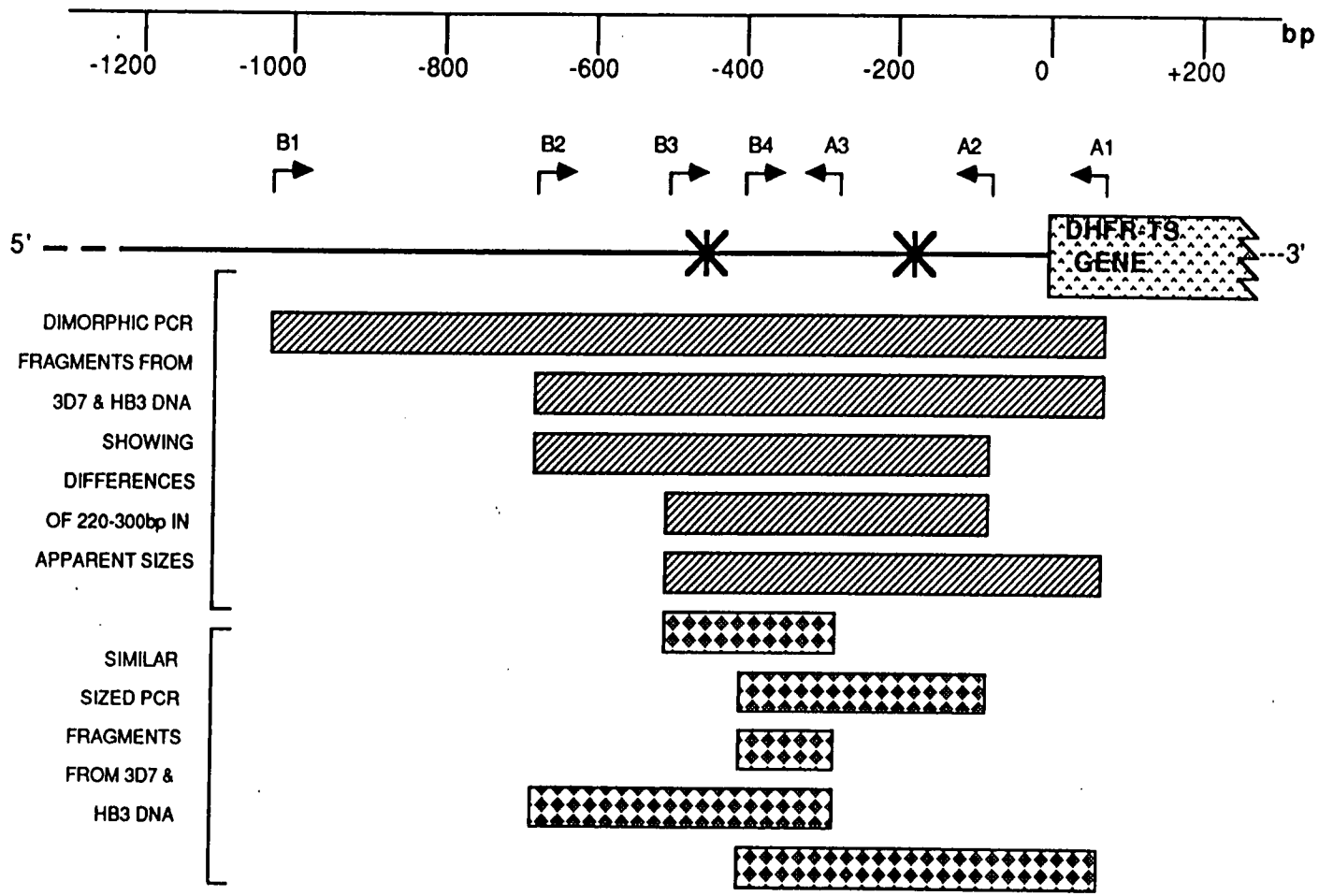


Fig. 5.22 PCR strategy to amplify fragments from the 5' non-coding region of DHFR-TS gene from *P. falciparum* clones 3D7 and HB3 using different combinations of 5' and 3' primers. The regions marked with asterisks are believed to contain sequences involved in interaction to generate secondary structure in 3D7 DNA.

Table 5.1 A list of the expected and apparent sizes of the PCR fragments amplified from different primers

	HB3		3D7	
Primers	Expected Size	Apparent size	Expected Size	Apparent Size
A1/B1	1068	950 830@	1030	1250 1150@
A1/B2	737	700 620@	709	920 830@
A2/B2	585	580	577	830
A1/B3	569	580	553	830
A2/B3	417	430	421	700
A1/B4	458	410	442	370
A3/B2	393	380	381	375 620*
A2/B4	306	310	310	310 565*
A3/B3	225	220	225	220 450*
A3/B4	114	125	114	125 320*

* detected as faint bands
@ derived from non-specific amplification products

Dimorphic fragments were amplified from primers A1/B1, A1/B2, A2/B2, A2/B3 and A1/B3. The ethidium bromide stained gels of the PCR products are shown in Fig. 5.23 (plate a). The specificity of these fragments were confirmed by probing with oligonucleotides B4 (plate b) or A3 (plate c). These results show that the source of dimorphism was contained within the region encompassed by oligonucleotide sequences A2 and B3.

However, similar sized fragments were obtained from 3D7 and HB3 DNA when amplified from primers B3/A3 or A2/B4. The primers A3 and B4 were positioned in between B3 and A2. The ethidium bromide stained gels of the PCR products are shown in Fig. 5.24 (plate a). The specificity of these fragments was confirmed by probing with oligonucleotides B4 (plate b) or A3 (plate c). These results illustrate that the dimorphism is dissociated when either A2 or B3 was used with an internal primer, suggesting that deletion of sequence was not a cause for the RFLP. The results also suggest that some sequences located between B3/B4 and A2/A3 were involved in interaction to generate secondary structure which causes the anomalous mobility of the 3D7 DNA in agarose gels.

If this is the case, similar sized PCR products are expected from 3D7 and HB3 DNA using primers A3/B4, A1/B4 or B2/A3 which encompassed only one or none of these two regions. As illustrated in Fig. 5.22 and shown in Fig. 5.24, fragments of similar sizes were amplified, confirming the idea that the regions between B3/B4 and A2/A3 contained sequences important in producing the secondary structure.

However, the dissociation was not complete. Note that in Fig. 5.24, faint bands of bigger sizes were also detected in the PCR products of 3D7 DNA when amplified from primers A3/B2, A2/B4, A3/B3 and A3/B4.

Fig. 5.23 Dimorphism in PCR fragments from 3D7 and HB3 DNA.

a) Ethidium bromide stained gel of PCR fragments.

b) Southern blot of gel probed with internal oligonucleotide B4.

c) Southern blot of gel probed with internal oligonucleotide A3.

Track 1: HB3 DNA
Track 2: 3D7 DNA


a
A 1
B 3
A 2
B 3
A 2
B 2
Markers
1 2 1 2 1 2
bp
861
738
615
492
369
A1
B1
A1
B2
1 2 1 2
bp
1353
1107
861
615
b
A 1
B 3
A 2
B 3
A 2
B 2
1 2 1 2 1 2
bp
861
738
615
492
369
A1
B1
1 2
bp
1353
1107
861
615
c
A 2
B 2
1 2
bp
861
738
615
492
369
A 1
B 1
A 1
B 2
1 2 1 2
bp
1353
1107
861
615

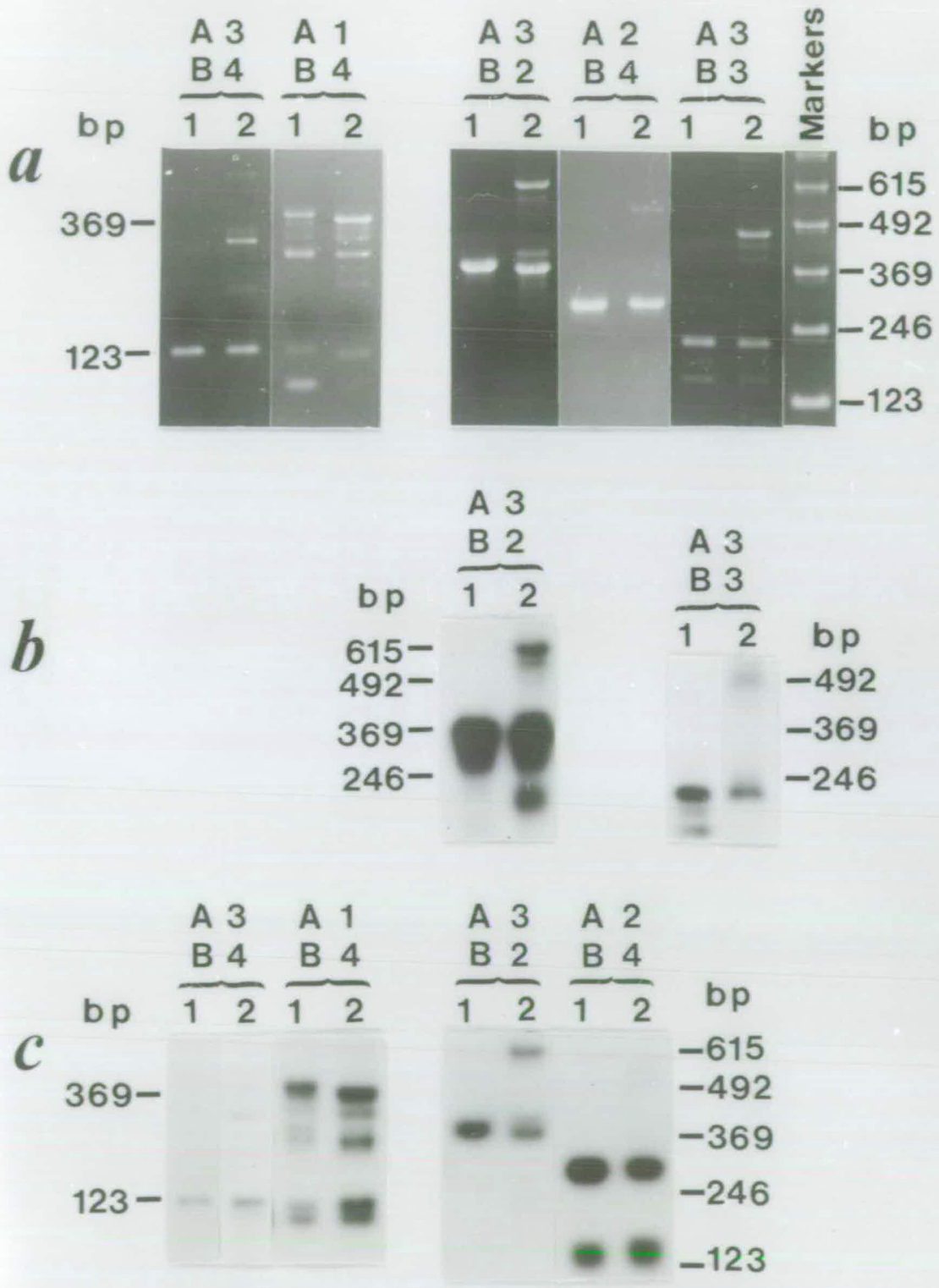
Fig. 5.24 Dissociation of dimorphism by PCR.

a) Ethidium bromide stained gel of PCR fragments.

b) Southern blot of gel probed with internal oligonucleotide B4.

c) Southern blot of gel probed with internal oligonucleotide A3.

Track 1: HB3 DNA
Track 2: 3D7 DNA


a
A 3
B 4
A 1
B 4
A 3
B 2
A 2
B 4
A 3
B 3
Markers
bp
1 2 1 2 1 2 1 2 1 2
369
123
615
492
369
246
123
b
A 3
B 2
A 3
B 3
bp
1 2
1 2
615
492
369
246
492
369
246
c
A 3
B 4
A 1
B 4
A 3
B 2
A 2
B 4
bp
1 2 1 2 1 2 1 2
369
123
615
492
369
246
123

These bands also cross-hybridized with the oligonucleotide probes suggesting that they were specific PCR products.

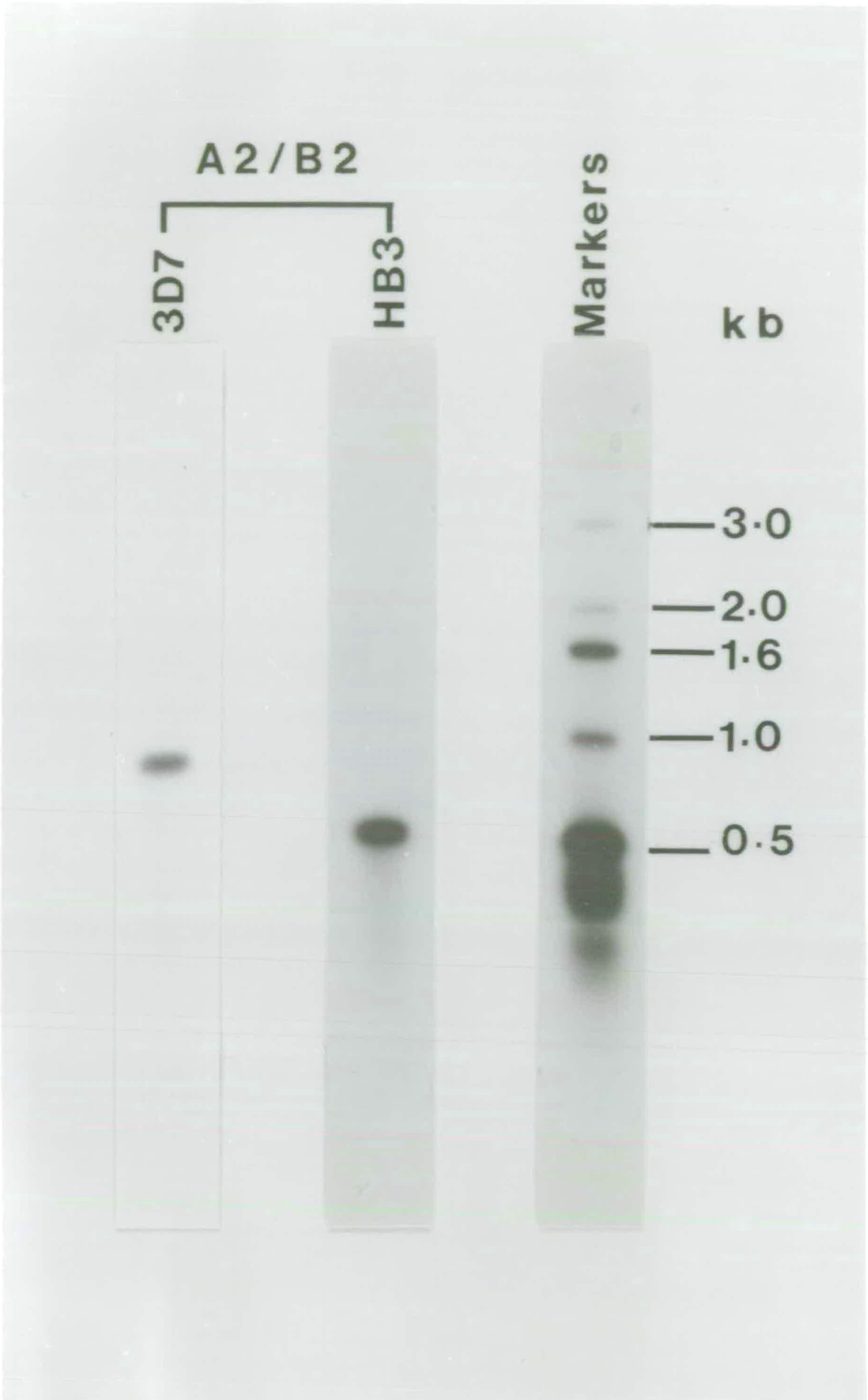
5.1.8.2 Alkaline Denaturing Gel: We cannot therefore exclude the possibility of the presence of a 250bp sequence in 3D7 DNA which was not sequenced due to the formation of a cruciform. To check whether there is such a loop in 3D7 DNA, the natural sizes of the DNA fragments were checked under denaturing conditions.

The PCR amplified fragments from 3D7 and HB3 DNA using oligonucleotide primers A2 and B2 were purified from agarose gel, dephosphorylated, end-labelled with [γ-^{32}P] dATP and then run on an alkaline denaturing gel at 60V for 7hrs (Section 2.4.28). The trichloroacetic acid fixed gel was dried and autoradiographed. The results are shown in Fig. 5.25. Dimorphic bands of 580bp and 830bp were still discernable for HB3 and 3D7 DNA segments respectively, suggesting that the natural sizes of the two DNA segments are 580bp and 830bp respectively.

It is therefore possible that a loop of 250bp was formed in 3D7 DNA which was bypassed by the sequencing enzyme at low temperature (37°C) although it is very unlikely that after bypassing a region of 250bp, the sequence obtained can still be as unambiguous as that obtained in this work. Furthermore, the conditions used for sequencing included 10% dimethylsulphoxide (DMSO) which should be sufficient to keep the DNA denatured during sequencing.

Although the results from these two experiments are conflicting, both suggest the formation of some unusual secondary structure in the 5' non-coding region of 3D7 DNA.

Fig. 5.25 Alkaline denaturing gel. Autoradiographs of end-labelled PCR fragments from 3D7 and HB3 DNA separated by alkaline denaturing gels.


A2/B2
3D7
HB3
Markers
kb
3·0
2·0
1·6
1·0
0·5

5.2 DISCUSSION

The prevalence of poly (dA), poly (dT), poly (AT) and A-T repeats in the 5' non-coding region of the DHFR-TS gene already makes it probable that this DNA region will form secondary structures. Comparison between gene sequence and gel mobility differences of DNA from the 5' non-coding region of the HB3 and 3D7 DHFR-TS genes suggests that secondary structures may exist in this region of the 3D7 genome. It could be postulated that a 250bp hairpin loop could exist in 3D7 DNA resulting in this region being bypassed by the sequencing enzyme such that it was not sequenced. However, if this is not the case, the apparent size difference must be caused by other secondary structure of 3D7 DNA resulting in its retardation during agarose gel electrophoresis. Here we consider bent DNA as a possible cause.

There is no reason to suppose that *P. falciparum* would be any difference from any other organisms in its possession of bent DNA. The ubiquitous occurrence of bent DNA from cellular to viral genomes implies its possible occurrence in parasitic protozoa (Marini *et al.*, 1982; Zahn & Blattner, 1985; Griffith *et al.*, 1986; Kawamoto *et al.*, 1989; Hagerman, 1990). Moreover, a highly repeated element of the *P. berghei* genome has been reported to exhibit bending character (Dore *et al.*, 1988).

The putative sequences generating the RFLP are limited to two regions. Within one region, an A stretch and a T stretch are found. Periodically repeated poly (dA) tract are often identified to be the locus of bending centre (Koo *et al.*, 1986; Wu & Crothers, 1984; Dore *et al.*, 1988). Poly d(A).poly d(T) sequence has been demonstrated to adopt an altered DNA configuration in physiological conditions by X-ray crystallography

(Arnott *et al.*, 1983; Alexeev *et al.*, 1987). Hence, it is possible to produce a bend at the junction of the B-form DNA and this altered DNA form as has been demonstrated for the junction between the B-form DNA and another DNA configuration, the A-form DNA (Selsing *et al.*, 1979).

Within the other region, a variation in the two sequences is detected. An insertion of four bases is present in the 3D7 sequence. Most noticeably, this insertion separates the above mentioned poly (dA) and poly (dT) from another poly (dT) stretch. The requirment of periodically repeated sequence in phase with the helix repeat to produce inphase bending (Trifonov, 1980; Trifonov & Sussman, 1980) implies that the insertion or deletion of short DNA sequences may disrupt the phasing of the bends and hence abolish DNA bending. In 3D7 DNA, the insertion of four bases may alter the phasing of the bends and hence significantly increase the magnitude of the bending in this DNA fragment to produce a macroscopic effect which can be visualized by gel electrophoresis.

Variation in other sequences e.g. poly (AT) and A_nT repeats may also play a part in contributing to DNA bending especially since poly (AT) has been shown to adopt an alternating B-conformation (Suggs & Wagner, 1986) which also has the potential to generate a bend at the junction with the conventional B-form DNA. However, their functional importance cannot be predicted from the present study.

These models are open to further testing (Wu & Crothers, 1984; Koo *et al.*, 1986). The cloned K1 DNA provides a very good system to study the nature of this RFLP since the cloned insert generates a doublet having the same size as the 3D7 and HB3 dimorphic DNA fragments and there is evidence to suggest that the smaller fragment derives from the upper one.

The two bands can be purified and individually sequenced. Any differences in the two sequences will pinpoint the variations causing the RFLP. Furthermore, sequencing reactions should be ideally performed at high temperature (72°C) using *Taq* polymerase. Under this condition, any cruciform structures should be removed.

CHAPTER 6
CHARACTERIZATION OF THE TRANSCRIPTIONAL UNIT OF THE DHFR-TS GENE OF *PLASMODIUM FALCIPARUM*

6.1 RESULTS

6.1.1 Approximate Mapping of the Transcriptional Start Sites of DHFR-TS Genes from 3D7 and HB3 by PCR

6.1.2 Accurate Mapping of the Transcriptional Start Sites of DHFR-TS Genes from 3D7 and HB3 by Primer Extension

6.1.3 Analysis of the Upstream Promoter Region

6.1.4 Determination of the Size of DHFR-TS Transcript

6.2 DISCUSSION

Because of our ignorance of transcriptional units of *Plasmodium* genes and as no functional test for putative promoter sequences of *Plasmodium* genes exists at present, prediction of putative promoter motifs of *Plasmodium* genes must initially rely on consensus analysis. Thus far, only the transcriptional unit of the *P. knowlesi* circumsporozoite (CS) gene is well characterized (Ruiz I Altaba *et al.*, 1987) though the 5' termini of the mRNA of the precursor of the major merozoite surface antigen (PMMSA) genes from *P. yoelii* (Lewis, 1990) and *P. falciparum* (Myler, 1990), the aldolase gene (Knapp *et al.*, 1990) and the calmodulin gene from *P. falciparum* (Robson & Jennings, 1991) have also been mapped. Dihydrofolate reductase (DHFR) is a functional gene. Mapping of its transcriptional unit could form the basis of consensus analysis of putative promoter elements of functional genes in *Plasmodium*.

Furthermore, there is an additional relevance in mapping the transcriptional unit of the DHFR-TS gene as it is possible that a pyrimethamine resistant mutant T9/94 (M1-1) possesses a mutation affecting transcription (see CHAPTER 4).

In this chapter, I describe the mapping of the transcriptional start sites of DHFR-TS genes from the cloned lines 3D7 and HB3. The start sites were first mapped approximately by PCR methods and then accurately by primer extension.

6.1 RESULTS

6.1.1 Approximate Mapping of the Transcriptional Start Sites of DHFR-TS Genes from 3D7 and HB3 by PCR

Preliminary studies were carried out using PCR to locate the regions where transcription starts. The rationale is illustrated in Fig. 6.1. As DNA is transcribed into mRNA which is then translated into protein, specific synthesis of certain cDNA can be initiated from an RNA preparation containing a population of mRNA by adding a downstream antisense oligonucleotide primer 'Pa' which is complementary to a short region of the mRNA. The reverse transcriptase activity of the *Taq* polymerase can then start synthesizing a complementary cDNA. Then, by adding an upstream primer 'Pb' or 'Pc' from the sense strand to the reaction mixture, the secondary DNA strand will be synthesized. If one of the upstream oligonucleotide primers 'Pd' is located to a region outside the transcriptional unit, there would not be any synthesis of the secondary DNA strand from the cDNA strand and hence no template for PCR. The start site of transcription can thus be located to within the two oligonucleotide sequences 'Pc' and 'Pd' where such a cut-off is observed.

Controls are very important in this experiment since amplification from contaminating DNA could give false positive results. Therefore, the RNA preparations were initially treated with DNase to destroy all DNA. The efficiency of the DNase was first tested by treating DNA preparations with DNase in parallel before being subjected to PCR. The absence of any amplified products from the DNA samples confirms that the DNase was active enough to destroy the DNA (data not shown).

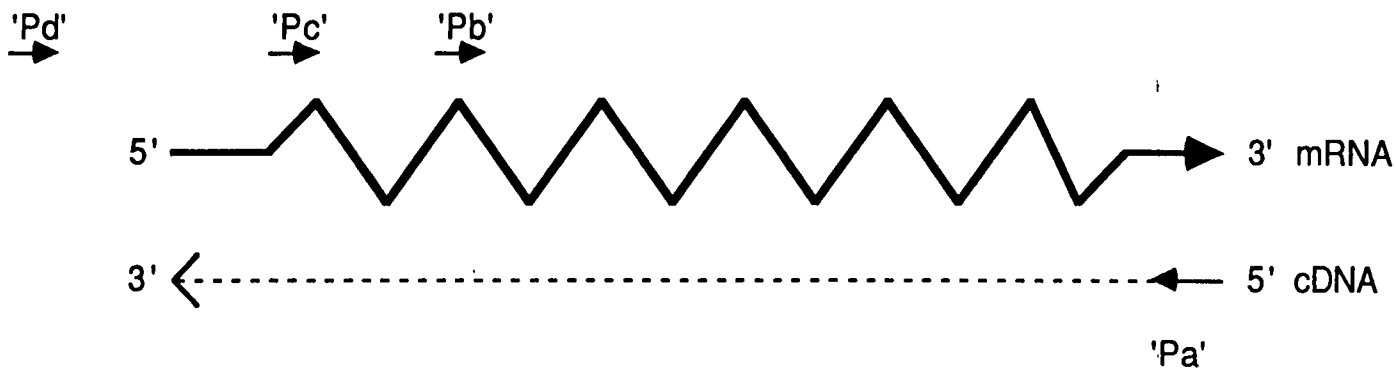


Fig. 6.1 The rationale of PCR from RNA to map the approximate location of the transcription start site. Specific synthesis of cDNA can be initiated by Taq polymerase from an RNA preparation by adding a downstream antisense oligonucleotide primer 'Pa' which is complementary to a region near the initiation codon of the gene. Then, by adding upstream primer 'Pb' or 'Pc' from the sense strand to the reaction mixture, the secondary DNA strand will be synthesized. If one of the upstream primers 'Pd' is located to a region outside the transcriptional unit, there would not be any synthesis of the secondary DNA strand from the cDNA strand and hence no template would be generated for PCR. The start site of transcription can thus be located within the two oligonucleotide sequences 'Pc' and 'Pd' where such a cut-off is observed.

The DNase-treated RNA preparations were then subjected to PCR using the antisense oligonucleotide A1 and the sense strand oligonucleotides B1, B2, B3 and B4 (see Fig. 5.16). The sizes and the specificity of the PCR fragments were checked on agarose gels. For each experiment a control reaction was carried out using RNA which had been treated with both DNase and RNase.

The results are shown in Fig. 6.2. No bands of the expected sizes were observed in the control reactions. However, in the experimental reactions, the patterns in the agarose gels reveal that specific fragments of the expected sizes were amplified from both 3D7 and HB3 RNA preparations using pairs of primers A1/B4 and A1/B3. With 3D7 RNA, fragments of 440bp and 800bp were obtained. With HB3 RNA, fragments of 440bp and 550bp were obtained. No amplified fragments were detected from either RNA samples using pairs of primers A1/B2 and A1/B1. This cut-off of PCR signal suggests the location of a transcriptional start site between oligonucleotide sequences B2 and B3.

When the sensitivity of the test was increased by Southern blot analysis of this gel, probing with the internal oligonucleotide A2, faint bands were also detected in tracks loaded with samples amplified from primers A1/B2 (Fig. 6.3). Signals from samples with primers A1/B4 or A1/B3 showed up readily after short exposure (overnight). Upon prolonged exposure (4 days), faint bands of 920bp and 730bp were also observed from 3D7 and HB3 RNA samples amplified from the primers A1/B2. Since the same amounts of RNA were put into each reaction, the difference in PCR efficiency might be explained by the existence of major and minor transcriptional start sites although it is also possible that this difference might be affected by the

Fig. 6.2 Ethidium bromide stained gels of PCR products from RNA samples using different combinations of primers.

a) 3D7 RNA

b) HB3 RNA

The oligonucleotides used in these reactions are given above their respective samples. For each set of oligonucleotides two experiments were carried out. Lane 1 shows the results with RNA treated with DNase only. Lane 2 shows the results with RNA which had been treated with both RNase and DNase.

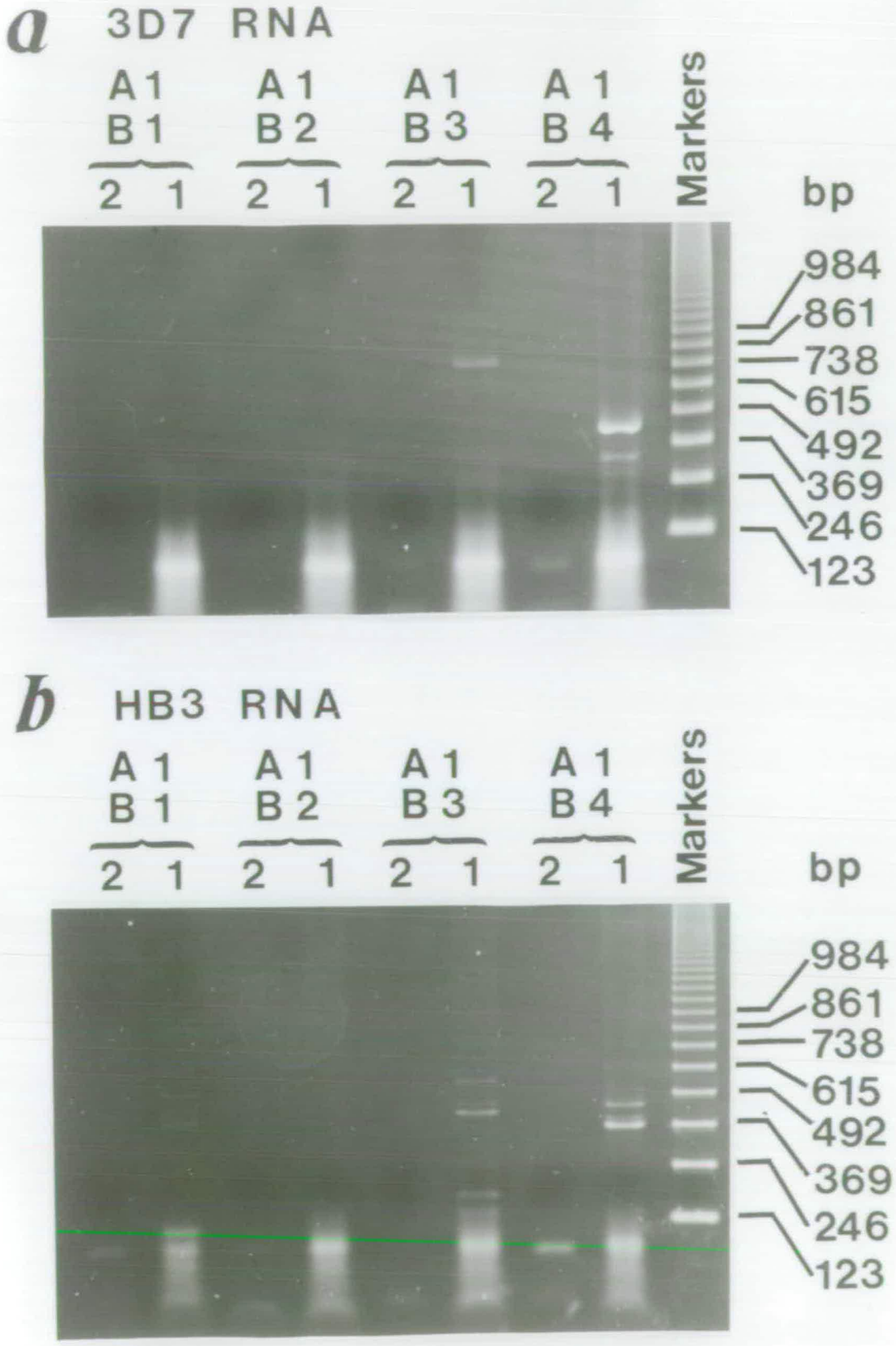
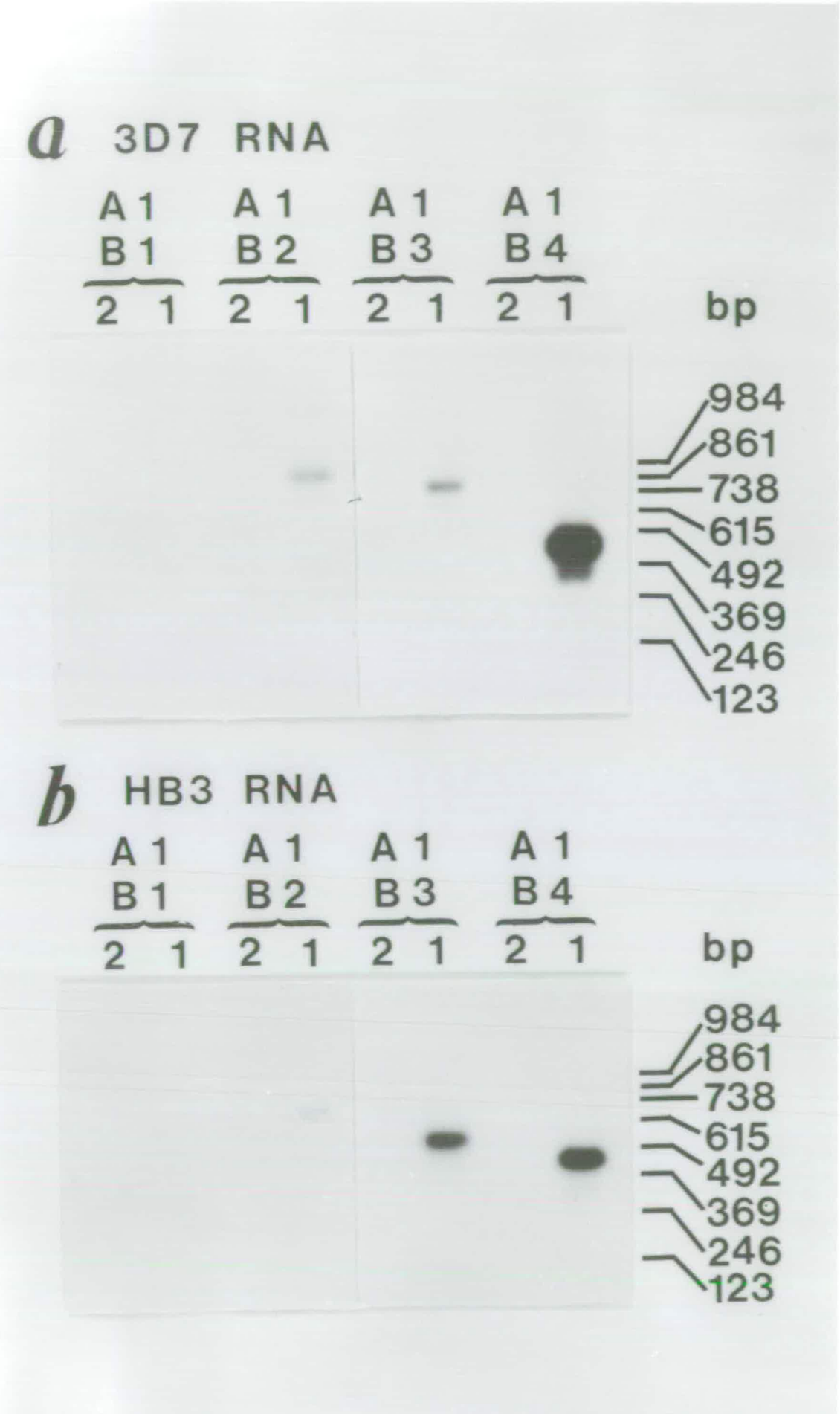

a
3D7 RNA
A1 B1
A1 B2
A1 B3
A1 B4
2 1
Markers
bp
984
861
738
615
492
369
246
123
b
HB3 RNA
A1 B1
A1 B2
A1 B3
A1 B4
2 1
Markers
bp
984
861
738
615
492
369
246
123

Fig. 6.3 Southern blot of gels in Fig. 6.2, probed with an internal oligonucleotide A2. The bands from A1/B3 and A1/B4 were detected readily after short exposure (overnight). The bands from A1/B2 were discernible upon prolonged exposure (4 days) of the blots.

a) 3D7 RNA

b) HB3 RNA


a
3D7 RNA
A1 B1 2 1
A1 B2 2 1
A1 B3 2 1
A1 B4 2 1
bp
984
861
738
615
492
369
246
123
b
HB3 RNA
A1 B1 2 1
A1 B2 2 1
A1 B3 2 1
A1 B4 2 1
bp
984
861
738
615
492
369
246
123

priming efficiency of various primers used. It should be noted that no signals were observed in lane 2 of the samples confirming the products were derived from RNA.

The measurements of the fragment size are tabulated in Table 6.1 and a diagrammatic representation of the results is illustrated in Fig. 6.4. In the figure, the locations of the major and minor transcriptional start sites are marked. As can be seen in the table, the PCR products from RNA still display size dimorphism when amplified from the primers A1/B3 and A1/B2 respectively but not from the primers A1/B4. This resembles the size dimorphism of the DNA fragments amplified from PCR as discussed in CHAPTER 5. Furthermore, the hybridization signal of the amplification products from 3D7 RNA using the primers A1/B3 is much weaker than that of HB3 RNA (Fig. 6.3). The less efficient amplification from 3D7 RNA using this pair of primers may imply the presence of secondary structure in this region, as has been discussed in CHAPTER 5.

With the approximate mapping of the transcriptional start sites achieved, I was able to proceed to mapping the sites more accurately using primer extension.

6.1.2 Accurate Mapping of the Transcriptional Start Sites of DHFR-TS Genes from 3D7 and HB3 by Primer Extension

To precisely locate the 5' termini of the DHFR-TS transcripts from HB3 and 3D7, end-labelled oligonucleotide primers were used to produce a cDNA complementary to the RNA templates (MATERIALS & METHODS Section 2.5.5). Analysis of the primer extended products by denaturing polyacrylamide gel electrophoresis provided information on the transcriptional

Table 6.1 The apparent sizes of the specific PCR fragments amplified from RNA preparations of 3D7 and HB3 using different combinations of primers

Primers	Apparent sizes (bp)	
	3D7 RNA	HB3 RNA
A1/B4	440	440
A1/B3	800	550
A1/B2	920	730
A1/B1	-	-

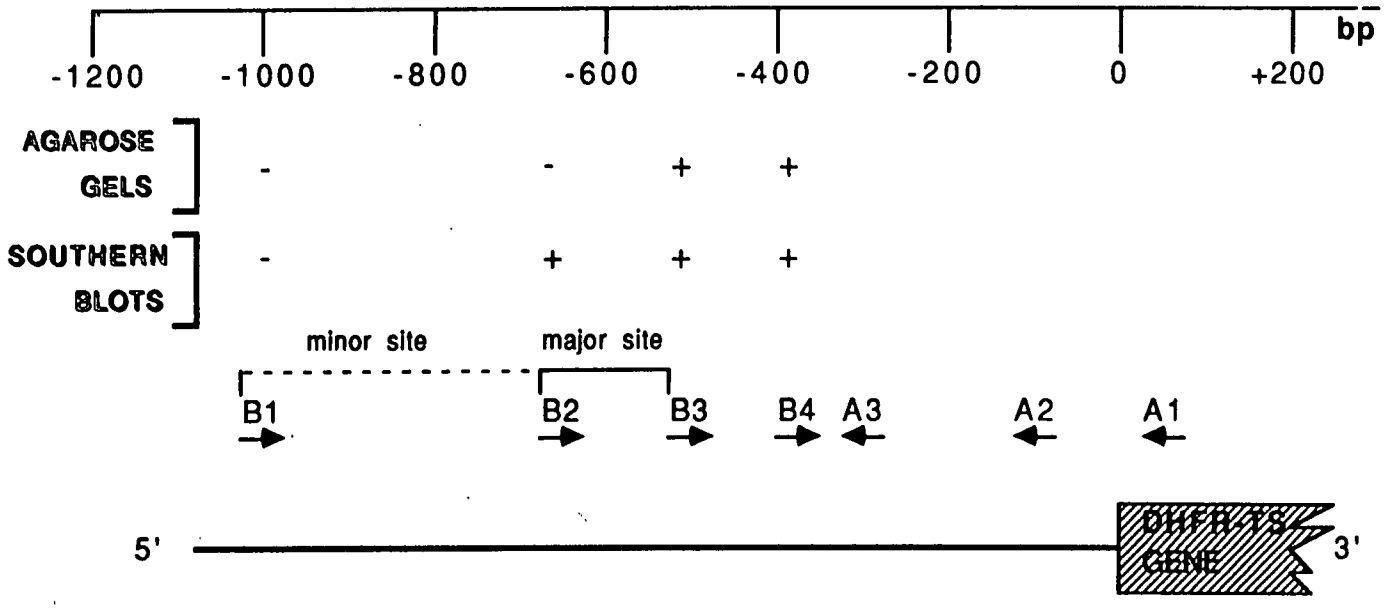


Fig. 6.4 A diagrammatic representation of the strategy used to amplify specific fragments from RNA preparations of 3D7 and HB3 using the primer A1 in combination with primers B1,B2,B3 or B4. The PCR products were detected by agarose gel electrophoresis and Southern blotting, probed with an internal oligonucleotide A2 . The presence or absence of specific amplified fragments is denoted as + or -. The cut-off of PCR signals suggests a major transcription initiation site between oligonucleotide sequence B2 and B3 and a minor site between oligonucleotide sequence B1 and B2.

start sites.

Since the major start site was located to a region between oligonucleotide sequences B2 and B3, the most proximal oligonucleotide A3 was chosen as the antisense primer in the extension reaction. Note that the nucleotide sequences for both HB3 and 3D7 genomic DNA are identical upstream of this region (see Fig. 5.20).

The results are illustrated in Fig. 6.5. Analysis of HB3 mRNA, produced a major fragment which mapped to position -552 corresponding to a C nucleotide and two minor fragments which were mapped to positions -557 and -543 corresponding to A and C bases respectively. The 5' termini were mapped to the same bases in 3D7. However, because of the slight variation of the 3D7 and HB3 sequences in the 5' non-coding region (see CHAPTER 5), the major fragment of the 5' termini is at position -536 in 3D7 and the minor fragments at positions -541 and -527. These results are in agreement with the position indicated for a transcriptional start site by PCR.

In order to see if a sub-population of mRNAs existed with an earlier transcriptional start site as also suggested by PCR, the samples were subjected to an extended run and the subsequent gel subjected to a prolonged exposure to reveal any bands occurring upstream. Fig. 6.6 illustrates a prolonged exposure from such an experiment which shows the occurrence of some faint bands in the upstream region. These may represent minor transcripts initiating from minor start sites 5' to the major start sites present at the bottom of the gel. It is difficult to map these minor start sites accurately from this experiment but a rough estimation has located these sites to ≈180, ≈230 and ≈265 bases from the major start sites which once again fall

Fig. 6.5 Autoradiographs of primer extension products using oligonucleotide A3 as primer. RNA preparations from the cloned lines 3D7 and HB3 were examined. Sequencing reactions from pUBS1 containing subcloned PCR fragments using the same primer were run alongside the primer extension products as markers. The arrows indicate a cluster of three start sites. The solid arrow represents the major signal whereas the two dotted arrows represent two minor signals.

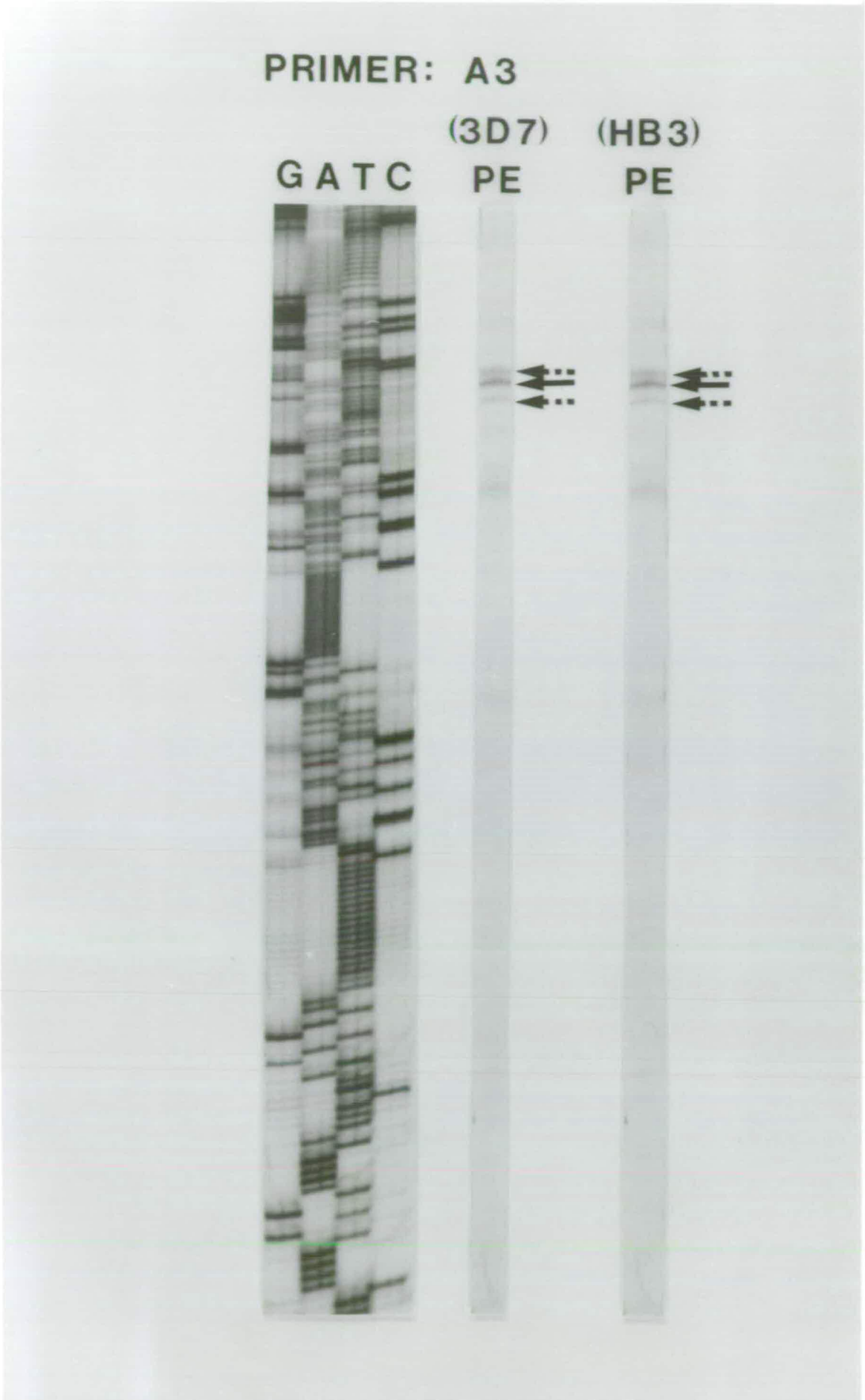
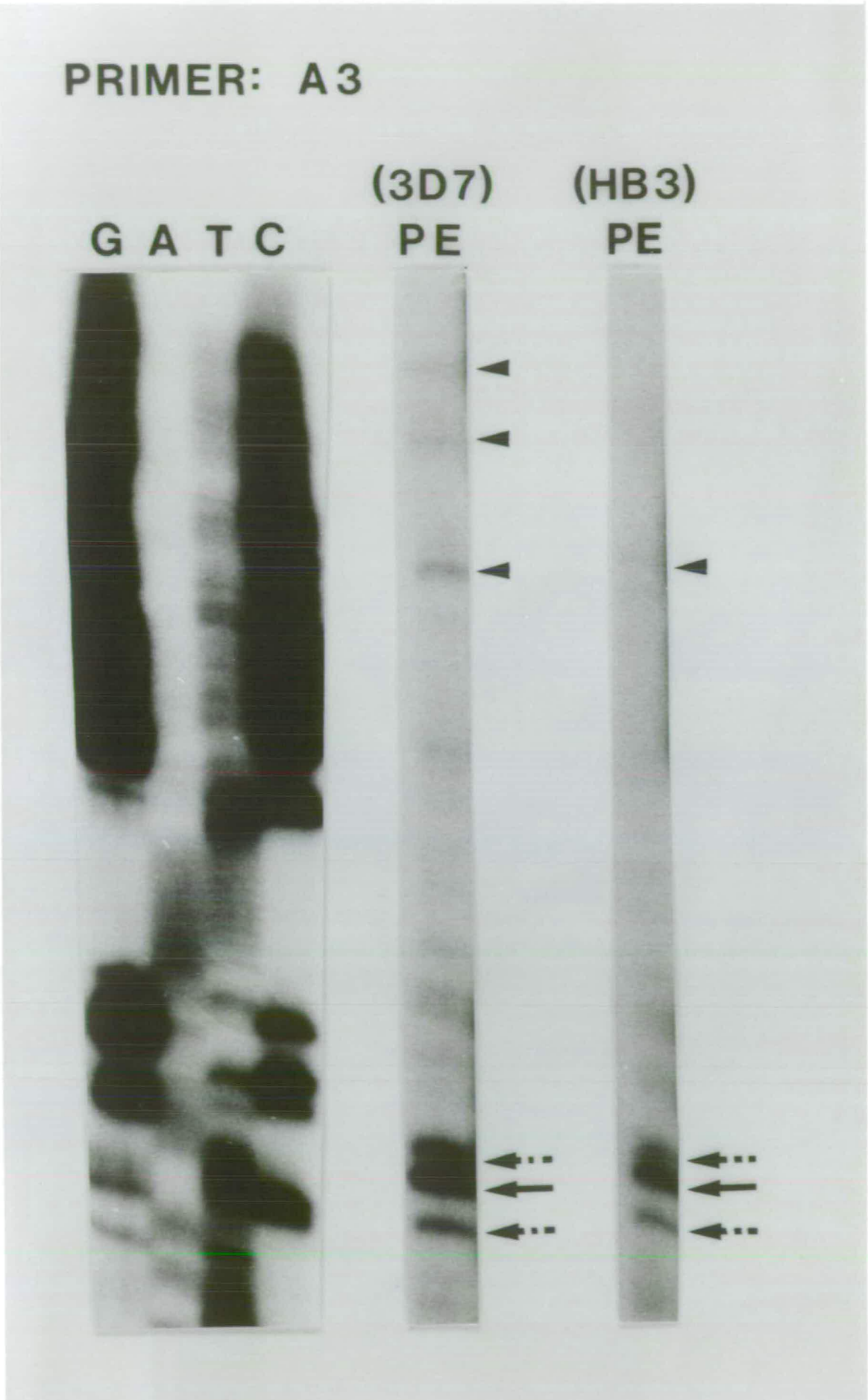

PRIMER: A3
(3D7)
(HB3)
G A T C
PE
PE

Fig. 6.6 Prolonged exposure of autoradiographs of primer extension products using oligonucleotide A3 as primer. Primer extended products from RNA samples of the cloned lines 3D7 and HB3 were subjected to an extended run and the subsequent gel subjected to a prolonged exposure. Sequencing reactions from pUBS1 containing subcloned PCR fragments using the same primer were run alongside the primer extension products as markers. The solid and broken arrows indicate a cluster of the major start sites (see also Fig. 6.5). The arrowheads indicate the minor start sites.


PRIMER: A3
(3D7)
(HB3)
G A T C
PE
PE

within the region defined by oligonucleotides B1 and B2 as predicted by PCR.

The results from primer extension therefore fit in with the previous PCR results which also predicted major and minor start sites. This is represented diagrammatically in Fig. 6.7. The major start sites mapped by primer extension are contained within a region also mapped to contain the major start sites by PCR. Both experiments also detected a possible upstream minor start site. The 5' non-coding sequences of DHFR-TS genes and its predicted transcriptional start sites are depicted in Fig. 6.8.

6.1.3 Analysis of the Upstream Promoter Region

Thus far, there is no functional test for *Plasmodium* promoter elements. Prediction of putative promoter sequences from the few genes analysed (Ruiz I Altaba *et al.*, 1987; Lewis, 1990; Myler, 1990; Robson & Jennings, 1991) relies completely on consensus analysis with known promoter motifs from other species.

When the 5' non-coding region of the *P. falciparum* DHFR gene is compared to mouse, human, Chinese hamster or *C. fasciculata* DHFR genes, no significant sequence homologies are found. Therefore, despite the similarity in the promoter sequences among the mammalian DHFR systems, it is not known which motifs are important in the protozoan system. The two kinetoplastids, *L. major* (Kapler *et al.*, 1987) and *C. fasciculata* (Hughes *et al.*, 1989) share striking homologies in the coding regions of the DHFR-TS genes, however, the non-coding regions vary significantly. Although closely related phylogenetically, they appear to have evolved different transcriptional regulation sequences. We cannot predict, therefore, the putative promoter

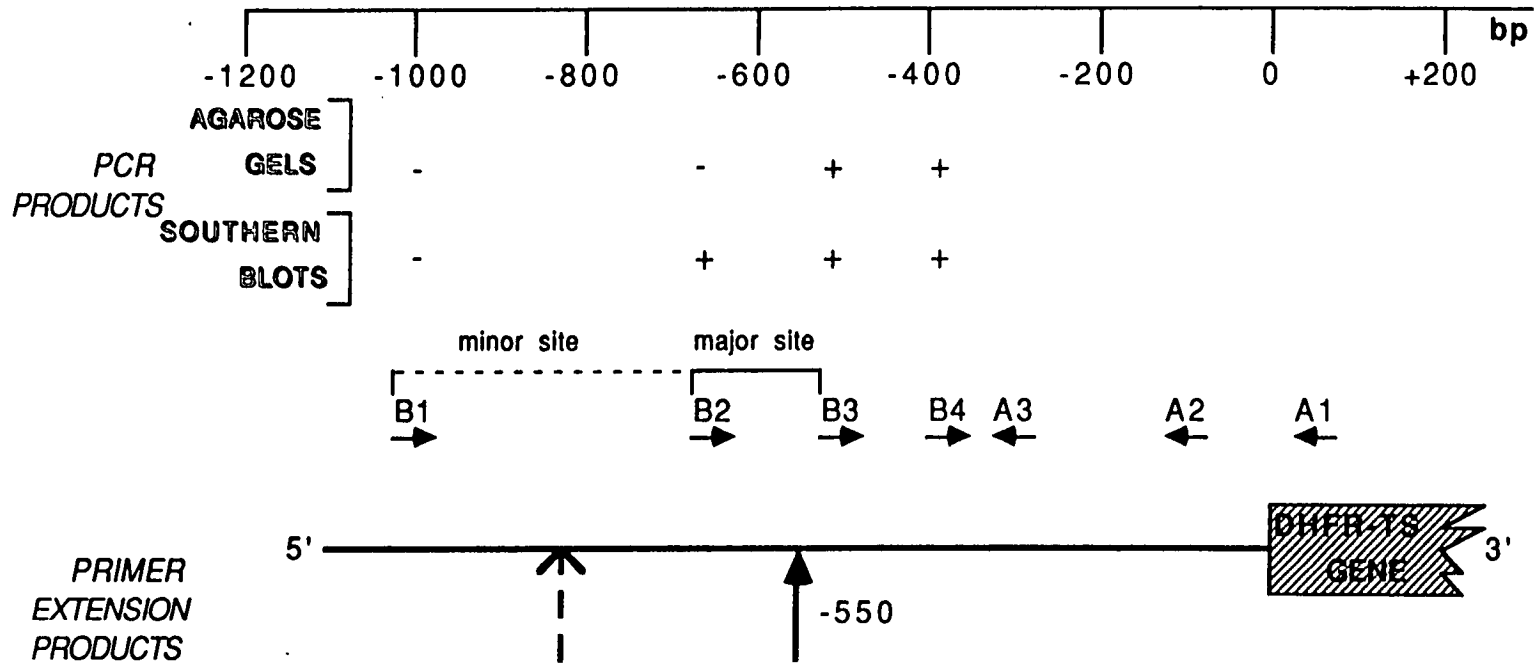


Fig. 6.7 A summary of the results obtained from PCR and primer extension. The major start sites (solid arrow) were mapped to about 550bp by primer extension, in a region mapped to contain the major start site by PCR. Both methods detected a putative minor start site in the 5' upstream region (dotted arrow and dotted line).

Fig. 6.8 The transcriptional unit of the DHFR-TS gene from 3D7 and HB3. The sequence of the 5' non-coding region of the gene from 3D7 is shown on top. The sequence begins behind the broken lines. The differences in the HB3 sequence are shown as either insertion of sequences or deletion of sequences (dotted line) in the bottom line.

Key:

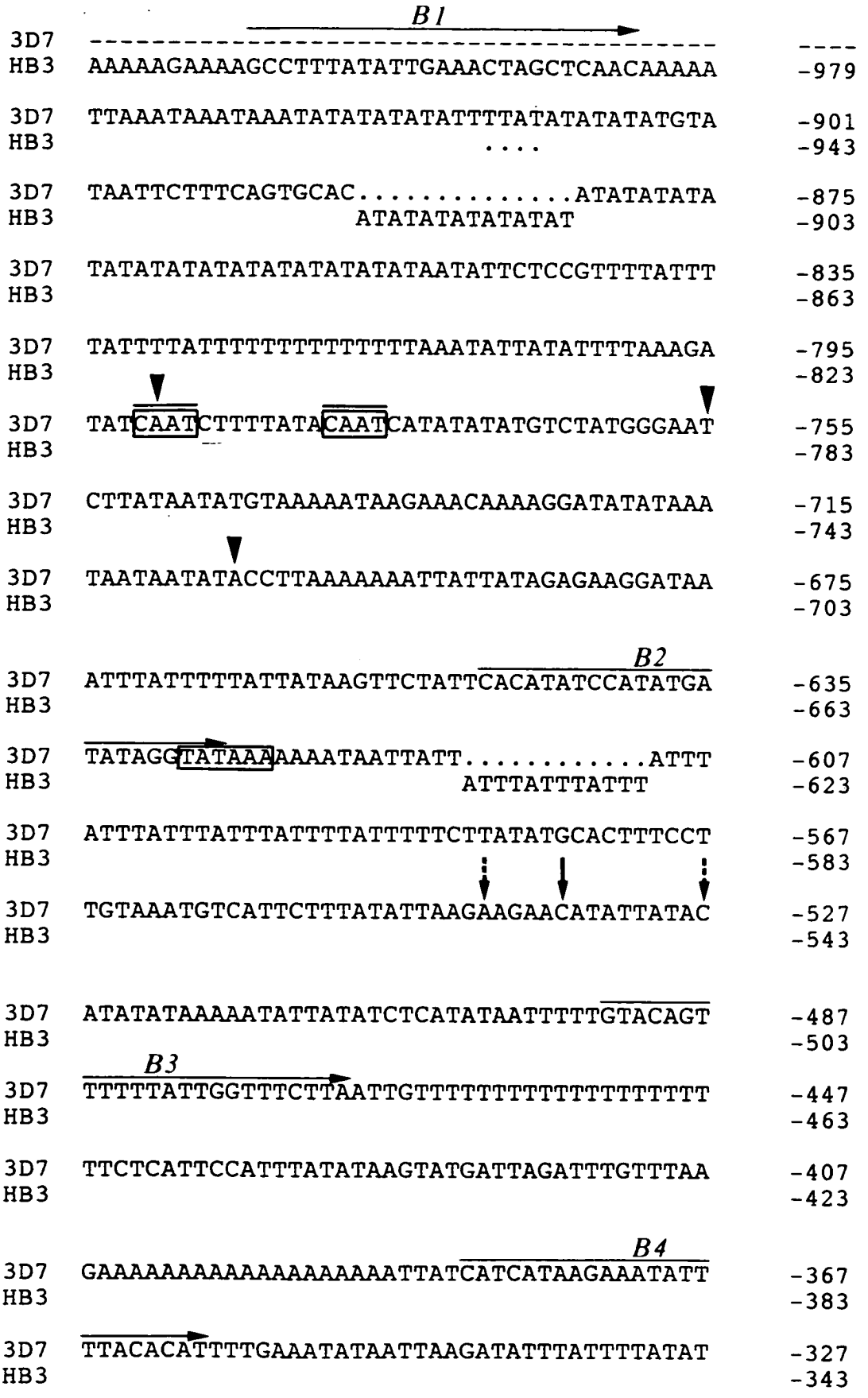
arrows: major transcriptional start sites as mapped by primer extension; solid arrow represents the major signal whereas the two dotted arrows represent two minor signals (see also Fig. 6.5)

arrowheads: approximate locations of minor transcriptional start sites as mapped by primer extension (see also Fig. 6.6)

boxed: 'TATA' boxes

boxed and overlined: 'CAAT' boxes

overlined: oligonucleotide primers, arrows indicate their orientations


B1
3D7 ---------- ----
HB3 AAAAAGAAAAGCCTTTATATTGAAACTAGCTCAACAAAAA -979
3D7 TTAAATAAATAAATATATATATATTTTATATATATATGTA -901
HB3 .... -943
3D7 TAATTCTTTCAGTGCAC.....ATATATATA -875
HB3 ATATATATATATAT -903
3D7 TATATATATATATATATATATAATATTCTCCGTTTTATTT -835
HB3 -863
3D7 TATTTTATTTTTTTTTTTTTTAAATATTATATTTTAAAGA -795
HB3 -823
3D7 TATCAATCTTTTATACAATCATATATATGTCTATGGGAAT -755
HB3 -783
3D7 CTTATAATATGTAAAAATAAGAAACAAAAGGATATATAAA -715
HB3 -743
3D7 TAATAATATACCTTAAAAAAATTATTATAGAGAAGGATAA -675
HB3 -703
B2
3D7 ATTTATTTTTATTATAAGTTCTATTCACATATCCATATGA -635
HB3 -663
3D7 TATAGGTATAAAAAAAATAATTATT.....ATTT -607
HB3 ATTTATTTATTT -623
3D7 ATTTATTTATTTATTTTATTTTTCTTATATGCACTTTCCT -567
HB3 -583
3D7 TGTAAATGTCATTCTTTATATTAAGAAGAACATATTATAC -527
HB3 -543
3D7 ATATATAAAAATATTATATCTCATATAATTTTTGTACAGT -487
HB3 -503
B3
3D7 TTTTTATTGGTTTCTTAATTGTTTTTTTTTTTTTTTTTT -447
HB3 -463
3D7 TTCTCATTCCATTTATATAAGTATGATTAGATTTGTTTAA -407
HB3 -423
B4
3D7 GAAAAAAAAAAAAAAAAAAATTATCATCATAAGAAATATT -367
HB3 -383
3D7 TTACACATTTTGAAATATAATTAAGATATTTATTTTATAT -327
HB3 -343

```
3D7   ATATTTAAATGAATAGATACATTTATTTATGAATCTGTTT      -287
HB3                                               -303
          A3
      AAAAGTTATATTAAGGGGATTATAATAAAAATATAATATA
3D7                                               -247
HB3                                               -263

3D7   TAAATAAATAAATATAAATAAATATATATATATATATATA     -207
HB3                           ....                -227

3D7   GTTTTTTTTTTTTTTTTTTTTTTTTTACTAGCCATTTTT      -167
HB3                                               -187

3D7   GTATTCCCAAATAGCTAGTTCATTTTAACTTACACAATAT     -127
HB3                                               -147

3D7   ATATTTTTCTTTAAATTTATAAAAATATATTCCAACATTT      -87
HB3                                               -107
      A2
      TCAAGATTGATACATAAAGATAATATATATATATATATAT
3D7                                               -47
HB3                                               -67

3D7   ATATATATATA.....................TTTATTTAT     -27
HB3                TATATATATATATATATTTA           -27

3D7   TTATATATTTATATTTTCTCCTTTTTATGATGGAACAAGT     14
HB3                                               14
                                          A1
3D7   CTGCGACGTTTTCGATATTTATGCCATATGTGCATGTTGT      54
HB3                                               54

      AAGGTTGAAAGCAAAAATGA
3D7                                               74
HB3                                               74
```

motifs in the more remote *P. falciparum* DHFR-TS gene by comparing with other protozoa especially when the AT content of its genome is so different.

We therefore look for sequences that appear to have frequent occurrence namely 'TATA' boxes (Breathnach & Chambon, 1981) and 'CAAT' boxes (Efstratiadis *et al.*, 1980; Grosveld *et al.*, 1982; McKnight *et al.*, 1985). A 'TATA' box is identified 90-100bp from the major start sites in the DHFR-TS genes presented here. Similar 'TATA' sequences are located more than 40bp away from the mRNA start sites in *P. knowlesi* CS gene (Ruiz I Altaba *et al.*, 1987) and *P. yoelii* PMMSA gene (Lewis, 1990). The location of the TATA sequence at this position resembles that of yeast in which the 'TATA' boxes are located at variable distances up to 100bp from the transcriptional start sites (Guarente, 1985). In yeast, the 'TATA' boxes are used to determine the level of transcription but not the precise position of transcription initiation (Guarente, 1985). The occurrence of heterogeneous start sites in *Plasmodium* genes may also imply a functional similarity of the 'TATA' boxes between the two species.

No 'CAAT' boxes are found close to the major transcriptional start sites of the *P. falciparum* DHFR-TS gene. However, two 'CAAT' sequences are located -816 to -819 and -804 to -807, which are ~70-80 bases from one of the minor start sites. 'CAAT' boxes are normally situated 40-110bp from the transcriptional start site in other species (Efstratiadis *et al.*, 1980; Grosveld *et al.*, 1982; McKnight *et al.*, 1985). In *Plasmodium*, 'CAAT' boxes also occur in some of the genes analysed e.g. the *P. knowlesi* CS gene (Ruiz I Altaba *et al.*, 1987), the PMMSA (gp195) gene (Myler, 1990) and the calmodulin genes of *P. falciparum* (Robson & Jennings, 1991), in positions 45-170 bases from the transcriptional start sites.

A number of potential Z-DNA forming sequences are located in the non-coding region of DHFR-TS gene (see Fig. 5.15). Formation of Z-DNA

has been evident to associate with transcriptional activities in eukaryotic, viral and protozoan systems (Nordheim & Rich, 1983b; Pardue *et al.*, 1983). Poly (CA) stretches, capable of adopting a Z-DNA configuration are located in the upstream region of *L. major* DHFR-TS gene (Kapler *et al.*, 1987). Short stretches of alternating purine and pyrimidine are also found in *P. knowlesi* CS gene (Ruiz I Altaba *et al.*, 1987) and *P. yoelii* PMMSA gene (Lewis, 1990).

The exceptionally high AT content in the *Plasmodium* genome may imply the possible utilization of a completely different regulatory sequence motif from the standard eukaryotic system. Sequences such as poly (AT) and oligo (dA), oligo (dT) tracts are commonly found in the *Plasmodium* non-coding regions. These sequences have the potential to induce conformational changes (Arnott *et al.*, 1983; Suggs & Wagner, 1986; Alexeev *et al.*, 1987), thus affecting the binding of regulatory proteins, resulting in activation or inactivation of the gene. Proteins such as histone H1, *lac* repressor, and proteins of unknown function from *Drosophila* and *Dictyostelium* have been shown to bind to AT rich DNA (Renz & Day, 1976; Klug *et al.*, 1979; Levinger & Varshavsky, 1982; Garreau & Williams, 1983).

6.1.4 Determination of the Size of DHFR-TS Transcript

Northern blot analysis was used to measure the size of the mRNA transcript. Total and poly A^- RNA was fractionated on formaldehyde denaturing gels and blotted before hybridizing with a probe derived from the DHFR-TS gene of the K1 isolate (Snewin *et al.*, 1989)

The results are shown in Fig. 6.9. A single transcript of 3.5kb from 3D7 total RNA was observed. A control track containing poly A^- RNA did not reveal any band. This message probably derived from the major

Fig. 6.9 Northern blot of total and poly(A$^-$) RNA from 3D7, probed with a BglII-EcoRI probe from the K1 isolate. The arrow indicates the major mRNA transcript of 3.5kb.

3D7
RNA:
total
poly(A⁻)
kb
3·7
3·5
2·1
1·7
a
b

transcripts. The absence of other bands derived from the minor transcripts may be explained by the small quantity of these transcripts which cannot be detected by Northern blotting.

Considering the size of the gene which is 1.8kb and the major 5' termini of the transcript mapped to about 550 bases from the AUG codon, this would imply a 3' terminus as long as 1.15kb.

6.2 DISCUSSION

Mapping of the transcriptional start sites to the same bases in the two cloned lines 3D7 and HB3 indicates conserved start sites in different parasite strains which are unaltered by their sensitivity to pyrimethamine.

Apart from the identified major start sites, putative minor upstream start sites are also predicted. The presence of two start sites may imply a functional significance as they can be used differentially at different stages during the cell cycle. It is believed that the enzyme DHFR is cell cycle regulated, following the same temporal relationship of DNA synthesis (Newbold *et al.*, 1982; Inselburg & Banyal, 1984; de Rojas & Wasserman, 1985). Although at present there is no evidence to suggest regulation of DHFR synthesis at the level of transcription, preliminary results on assaying the quantity of the enzyme suggest the stage-specific expression of the enzyme (not shown). The rate of enzyme synthesis is greatest at the early schizont stage. Similarly, it is suggested that differential expression of the *P. falciparum* β-tubulin gene in different developmental stages involves differences in the 5' or 3' sequences (Delves C.J. *et al.*, 1990). Examples are also known in eukaryotes. Young *et al* (1981) have shown that a single mouse α-amylase gene encodes two tissue specific mRNAs that differ only in

5' non-coding sequences. In the non-mammalian eukaryote, *Drosophila melanogaster*, the alcohol dehydrogenase gene contains two 'TATA' boxes which are used differentially in the larval and adult stages (Benjayati *et al.*, 1983; Posakony *et al.*, 1985).

In contrast to other eukaryotic genes whose 5' leader sequences are relatively short, the 5' non-coding sequence of the major mRNA of the *P. falciparum* DHFR-TS is 550 bases in length. This is in a range similar to that of other *Plasmodium* genes i.e. the *P. knowlesi* CS gene (≈270 bases) (Ruiz I Altaba *et al.*, 1987); the *P. falciparum* histidine-rich-protein (HRP) gene (≈700 bases) (Ravetch *et al.*, 1984); the *P. yoelii* PMMSA gene (≈400 bases) (Lewis, 1990); the *P. falciparum* gp195 gene (≈520 bases) (Myler, 1990) and the *P. falciparum* aldolase gene (≈322 bases) (Knapp *et al.*, 1990). The functional significance of a long untranslated region in *Plasmodium* is unknown but information important for gene control may be contained in this region. It is known that elements modulating translation are contained in sequences within the 5' untranslated regions of the *Drosophila* heat-shock gene *hsp22* (Hultmark *et al.*, 1986), the human ferritin mRNA (Aziz & Munro, 1987; Hentze *et al.*, 1987a; b) and the yeast regulatory protein *GCN4* (Fink, 1986; Mueller & Hinnebusch, 1986).

Similar to the 5' untranslated region, the long 3' untranslated region may reflect a functional significance in regulating gene expression e.g. sequences influencing translation, stability and the levels of the mRNA may be contained in this region (Casey *et al.*, 1988; Ross, 1988).

Prediction of promoter elements relies on consensus sequence analysis with other known eukaryotic promoter motifs. However, the exceptionally high AT content of the *Plasmodium* genome may imply the possible utilization of completely different regulatory sequence motifs from the standard eukaryotic system. Ideally, consensus analysis should be performed

among functional genes of the same species. The present study provides sequencing data and information about the transcriptional start sites of DHFR-TS gene from two cloned lines of *P. falciparum*, which will form the basis for consensus analysis when information about the transcriptional units of other *P. falciparum* functional genes are available. Another functional gene encoding the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) important in the purine synthesis in *P. falciparum* has already been cloned in our laboratory and it would be interesting to map the transcriptional start site for this gene.

The development of a functional test would prove invaluable in prediction of promoter motifs although this is as yet unavailable for the *Plasmodium* system as it requires the development of a transformation system. It may be that analysis of expression mutants selected *in vitro* from a genetically pure parent (see CHAPTER 4) offers another means to dissect the control of transcription in *P. falciparum*. Mutants that have an altered rate of gene expression may possess a mutated promoter which could be defined by simple sequence analysis.

CHAPTER SEVEN
CONCLUSIONS

The present study provides genetic confirmation that the target for the antimalarial drug, pyrimethamine is the enzyme dihydrofolate reductase-thymidylate synthetase (DHFR-TS). It also opens up the possibility of over-production of this enzyme as a means of resistance.

From previous sequencing and biochemical studies (McCutchan *et al.*, 1984b; Sirawaraporn & Yuthayong, 1984; Dieckmann & Jung, 1986a; Walter, 1986; Chen *et al.*, 1987; Cowman *et al.*, 1988; Peterson *et al.*, 1988; Snewin *et al.*, 1989; Zolg *et al.*, 1989; Sirawaraporn *et al.*, 1990), the changes responsible for resistance to pyrimethamine in a number of naturally occurring parasites have been pinpointed to specific base changes in the coding region of the gene encoding dihydrofolate reductase (DHFR), resulting in a structural alteration of the enzyme with reduced affinity for pyrimethamine. The present study provides genetic confirmation that the target for pyrimethamine is DHFR. The DHFR-TS allele from the pyrimethamine resistant clone HB3, tagged by a restriction-fragment-length-polymorphism (RFLP) marker, is shown to co-segregate with the pyrimethamine resistance phenotype in the progeny of a genetic cross with the pyrimethamine sensitive clone 3D7. No other changes i.e. over-production of the enzyme, amplification of the DHFR-TS gene were detected in HB3. It therefore seems that the single base difference previously noted between these two clones is the only event responsible for resistance to pyrimethamine in HB3. Evidence that a single base change is causing pyrimethamine resistance in HB3 is also demonstrated by the absence of intermediate levels of resistance in the progeny of the genetic cross (Walliker *et al.*, 1987).

However, the existence in nature of a wide range of resistance to pyrimethamine, with MIC values varying by up to a factor of 10^6 from 10^{-10}M to 10^{-4}M (Thaithong *et al.*, 1983), suggests the operation of additional or alternative mechanism(s). In this study an *in vitro* selected pyrimethamine

resistant mutant, derived from a sensitive parent, is shown to contain twice the amount of enzyme as the parent clone. It is argued that this may play a role in the drug resistance observed. As there is no evidence of gene amplification and the sequence of the DHFR gene remains unaltered, it is possible that the mutation(s) giving rise to over-production of the protein may be at the transcriptional level.

The ease with which mutants are selected *in vitro* which over-produce the enzyme implies that they may occur in the field isolates more frequently than was thought. Preliminary results have already suggested over-production of the protein in a naturally occurring pyrimethamine resistant cloned line, PR70/CB3. A thorough and comparative quantification of the protein levels in naturally occurring parasite strains will be needed to verify this point.

A lot of interesting questions remain. For example, is over-production of the enzyme caused by an increase in the rate of transcription? Is the molecular mechanism causing such a change the same in naturally occurring parasite strains and *in vitro* selected mutants? How common is this mechanism in nature? The results of the present study form the basis for further investigation of these questions.

Identification of a putative expression mutant prompted a further study on the regulatory unit of the DHFR-TS gene. As a first step towards understanding the role played by transcriptional control, the 5' flanking region of the gene from two parasite cloned lines with different sensitivity to pyrimethamine were sequenced and the transcriptional start sites mapped. It seems that the transcriptional start sites are conserved in spite of the minor variations in the upstream sequences and the difference in sensitivity to pyrimethamine.

Further studies will be focused on comparative analysis of the *in vitro* mutant T9/94 (M1-1) with the parent T9/94 since this provides a well-

defined system for pinpointing the mutation(s) giving rise to pyrimethamine resistance and for studying transcriptional regulation in this organism. However, further tests need to be performed to characterize the nature of the mutation giving rise to T9/94 (M1-1) to see whether this mutation represents an alteration of the parasites transcriptional machinery. As a start the level of the mRNA should be quantified by Northern blotting. The upstream region of the pyrimethamine resistant mutant T9/94 (M1-1) should be sequenced and compared to that of the pyrimethamine sensitive parent clone T9/94.

Mutants that have an increased transcription rate may involve an altered promoter or possibly other changes such as mutation(s) in transcription factors. Mapping of the transcriptional start sites and comparative sequence analysis of the upstream regions from mutants and parent parasites would not only pinpoint any changes contributing to pyrimethamine resistance but could also help to predict elements which are significant in gene transcription. This in turn would offer a means to dissect the promoter machinery of *P. falciparum*. At present, however, there is no functional test for the *Plasmodium* transcription system and only few *Plasmodium* genes with known transcriptional units are available for consensus analysis. The development of a functional test ultimately relies on the development of a transformation system for *P. falciparum.*

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APPENDIX

Some of this work has already been published:

Chan S.-W., Walliker D., Snewin V., Hyde J.E., Beale G. & Scaife J.G. (1989). Aspects of pyrimethamine resistance in *Plasmodium falciparum*. In: *Immunological & Molecular Basis of Pathogenesis in Parasitic Diseases. Proceedings of the 2nd Southeast Asian Symposium on Medical Parasitology.* Ko R.C. (ed).

11. Aspects of pyrimethamine resistance in *Plasmodium falciparum*

*S.-W. Chan[1], D. Walliker[2], V. Snewin[3], J.E. Hyde[3], G. Beale[2] and J.G. Scaife[1]**
[1]Department of Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR, U.K.

The control of malaria remains a major priority in the health programmes of many tropical countries. Despite recent efforts to develop a subunit vaccine against the disease, we still rely on drugs to combat malarial infections.

Chemotherapy has one major drawback. Extensive use of popular anti-malarial drugs has selected resistant parasites. In certain parts of the world some drugs can no longer be used against *Plasmodium falciparum*. For example, in S.E. Asia the majority of parasites are resistant to chloroquine (chl^R) and pyrimethamine (pyr^R), so many patients must now be treated with drugs which are more expensive or have significant side effects.

Despite this, little is understood about the mechanisms underlying drug resistance in malaria. For example, the precise target for chloroquine is still unknown. In fact this drug may have multiple targets in *Plasmodium*, since in at least one case, a chl^R parasite has been found to have more than one mutation (Padua, 1981). Other studies suggest that the chl^R phenotype may also arise by a different mechanism. Tumour cell lines can become drug resistant by activation of a verapamil-sensitive, Ca^{++}-dependent pump which removes the drug from the cell (Rogan *et al.*, 1984; Fojo *et al.*, 1985; Chen *et al.*, 1986). A similar system may operate for some chl^R mutants of *P. falciparum* (Martin *et al.*, 1987).

In the present study our interest focuses on pyrimethamine. Resistance to this drug has a different perspective. It is an analogue of dihydrofolate and its target is thought to be the enzyme, dihydrofolate reductase (DHFR). The product of the enzyme, tetrahydrofolate, and derivatives of this compound participate in several vital steps of parasite metabolism. Perhaps the most important of these is catalysed by the enzyme thymidylate synthase (TS) which uses N^5, N^{10} methylene tetrahydrofolate to convert dUMP to dTMP for use in DNA synthesis. Remarkably, in *P. falciparum*, as in certain other protozoa, a single protein molecule carries both DHFR and TS functions (Ferone, 1970; Garrett *et al.*, 1984; Bzik *et al.*, 1987).

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[2] Department of Genetics, University of Edinburgh.
[3] Department of Biochemistry and Applied Molecular Biology, UMIST, P.O. Box 88, Manchester M60 1QD.
* To whom all correspondence should be addressed.

The gene for DHFR-TS has been cloned from *P. falciparum* and sequenced. The amino-acid sequence encoded by the gene shows a N-terminal segment (amino-acids 1 to 228) homologous to DHFR sequences in other organisms. At the C-terminus (amino-acids 323 to 608) the sequence shows homology with TS. Between the two (amino-acids 229 to 322) is a peptide which presumably functions as a linker (Bzik *et al.*, 1987; Peterson *et al.*, 1988; Snewin *et al.*, 1989).

This extensive molecular information helps us formulate models about the mechanism underlying resistance to pyrimethamine. They lead to clear predictions, some of which will be tested in this paper.

We can envisage at least three basic mechanisms which could contribute to pyrimethamine resistance in malaria parasites. The target enzyme could be changed in structure to reduce binding of the drug. Mutations of this type have been documented in other organisms which have acquired resistance to antifolates such as methotrexate (Pan *et al.*, 1983). Equivalent changes have been reported for *P. falciparum* (Inselburg *et al.*, 1987). The mutant could make an excess of the enzyme so that the drug cannot completely block the synthesis of tetrahydrofolate. A mutant of *P. falciparum* selected *in vitro* appears to function in this way (Inselburg *et al.*, 1987). However, there may be mutants which are resistant to the drug, not because the enzyme is changed but, because uptake or subsequent processing of the drug in the parasite is changed. To date, pyr^R mutations of this kind have yet to be found in *P. falciparum*.

In this paper we shall present genetic and molecular biological studies on two clones of *P. falciparum*. One of them is naturally resistant to pyrimethamine. Our data throw light on the nature of the pyr^R mutation in this clone.

DHFR AND PYRIMETHAMINE IN MALARIA PARASITES

Biochemical studies on both *P. falciparum* and *P. berghei* provide direct evidence that pyrimethamine blocks DHFR *in vivo* (Sirawaraporn and Yuthavong, 1984; Walter, 1986). When partially purified enzyme prepared from a pyr^S parasite (3D7) is compared with that extracted from a pyr^R parasite (HB3), the enzyme from the latter is significantly less inhibited by the drug *in vitro* (Chen *et al.*, 1987). This result suggests that drug sensitivity of the parasite may be attributed to inhibition of DHFR. However, this conclusion should be confirmed *in vivo*. For example, one of the other factors listed above could contribute to the resistance of HB3.

This possibility is open to genetic test. If the sole cause of drug resistance in HB3 is a change in the structure of its DHFR we should be able to localise the responsible mutation in the gene for DHFR.

Mapping determinant for pyr^R in cross between clones

One way to find the genetic determinant for pyrimethamine resistance in HB3 is to use a genetic cross. We can mate a pyr^S clone of the parasite with the pyr^R mutant, and screen the progeny to discover whether the allele of the DHFR gene from the pyr^R parent is always found in those progeny which have inherited resistance to the drug. In our experiments we tagged this allele by defining a restriction target in the adjacent parasite DNA.

The inheritance of this DNA marker was then correlated with inheritance of resistance to the drug in cloned progeny parasites.

Cross between different clones

In the study presented here we exploit the progeny from an experimental cross already performed between two genetically pure clones of the parasite which had been maintained in culture *in vitro* (Walliker *et al.*, 1987). Two parasite lines were chosen which differed in a range of characters including antigens, repetitive DNA and drug resistance (Table 1). The haploid blood stages were cultured separately *in vitro*, mixed and fed to mosquitoes (Fig. 1). In the mosquito gut the sexual stages of the parasite form diploid zygotes. Haploid

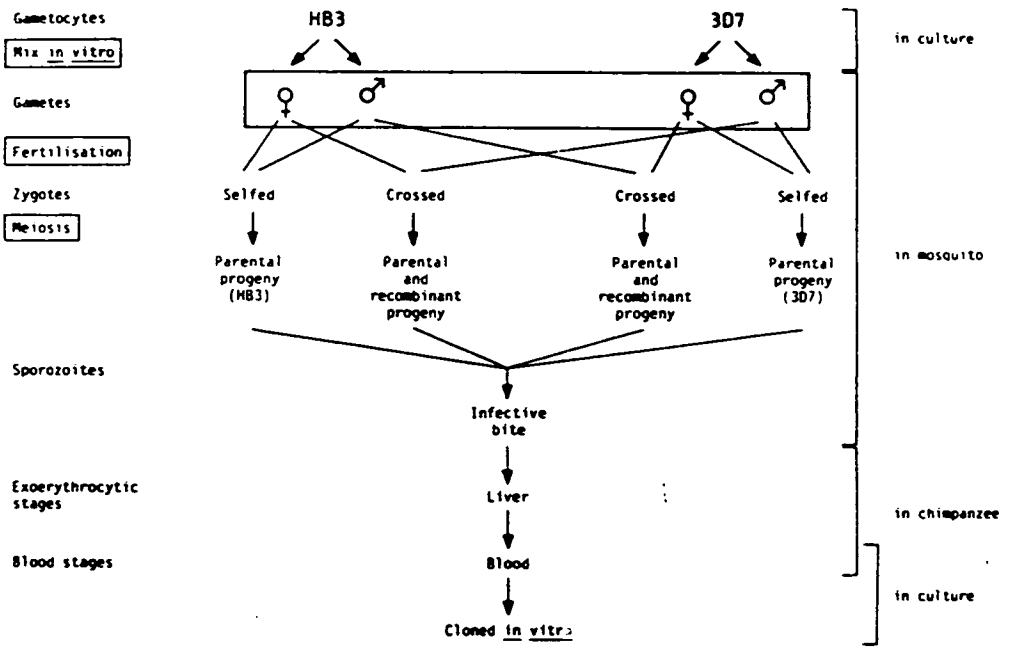

Fig. 1. Cross between two clones of *P. falciparum*, HB3 and 3D7. The diagram shows the steps leading to selfed and crossed zygotes and how these are manipulated to obtain pure progeny clones for testing. Note that genetic makers of HB3 and 3D7 appear in Table 1.

progeny develop and appear in the salivary glands as sporozoites. During this process, recombination occurs, both by reassortment of whole chromosomes and by crossovers between them. As a result, in many of the progeny the characters of the two parents are reassorted. In the experimental cross the progeny were scored as follows. Firstly, the sporozoites were introduced into a chimpanzee and allowed to develop in the liver. These ultimately gave rise to asexual stages in the blood which were subsequently cloned *in vitro* and tested for the markers of interest.

Restriction fragment length polymorphism in vicinity of gene for DHFR

Like other organisms, *P. falciparum* shows minor variations in the DNA surrounding a

particular gene. These can be used as markers which distinguish the alleles of a given gene in a cross. We found a restriction enzyme, *Acc*I (Fig. 2), which cuts the DNA in the neighbourhood of the DHFR gene in the two parents of the cross. The pyr^S parent (3D7) gives a DHFR fragment 2.4 kb long, whilst the same enzyme yields a DHFR fragment of 2.15 kb in the DNA of the resistant parent (HB3). The difference can be seen in Fig. 3, where the DHFR-containing fragment of HB3 migrates to a lower position on the gel than that of 3D7. These and neighbouring fragments have been visualised in the following way. DNA containing the DHFR-TS gene of *P. falciparum* had previously been inserted into bacteriophage lambda in order to sequence the gene (Snewin *et al.*, 1989). The structure of the gene and the fragments inserted in the phage vector are shown in Fig. 2. Insert DNA from the recombinant phage λ contains all the DHFR domain of the gene together with the sequence just upstream from the coding sequence (Fig. 2). This insert, labelled with ^{32}P, was used to visualise the fragment in Southern blots of parasite DNA.

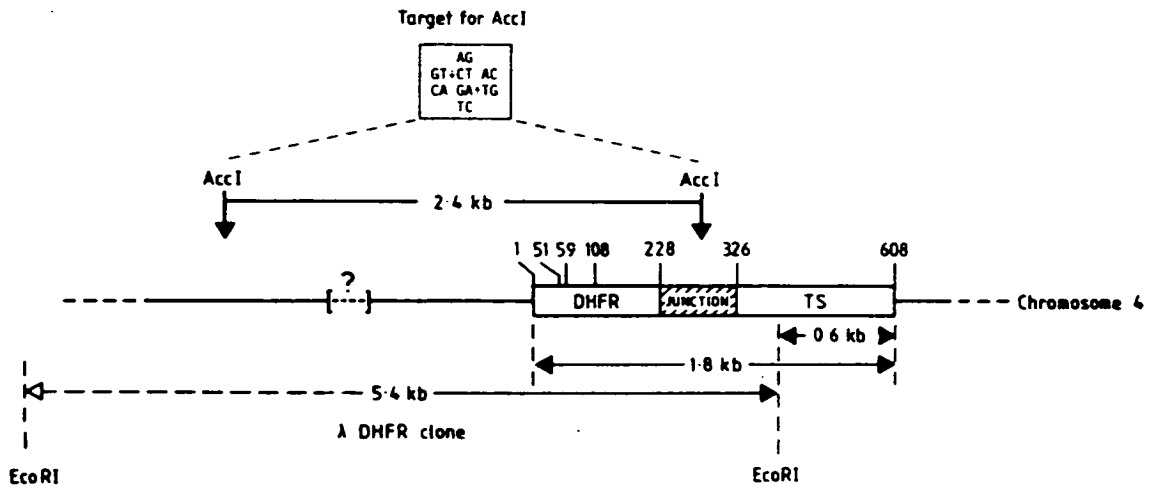

Fig. 2. Organisation of the gene for DHFR-TS. Targets and sequence recognised by the restriction enzyme *Acc*I in DNA are shown. Important amino acid positions are numbered on the gene (see text). Positions 51, 59 and 108 are candidate sites for amino acid changes affecting pyrimethamine sensitivity (Peterson *et al.*, 1989; Snewin *et al.*, 1988). The origin of the EcoRI λ DHFR clone is described by Snewin *et al.* (1988). The position of a putative deletion responsible for the *Acc*I RFLP is indicated: [ ].

The difference between the fragments detected in the DNA from HB3 and 3D7 by the probe thus defines a restriction fragment length polymorphism (RFLP) for the DHFR of these two parasite lines which can act as marker for the two alleles of the gene.

Segregation of DHFR gene in progeny of cross

Blood from the chimpanzee infected with parasites from the cross described above was used as an inoculum for parasite culture *in vitro*. The parasites established in culture were then cloned by the end-point dilution method and by micromanipulation. A total of 29 independent clones was obtained in this way. These were then screened for the markers in the cross (Table 1). Note that three of the clones had a combination of markers identical

Table 1. Linkage of RFLP with pyr^R in the progeny of a cross

	RFLP[a]	$Pyr^{R/Sb}$	MAb7.3[c]	MAb9.2[c]	MAb12.3[d]	Progeny Phenotype[e]
3D7	1	S	-	+	+	
HB3	2	R	+	-	-	
X2	2	R	+	-	+	rec
X4	2	R	+	-	-	rec*
X6	2	R	-	+	-	rec
X8	1	S	-	+	+	par
X10	1	S	+	-	-	rec
X11	2	R	+	-	-	rec*
X12	2	R	+	-	-	rec*
X30	1	S	+	-	+	rec
X33	2	R	+	-	+	rec
X34	2	R	-	+	-	rec
X35	2	R	-	+	-	rec
X36	1	S	-	+	+	par
X37	1	S	-	+	+	par
X41	2	R	-	+	-	rec
X44	1	S	+	-	+	rec
X45	1	S	-	+	-	rec
X47	1	S	+	-	-	rec
X48	1	S	-	+	-	rec
X50	1	S	-	+	-	rec
X51	1	S	+	-	-	rec
X52	1	S	+	-	-	rec
X58	1	S	+	-	-	rec
X63	1	S	-	+	-	rec
XP1	2	R	+	-	-	rec*
XP3	2	R	+	-	-	rec*
XP5	2	R	+	-	-	rec*
XP7	2	R	+	-	-	rec*
XP8	2	R	+	-	-	rec*
XP9	2	R	+	-	-	rec*

[a] The 2.4 kb fragment of 3D7 = 1; the 2.15 kb fragment of HB3 = 2. [b] Sensitive to 10^{-7} M pyrimethamine or more; resistant to 10^{-5} M pyrimethamine or less. [c] Monoclonal antibodies against a 195 kDa schizont surface antigen. [d] Monoclonal antibody against a 40 kDa schizont surface antigen. [e] Progeny are designated either parental (par) from selfed zygotes or recombinant (rec) from crossed zygotes. * These progeny are recombinants as judged by other criteria (segregation of proteins on 2D gels).

with that of the 3D7 parent. We had to exclude these from the subsequent analysis because they could have derived from selfed zygotes in which the markers would not be able to recombine (see Fig. 1). The remainder, which must have developed from cross-fertilised zygotes, were screened for recombination between pyr^R and the RFLP marker in the DNA in the following way.

The confirmed recombinant progeny were tested for their sensitivity to pyrimethamine. Note that the results are simple (Table 1). In the drug test, all of the progeny behave like one or other of the parents - either resistant (able to survive up to 10^{-5} M) or sensitive (killed at 10^{-7} M or more). No progeny of intermediate resistance were found.

At the same time their DNA was extracted, cut with *Acc*I and checked with the DHFR probe. A clear difference is seen between the digests of the sensitive and resistant progeny. The results are illustrated in Fig. 3 and summarised in Table 1. All of the progeny with the sensitive phenotype have the 2.4 kb DNA fragment characteristic of the sensitive parent. The remainder have that of the resistant parent (2.15 kb). Thus, in our experiment, pyrimethamine-resistance is always linked to the gene for DHFR tagged by the RFLP marker. Our results suggest that the mutation conferring drug-resistance on the strain HB3 is linked to the DHFR gene.

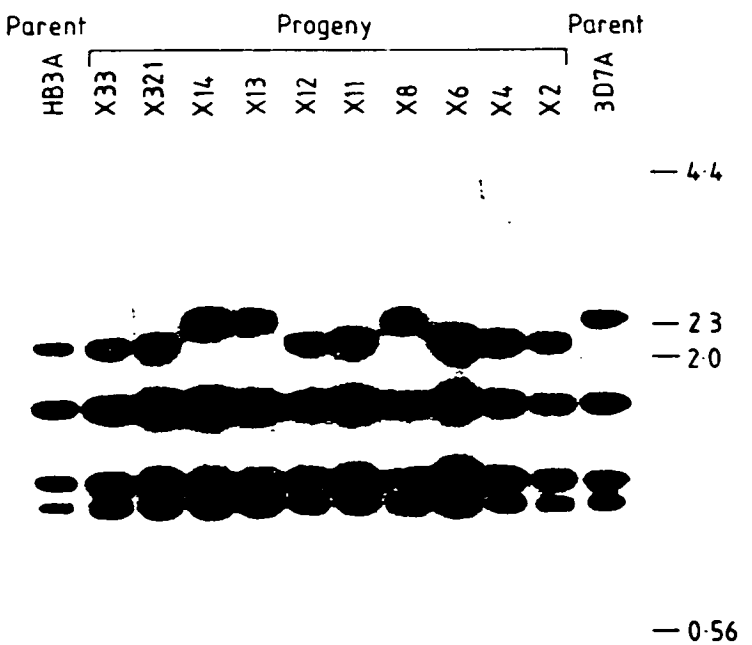

Fig. 3. RFLP marker revealed when the DHFR-TS DNA of the parasite is cut with *Acc*I. Note the difference in the upper band between the pyrimethamine sensitive (3D7) and resistant (HB3) parents. The lower bands are adjacent fragments which hybridise to the probe (see Fig. 2). Note that the progeny clones have only one of the two possible RFLP's. Bands were visualised by blotting to a Hybond nylon filter, denatured and probed with ^{32}P-labelled DHFR DNA (see Fig. 2).

The simple interpretation of our results is that the mutation is in the DHFR itself. This conclusion would be strengthened if we could test a larger number of progeny without the laborious isolation of individual clones used above. To this end we devised the following experiment. Uncloned progeny parasites were cultured in the presence of pyrimethamine long enough to select the resistant parasites from the cross. DNA was then extracted from the resulting population, which were a mixture of parasites all resistant to the drug. Firstly, this was compared with the DNA of a parallel mixture of parasites, not selected for pyrimethamine resistance. In addition, we calibrated the sensitivity of our test by mixing known amounts of 3D7 and HB3 DNA. The DNA samples cut with *Acc*I and probed for DHFR appear in Fig. 4. As expected, the unselected progeny from the cross, unchallenged

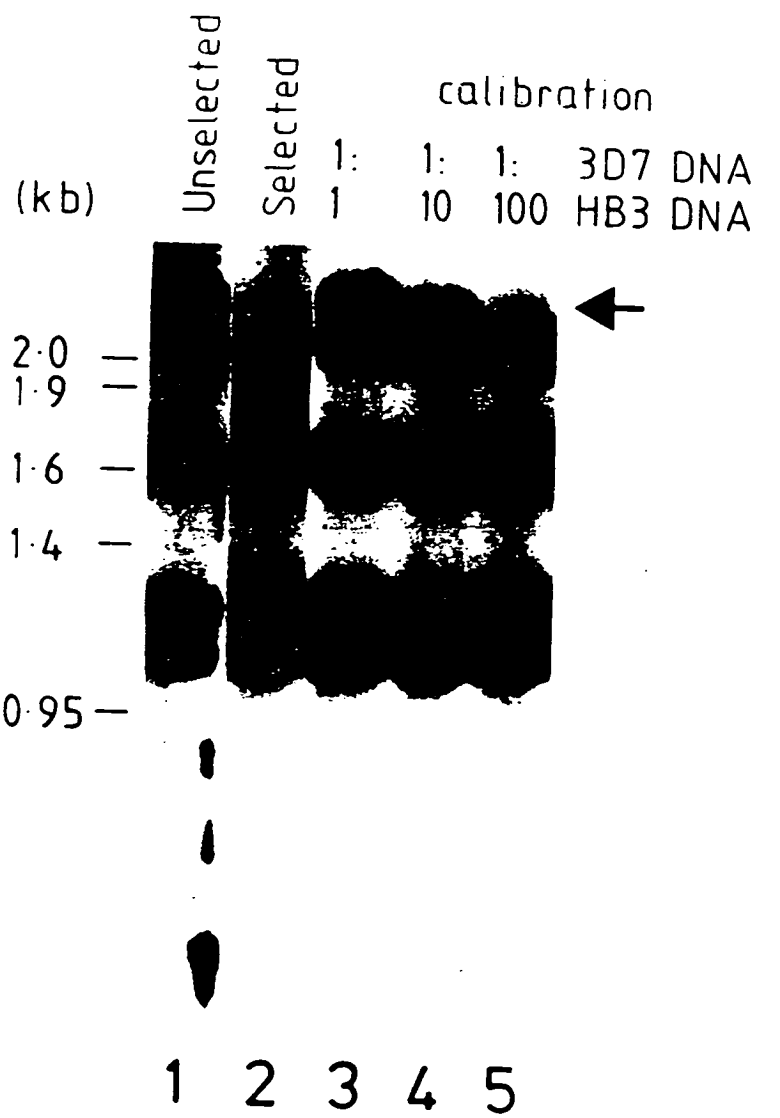

Fig. 4. Detection of the RFLP for DHFR in uncloned progeny DNA. Each track contains 3 μg of total DNA cut with *Acc*I. We show DNA from (track 1) unselected progeny and (track 2) progeny selected for growth in 10^{-6} M pyrimethamine. Tracks 3, 4 and 5 contain artificial mixtures of DNA from sensitive (3D7) and resistant (HB3) parasites to assess the minimum amount of the 3D7 RFLP detected.

by the drug, yield DHFR fragments characteristic of both the sensitive and resistant alleles in approximately equal proportions (track 1). By contrast, those grown up in the presence of pyrimethamine had only one DHFR allele - that characteristic of the resistant parent (track 2). In this test, the sensitivity is significantly higher. The mixtures show that if for a hundred resistant parasites there were only one 'aberrant' sensitive DHFR allele we would have detected it (tracks 3, 4 and 5; see arrow). Thus, our evidence indicates that the mutation to pyrimethamine resistance in the strain HB3 is located in, or at least close to, the DHFR gene.

DHFR gene not amplified in HB3

The mutation affecting the DHFR gene in HB3 could change the coding sequence and thus reduce the impact of pyrimethamine on the function of the enzyme. Alternatively, or in addition, the gene could be amplified, causing overproduction of the enzyme in the parasite. To test this possibility we have used dot blots (Fig. 5). Measured amounts of the DNA from both the resistant and sensitive clones were spotted on to nitrocellulose and probed with the DHFR fragment. Control spots probed with another gene (for ribosomal RNA) confirm that equal amounts of total DNA from each parasite was loaded in this experiment. If the gene in the resistant clone, HB3, had been amplified, we would have expected to see more DHFR copies in its DNA than in the DNA of the sensitive clone, 3D7. In fact, the two DNA samples hybridise with equal intensity to the probe, implying that the resistant strain has the same number of copies of this gene as the sensitive one.

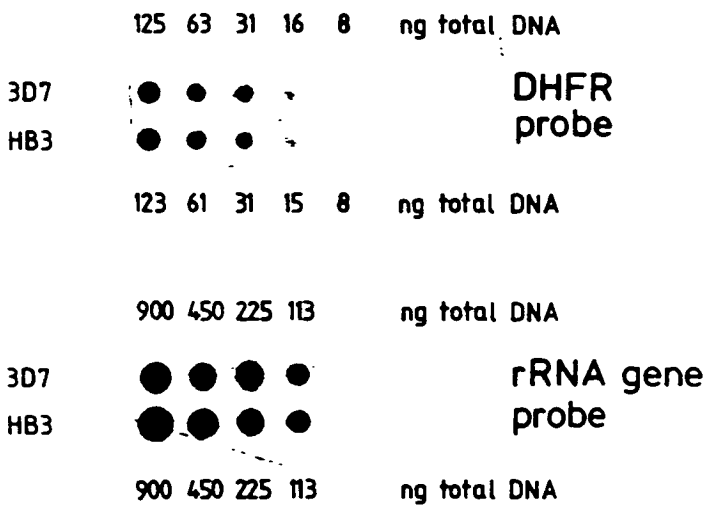

Fig. 5. Dot blot analysis of DNA from pyrimethamine sensitive (3D7) and resistant (HB3) clones of *P. falciparum*. Dilutions of DNA from each parasite were spotted directly to two Hybond nylon filters and denatured. Estimated quantities of DNA are shown. Bonded DNA was probed with ^{32}P-labelled DHFR probe (see Fig. 2) and a fragment of the rRNA genes (prib2; Langsley *et al.*, 1983).

CONCLUSION AND DISCUSSION

In a cross between two *P. falciparum* strains pyr^R behaved as a single mutation. The mutation was linked to the gene for DHFR and is probably located close to the gene or within it. No evidence for gene amplification was found.

Our results provide genetic confirmation for the conclusion recently drawn from localised sequencing studies reported by Peterson *et al.* (1988), which showed that HB3 has a base change in the DHFR gene, changing a serine at position 108 in the polypeptide chain to an asparagine. Comparisons have been made between the primary amino acid sequences of the enzyme DHFR from several different organisms. The crystal structures of the enzymes from *Escherichia coli* and *Lactobacillus casei* combined with these sequence comparisons allow us to pinpoint to the likely places in the polypeptide chain of the *P. falciparum* enzyme where pyrimethamine could bind. The serine at position 108 is in such a place (Peterson *et al.*, 1988; Snewin *et al.*, 1989).

The present studies leave a number of interesting questions unanswered. Parasites isolated from nature have a wide range of resistance to pyrimethamine. Some parasites can tolerate as much as 1,000 times more of the drug than the sensitive organism (see Walter, 1986; Thaithong, S., pers.comm.). By contrast those isolated following selection in culture in the laboratory show relatively low levels of resistance to the drug (Banyal and Inselburg, 1986). This suggests that there may be several other mechanisms which contribute to resistance. These may include the changes in drug uptake and targetting discussed above in addition to changes in the regulation of DHFR gene expression which could give more enzyme without amplification of the gene itself.

SUMMARY

Current therapy and prophylaxis of malaria are limited by the spread of parasites resistant to the commonly used drugs. An understanding of the genetic basis of resistance could help in the design of new drugs and the prediction of its mode of spread through a parasite population. We are studying resistance to pyrimethamine, whose target is the enzyme dihydrofolate reductase. Mutants (pyr^R) resistant to pyrimethamine can have an altered dihydrofolate reductase (dhf). However, other factors may participate in resistance to this drug e.g. mutation affecting uptake could contribute to resistance.

Analysis of pyr^R inheritance in a cross between sensitive and resistant clones of *Plasmodium falciparum* could throw light on this question. The dhf gene cloned in phage lambda has been used to identify a restriction fragment length polymorphism (RFLP) in the DNA of the resistant parent of the cross (HB3). This specifically distinguishes it from the DNA of the sensitive parent (3D7). The RFLP is being used as a marker for inheritance of the dhf gene from the resistant parent. If this is the sole determinant of resistance to the drug, we should find that all of the pyr^R progeny have this RFLP.

In parallel studies we are using the cloned gene to seek dhf sequence changes associated with resistance.

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