

**EPITOPE MAPPING STUDIES ON A HIGHLY
CONSERVED RHOPTRY ANTIGEN FROM
PLASMODIUM FALCIPARUM.**

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PhD THESIS

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1993



Declaration

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

Dedication

MUM. *In memoriam*

DAD.

and my supervisor Prof. JOHN G. SCAIFE. *In memoriam*

Acknowledgements

During my study at Edinburgh University, it seems to me that there is no better place where I can enjoy studying, living and traveling at the same time. Of course, this is not because the beauty of the Scottish landscape but also people whom I met here.

I owe tremendous debt of gratefulness to my supervisor, Professor John G. Scaife, for his understanding, encouragement and advice. He led me into the fascinating world of Molecular Biology and also taught me "what to think" and "how to think" to solve a scientific problem to which I never followed him quick enough.

I also thank to my second supervisor, Dr Robert Ridley for his support and advice. This thesis can be finished because of his untiring correction and criticisms. I would like to thank Dr Brian Kilbey and Dr David Finnigan who gave me their advice, help and support. Special thanks to Dr Jana McBride for her attention, help, valuable data and samples. I wish to thank Richard Moon and Sandy Edgar who also help me to confirm the sequence of the PCR product. Thanks to Alan Cuthbertsow who gave me his advice on peptide synthesis works.

Thanks to all my teachers Dr Sumalee Pitchyangkul, Mike Goman, Chris Delves and Shahabuddin Mohammad for their support and teaching. I also wish to thank all former and present member of my group Beth Margutti-Pinto, Anjam Khan, John White, Sandy Cheesman for giving me good memories during my study. Thanks to Mike Dyer, Graham Harold, Annie Wilson, Delia Johnson and Sybil McAleese for their hospitality. Thanks to Frank Johnston and Graham Brown for their help on photography work and also to Jean Ramsay for her work in media room. I also would like to express my appreciation for supports and friendships from all Thai students in Edinburgh.

I wish to thank the IPST institute for financial support during my study and for their understanding to my situation.

Finally, I thank to my father who always told me that "Keep on smiling, it hurts nobody" and to my mother who teach me everything in my life and told me how to achieve my PhD.

Epitope mapping studies on a highly conserved rhoptry antigen from *Plasmodium falciparum*.

Abstract.

Malaria is one of the most widespread parasitic disease. It is caused by *Plasmodium* protozoa. *Plasmodium falciparum* infection leads to the most virulent symptoms in human. Researchers have been devoted to develop a malaria vaccine for malaria eradication, especially against *P. falciparum*. Rhoptry associated protein-1 (RAP-1) is one of the *P. falciparum* vaccine candidates because it protected monkeys in an immunization experiment and some monoclonal antibodies raised against this protein can inhibit parasite growth *in vitro*.

This thesis describes work in which the epitopes of the inhibitory monoclonal antibodies were mapped by using a combination of recombinant DNA technology and peptide synthesis. The part of *rap-1* gene, which contains epitopes of inhibitory monoclonal antibodies, was subcloned and expressed in *Escherichia coli* in a β -galactosidase fusion form. The use of restriction enzymes and exonuclease III to generate different fragments of *rap-1* gene suggested that the epitopes of these monoclonal antibodies cluster near a proteolytic cleavage site on the protein (between A₁₉₀ and D₁₉₁). This was confirmed when the epitopes of all the inhibitory monoclonal antibodies were located on a TLTPLEELYP₂₁₀ peptide generated as one of a series of overlapping decapeptides.

The study of RAP-1 protein immunogenicity in rabbit and humans showed that both species are also able to recognize this 'inhibitory epitope'. The epitope mapping of another set of monoclonal antibodies raised against a recombinant RAP-1 protein lacking its 'native' conformation was carried out. The results suggested that this expressed protein can stimulate immune responses against the 'inhibitory epitope' but two additional epitopes are also recognized by these monoclonal antibodies.

Sequence analysis of the *rap-1* gene from a number of isolates and clones demonstrated that the RAP-1 protein is highly conserved in *P. falciparum* from several parts of the world. This finding verified the potential of the RAP-1 protein as a malaria vaccine candidate.

Abbreviations

ATP	Adenosine-5'-triphosphate
BCIP	5-Bromo-4-Chloro-3-indolyl-phosphate
°C	degrees centigrade
Ci	Curie (s)
cm	centimetre (s)
CTP	Cytidine-5'-triphosphate
ddATP	2', 3'-dideoxyadenosine-5'-triphosphate
ddCTP	2', 3'-dideoxycytidine-5'-triphosphate
ddGTP	2', 3'-dideoxyguanosine-5'-triphosphate
ddTTP	2', 3'-dideoxythymidine-5'-triphosphate
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
DMF	NN-dimethylformamide
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
g	gram (s)
GTP	Guanosine-5'-triphosphate
IPTG	Isopropylthio- β -D-galactoside
kb	kilobase
kDa	kilodalton
μ Ci	microcurie
μ l	microlitre
M	Molar
mA	milliampere (s)
mAb	Monoclonal antibody
mg	milligram (s)

mins	minutes
ml	millilitre (s)
mM	millimolar
NBT	Nitro blue tetrazolium
ng	nanogram (s)
nm	nanometre (s)
NP40	Nonidet40
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
pg	picogram (s)
RNA	Ribonucleic acid
RpAb	Rabbit polyclonal antibody
SDS	Sodium dodecyl sulphate
secs	seconds
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris-Cl	Tris(hydroxymethyl)aminomethane buffer
UV	Ultraviolet

Amino acid abbreviations.

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

Names used for recombinant plasmids and clones in this thesis.

The naming system used for each plasmids and recombinant clones in this thesis are explained below. The details of these plasmids and clones are described in Chapter 3.

UR, MS	bacteria clone containing pUR278 or pMS1S plasmid.
URES, MSES	recombinant clone which possesses pUR278 or pMS1S plasmid containing <i>EcoRI-SalI</i> fragment.
pURES, pMSES	recombinant pUR278 or pMS1S plasmid which containing <i>EcoRI-SalI</i> fragment.

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Chapter 1

Introduction

1.1.Malaria Parasite.

1.1.1. Classification and general characteristics.

Malaria is caused by protozoan parasites of the genus *Plasmodium*, which is classified into the phylum Apicomplexa (Schmidt and Roberts,1989). Other genera containing important parasites in this phylum are *Eimeria*, *Toxoplasma* and *Theileria* (Fig.1.1). All of the protozoa in this phylum share some characteristics. The most common and important feature is the presence of an apical complex at some stage of their life cycle. The apical complex typically includes a polar ring, micronemes and rhoptries. The polar ring is an electron-dense structure under the cell membrane, which encircles the anterior tip. The rhoptries are electron dense pear shaped organelles. Two to several rhoptries, depending on the species, share a common duct toward the cell membrane inside the polar ring structure. Micronemes are smaller but more abundant, elongate organelles, which are also seen as electron-dense structures under the electron microscope. The rhoptries and the micronemes are thought to play a crucial role in host cell invasion through secretion of their contents into the host cell during invasion (Bannister and Mitchell, 1989).

The specific characteristics of the genus *Plasmodium* amongst the apicomplexa are (i) its complex life cycle in which it undergoes asexual reproduction in both vertebrates and invertebrates and sexual reproduction in invertebrates; (ii) the female macrogametes and male microgametes develop independently; (iii) cilia and flagella are absent except for the microgamete; (iv) the zygote is motile.

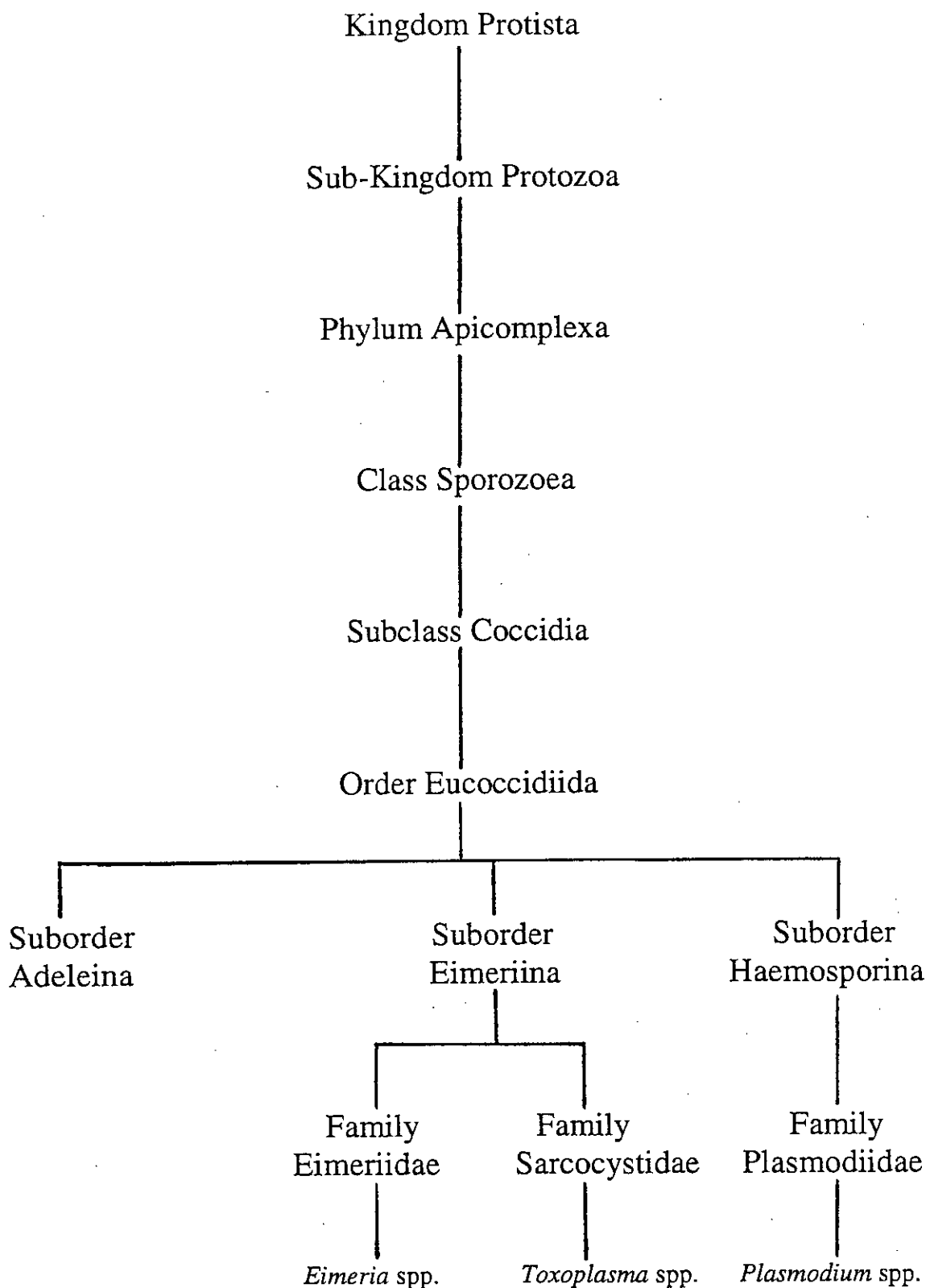


Figure 1.1. Classification of *Plasmodium falciparum* and related genera (Schmidt and Roberts, 1989).

1.1.2. Human malaria parasite.

There are many species of *Plasmodium* but only four of them can cause the malaria disease in humans. Those species are *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium vivax*. Each species can be distinguished by its morphology and pathology. *Plasmodium falciparum* is responsible for the most virulent form of malaria, known as malignant tertian. It is responsible for most of the malaria deaths.

1.1.3. Life cycle of *Plasmodium* spp.

The life cycle of *Plasmodium* spp. is outlined in Fig. 1.2. For a clear description, the malaria life cycle is divided into two stages; the asexual stage in human and the sexual stage in the mosquito.

1.1.3.1. Asexual stage.

1.1.3.1.1. Exoerythrocytic stage.

The malaria parasites are transmitted by female mosquitoes of the genus *Anopheles*. Infection is caused by injection of the sporozoite form of the parasite into the bloodstream along with mosquito saliva during blood meal ingestion. The sporozoites enter parenchyma cells in the liver shortly after injection. After invasion, the parasite develops into an irregular shaped exoerythrocytic schizont which grows asexually and develops into merozoites within the cell. In *P. falciparum* the mature schizont ruptures after about 5.5 days, releasing up to 30,000 exoerythrocytic merozoites into the bloodstream (Schmidt and Roberts, 1989) which are capable of invading red blood cells and initiating the erythrocytic cycle.

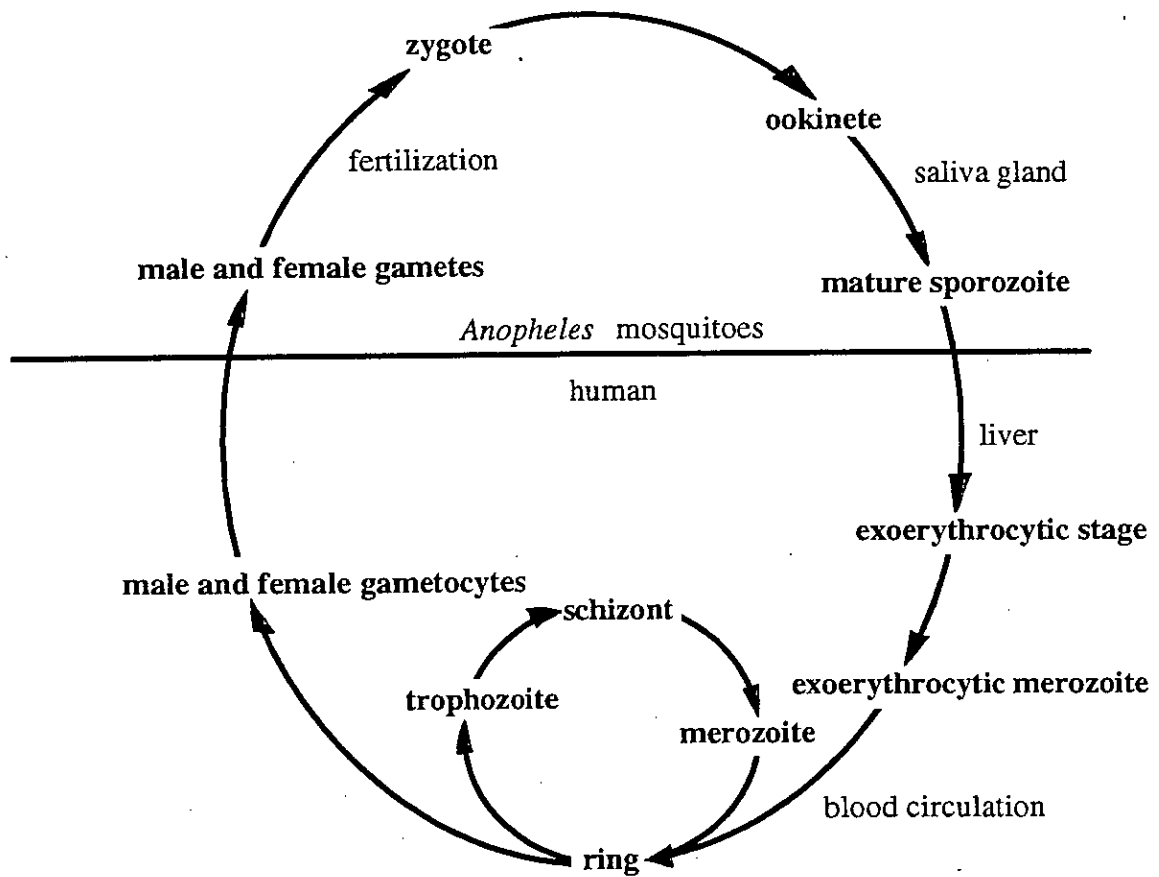


Figure. 1.2. The life cycle of *Plasmodium falciparum* malaria parasite (see text for detail).

1.1.3.1.2. Erythrocytic stage.

After release from the liver, the exoerythrocytic merozoite binds and enters a red blood cell. Once in the red blood cell, the parasite, surrounded by the parasitophorous vacuole membrane, develops through three different stages, ring, trophozoite and schizont, which can be distinguished under a light microscope (Schmidt and Roberts, 1989). The infected red blood cell and parasitophorous vacuole membrane are modified during these developments. During the ring stage, the merozoite structure begins to dedifferentiate as the merozoite-specific organelles disappear (Aikawa, 1971). Although, ribosomes are not seen until the trophozoite stage the protein synthesis actually starts in the ring stage of the parasite (Brown *et al*, 1982). The parasite obtains amino acids from digestion products of haemoglobin, which is endocytosed, from the plasma and from *de novo* synthesis from carbon sources (Sherman, 1979). At the schizont stage, the parasite undergoes nuclear division and certain cytoplasmic organelles are developed. At the end of schizogony, mature erythrocytic merozoites can be seen in the infected red blood cell. Each schizont produces 8-24 merozoites in *P. falciparum*, 12-24 merozoites in *P. vivax*, 6-12 merozoites in *P. malariae* and *P. ovale* (Kreier and Baker, 1987). The mature merozoites are released into the blood stream and invade other red blood cells to continue the erythrocytic cycle. The time required for each cycle of development varies between species, 72 hours in *P. malariae*, 48 hours in *P. falciparum*, *P. vivax*, *P. ovale* (Kreier and Baker, 1987). This period also coincides with the appearance of fever, the clinical symptom of malaria. It has been suggested that this may be caused by soluble exo-antigens released by which subsequently induce tumour necrosis factor, TNF (review in Playfair *et al*, 1990).

It was previously thought that the preerythrocytic merozoites can reinvade hepatocytes and cause the relapse of malaria in *P. vivax* and *P. ovale*. Now, evidence

suggests that relapses may be caused by a dormant form of the liver schizont (Krotoski *et al*, 1982).

1.1.3.1.3. Gametogony.

During the erythrocytic cycle, some merozoites develop into male microgametocytes or female macrogametocytes after entering a red blood cell. The mechanism, which 'switches on' this process, is still unknown. In *P. falciparum* gametogony takes about ten days during which time mature gametocytes develop in the blood spaces of the spleen and bone marrow and later, appear in the blood stream (Schmidt and Roberts, 1989). The shape and size of gametocytes within the infected cells are specific for each species. The gametocytes remain in the red blood cell until they die, are destroyed by the host immune system, or are taken up by a mosquito.

1.1.3.2. Sexual stage in the mosquito.

1.1.3.2.1. Sexual stage.

When the gametocytes are ingested by the *Anopheles* mosquito, both microgametocytes and macrogametocytes are released from the red blood cell membrane and develop into microgametes and macrogametes respectively. There is no significant difference in the characteristics of microgametocytes and macrogametes. The microgametocyte, however, rapidly divides its nucleus to form six to eight nuclei. Each nucleus migrates into a flagellum, which buds from the microgametocyte. The exflagellation process is completed when the flagella immediately break free into the plasma of the blood meal.

Fertilization occurs when the microgamete penetrates through the macrogamete membrane and both nuclei fuse to form a diploid zygote. The zygote

quickly undergoes meiotic division and develops into a motile ookinete. The ookinete then penetrates through the epithelial cell layer of the intestinal wall of the mosquito. At the basement membrane, the parasite transforms into an oocyst and is covered by an electron-dense capsule. The size of the oocyst becomes larger projecting into the body cavity as the parasite grows rapidly inside.

1.1.3.2.2. Sporogony.

Mitotic division and differentiation within the oocyst generates thousands of sporozoites, the infective form of the parasite. Two weeks after the mosquito was infected, the oocyst bursts to release mature sporozoites. The released sporozoites move toward the salivary glands of the mosquito where the parasites can be transferred into the vertebrate hosts during blood meal ingestion.

1.1.4. Erythrocytic invasion process.

Because the protein under investigation in this project may relate to the erythrocyte invasion process, this process will be explained in more detail. However, it should be noted that the invasion process of the sporozoite shares common features with the phenomenon of the merozoite because of the presence of the apical complex structures in both cells. Though the sporozoite and the merozoite forms of parasite have a different target cell and also contain rhoptry proteins specific to each stage, the entry of parasite in both forms occurs only after the apical complex is attach to the host cell surface.

During the malaria life cycle, merozoites originate from two different cells, from hepatocytes -so called exoerythrocytic merozoites, and from red blood cells -so called erythrocytic merozoites. From an avian malaria parasite study, Aikawa noticed some characteristics different between these two merozoites (Aikawa, 1971).

However, for the purposes of this discussion, it has been assumed that the invasion process in both kinds of merozoite are the same. Here, the invasion process of erythrocytic merozoite is described as a model of erythrocyte invasion by the malaria parasite.

The merozoite initiates invasion of the erythrocyte through the recognition by a parasite surface protein(s) of receptor molecules on the red blood cell membrane. The major receptors on the red blood cell membrane are believed to be the major red cell sialoglycoproteins, glycophorins A, B and possibly C (Cox, 1983) in *P. falciparum* and the Duffy blood group in *P. vivax* and *P. knowlesi* (Miller *et al*, 1977). Following successful attachment, the parasite reorientates until its apical end contacts the red blood cell membrane (Dvorak *et al*, 1975). Initially, the erythrocyte membrane is slightly raised and then invagination occurs at the junction, which thickens, increasing to about 15 nm (Aikawa *et al*, 1978).

As the parasite enters the red blood cell, the parasitophorous vacuole is formed. The mechanism of parasite interiorization into the red blood cell is still unclear. However, the ability of Cytochalasin B, a glucose transport inhibitor and microtubule disruptive agent, to inhibit entry movement, but not parasitophorous vacuole formation (Aikawa *et al*, 1981), reveals that the mechanisms of these two processes are different. Electron microscope studies suggest that the invagination of the red blood cell membrane is generated by rhoptries and micronemes (Bannister and Mitchell, 1989). Many electron micrographs show an electron-opaque projection which connects the opening duct of the rhoptries to the rhesus erythrocyte membrane during invasion by *P. knowlesi* (Aikawa *et al*, 1978, Bannister and Mitchell, 1989). Moreover, the study of parasitophorous vacuole origin suggested that the parasitophorous vacuole membrane is derived wholly or partly from the merozoite (Dluzewski *et al*, 1992). These facts together with the appearance of lamellar membranous material associated with rhoptries, and its secretion into the parasitophorous vacuole during invasion, lead to the conclusion that the rhoptries may

have an important role in invasion (Bannister *et al*, 1986). Several rhoptry proteins have been identified and roles for them in the invasion process have been proposed. These proteins will be described later (see section 3.3.2.4.).

During the entry process, the surface coat of the parasite is lost. In *P. berghei yoeli* the surface coat accumulates on the surface of the merozoite which still remains outside the red blood cell (Ladda *et al*, 1969). Invasion is complete when the parasite is totally internalized and the erythrocytic membrane fuses behind it in an iris diaphragm-like manner (Aikawa *et al*, 1978). The whole invasion process may last only 20 seconds (Wilson, 1982).

1.2. Global Malaria Situation.

1.2.1. Spreading of malaria disease.

Human malaria is spreading in a vast area between latitudes 64 degrees North and 31 degrees South (Fig. 1.3.). However, it is not found in some areas, for example arid and very high altitude areas where conditions are unsuitable for the vectors or the parasites. The fact that transmission of malaria requires an average temperature above 15 degrees centigrade for at least one month in a year (Knell, 1991), raises the possibility of the malaria spreading at the higher latitudes if the global temperature increases because of the greenhouse effect.

Malaria, like some other blood diseases, can be transmitted by blood transfusion, blood cross contamination among drug addicts, congenital infection etc.. In America, the average annual rate of malaria transmission by blood transfusion was 0.25 cases per million units of blood collected during 1972 to 1988 (Nahlen *et al*, 1991). Seventeen cases of congenital infection were also recently reported in the Southern province in Zambia during the rainy season (Larkin and Thuma, 1991). These

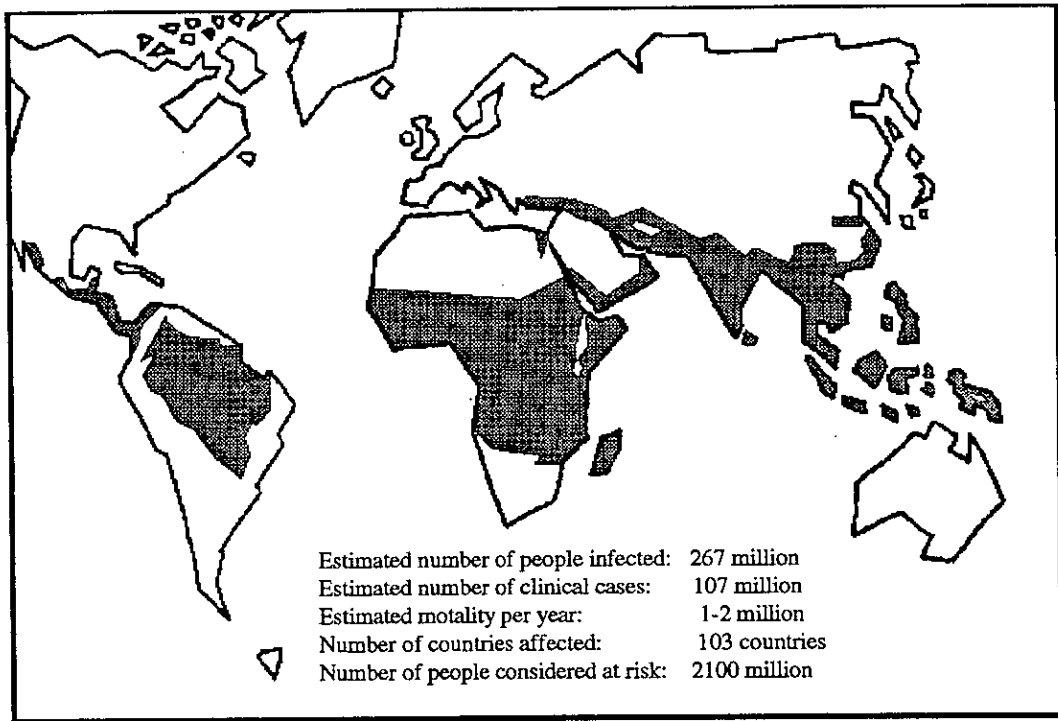


Figure. 1.3. World map diagram shows approximate distribution of malarious regions and some estimated data (adapted from UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Disease-WHO Division of Control of Tropical Diseases, 1990).

incidences, even at very low number, may help the malaria disease to spread into a malaria-free area.

Improvements in transportation can also play an important role in the spreading of malaria. In Britain, imported malaria cases have been reported for many years. The number of cases have increased from 101 in 1970 to 2,212 in 1985. Most of these are travellers from endemic areas in Asia and Africa (Knell, 1991). Imported malaria may have a significant impact in the 'anophelism without malaria' areas, *i.e.* areas contain *Anopheles* mosquitoes which are capable of transmitting the malaria but are uninfected with malaria parasite.

The outbreak of malaria in South America is an example of the human contribution to the increasing spread of malaria in some developing countries. Deforestation in malaria endemic areas puts workers at risk of malaria infection and enlarges the human reservoirs of the malaria parasite.

The World Health Organization estimated that there are one hundred million cases of malaria annually. Among those patients, approximately 1 to 2 million people will die each year, mostly from *P. falciparum* infection (Knell, 1991). These figures do not reflect the total impact of malaria on global health. In 1991, Brinkmann reported that malaria accounted for 20%-50% of all admissions in African health services, although these are only 8%-25% of total malaria cases (Brinkmann and Brinkmann, 1991). He also documented the rapidly increasing incidence of malaria in Gambia (7.3%), Togo (10.4%) and Rwanda (21.0%).

1.2.2. Effect of Malaria disease.

Economically, the main impact of the malaria disease may be visualized in the terms of financial loss and labour loss. However, in the real situation, the malaria disease also has a more subtle impact on human by affecting the human gene pool. It is known that malaria can play an important role as a selective pressure on some genetic

blood disorders, such as sickle-cell anaemia and thalassaemia, in some parts of the world (Knell, 1991). This selective pressure increases the survival chance of the disorder gene carriers compared to the rest of the population and so the variant genes are more likely to be carried over to the next generation.

Malaria also has an indirect effect on medical services. Not only does it weaken the immune system in the case of chronic infection, but it also affects other aspects such as the blood banking industry. In America, travellers who have had malaria or taken antimalaria chemoprophylaxis are excluded for three years from blood donation (Nahlen *et al*, 1991).

1.2.3. Malaria control and the problems.

The life cycle of the malaria parasite is complex. The parasite is transferred between human and the *Anopheles* mosquito. This complexity increases the difficulty of malaria control programmes. On the other hand, the host and vector specificity raise hopes for malaria eradication. Here, some of the methods used in control programmes and their problems are discussed

1.2.3.1. Vector eradication.

The human malaria parasite has to spend a part of its life cycle in the *Anopheles* mosquito. The eradication of this mosquito will decrease the incidence and spread of the malaria disease. Many methods are used to eliminate the mosquito, for example, the use of insecticides, environmental control and biological control.

Insecticides have been used as a tool for fighting malaria for many years. The most well known synthetic insecticide is DDT (Dichloro-diphenyl-trichloroethane). Many other insecticides have also been developed to improve the insecticidal effect and to reduce undesirable impacts on the environment. After

prolonged use of these insecticides, some problems frequently occur: (i) to effectively control the vector, vast areas have to be treated indefinitely; (ii) a huge amount of money is required; (iii) damage to the environment and/or the accumulation of insecticides in the food chain may occur; (iv) development of resistance to the insecticides among the *Anopheles* mosquito population.

Control of the malaria insect vector by destroying its breeding site is used in some endemic areas. The use and effectiveness of environmental control methods is limited by geographic conditions and lack of trained personal to plan, carry out and monitor the process.

Up to now, the biological control of the mosquito is only used in a small scale (Knell, 1991). The mosquito is either eliminated by a natural predator or a pathogen. For example, guppy fish are considered to be a suitable predator for the mosquito larvae in enclosed collections of water, though its use is restricted by appropriate habitat requirements. Bacterial pathogens such as *Bacillus thuringiensis* and *Bacillus sphaericus* are under investigation by several researchers. They are known to produce a toxin after being eaten by mosquito larvae (Knell, 1991), therefore they are considered as control agent. However, the use of these bacteria in nature may have an adverse impact on the environment and more study is required.

1.2.3.2. Human protection.

In principal, the ultimate aim of much malaria related research is to protect man from malaria infection. Human protection from the malaria parasite can be achieved by a combination of physical and biological methods. These protection methods need the individual responsibility of people for taking appropriate precautions, so the most important factor is to ensure that people in endemic areas have been educated about malaria infection.

Utilization of a bednet and a mosquito screen are among the oldest and most effective physical protection methods. Because the mosquito feeds through the late evening to early in the morning, sleeping under the cover of the bednet or the mosquito screen seem to be the best solution. The protectivity of bednets, which may fail to protect if it is damaged or improperly used, can improve upon impregnation with insecticides. In Gambia, bednets treated with permethrin, an insecticide, were shown to protect people from *Anopheles gambiae* bloodfeeding. At 500 mg of the permethrin per square metre the blood feeding rate is reduced by 91% compared with untreated bednet (Linsay *et al*, 1991). For outdoor protection, the use of an insect repellent and sensible clothing are the most convenient methods.

Biological methods of protection include prophylaxis treatment and drug cure. Amongst the oldest anti-malaria drug known to man is an extract obtained from the bark of the *Cinchona* tree. It has been used since the seventeenth century. The active alkaloid in the extract, quinine, was identified and is still among the best antimalarial compounds in used today. Many anti-malarial drugs have been discovered over the years from chemically synthesized drugs to herb extracts; for example, chloroquine, proguanil, mefloquine, pyrimethamine, sulphonamides, primaquine, halofantrine, atovaquone, artemisinin (active ingredient in *Artemisia annua*, Linn. or Qinghao) and its derivatives. The activities of these drugs are directed against different molecular targets of the malaria parasite (see Table 1.1). However, it was found that some malaria parasite strains have developed resistance to the well established anti-malarial drugs. In 1961, the first report of two patients who had chloroquine-resistant *P. falciparum* from Columbia was published (Moore and Lanier, 1961). The discovery of this chloroquine-resistant strain alerted all the malaria endemic countries. In 1963, a case of multidrug-resistant *P. falciparum* strain was reported in Thailand (Young *et al*, 1963). Thirteen years later, the chloroquine-resistant *P. falciparum* was found in Africa and is now spreading at a very high rate (Peters, 1985). Recently, resistance strains of halofantrine, a new malaria drug which

Class	Drug(s)	Proposed activity
<i>Cinchona</i> Alkaloid	Quinine	Inhibits haem polymerase enzyme activity (Slater and Cerami, 1992).
Aminoquinolines	Chloroquine	Inhibit DNA synthesis (Polet and Barr, 1968). Binds to DNA. RNA (Howells <i>et al</i> , 1970). Retains lytic capacity of ferriprotoporphyrin IX (Howells, 1985). Inhibits haem polymerase enzyme activity (Slater and Cerami, 1992).
Aminoquinolines	Mefloquine	Unknown probably by : Inhibits host cell digestion (Ginsburg, 1991). Retains lytic capacity of ferriprotoporphyrin IX (Howells, 1985). Interacts with phospholipids (Howells, 1985). Inhibits haem polymerase enzyme activity (Slater and Cerami, 1992).
Aminoquinolines	Primaquine	Disrupting respiratory chain of the parasite (Gutteridge, 1991). Inhibits dihydroorotate dehydrogenase (Howells, 1985).
Biguanide	Pyroquanil Cycloquanil	Inhibits dihydrofolate reductase (Walter, 1991).
Hydroxynaphtho-quinone	Atovaquone (566c80)	Disrupting respiratory chain of the parasite (Howells, 1985).
Phenanthrenemethanol	Halofantrine	Unknown probably the same as mefloquine (Peters, 1985).
Pyrimidine	Pyrimethamine	Inhibits dihydrofolate reductase (Walter, 1991).
Sesquiterpene lactone	Artemisinin	Unknown probably by : Inhibits of protein synthesis (Howells, 1985). Causes membrane degeneration (Klayman, 1985).
Sulfonamides and Sulfones	DDS (4.4' -diaminodiphenyl sulfone)	Inhibits dihydropteroate synthase (Walter, 1991).

Table 1.1. shows summary of antimalaria drugs in current uses and their possible modes of action.

is not yet commercially available in Thailand, were identified by a group of Thai researchers (Wongsrichanalai, 1992).

Failure of vector eradication and human protection, as described previously, forces scientists to find alternative ways for malaria eradication. Vaccination probably is the best long term solution if an effective vaccine can be developed. The success of the WHO smallpox vaccination programme gives encouragement to this approach.

1.3. Malaria vaccine.

Here, some basic principles related to human immune response and some vaccine candidates are described.

1.3.1. Antibody stimulation in human.

Human immunity can be functionally divided into two types, an innate (natural) immunity and an adaptive (acquired) immunity (Roitt *et al*, 1985). Innate immunity is non-specific and is mediated by phagocytes and natural killer (NK) cells. It acts as a first barrier against all foreign antigens. Adaptive immunity is a specific immunological reaction, which involves other types of leukocytes (white blood cells), called lymphocytes. Adaptive immunity can be artificially stimulated by an appropriate part of an antigen using vaccination. This discussion will only concentrate on the adaptive immunity.

Stimulation of the adaptive immune response can be divided into two steps, a T cell activation and a B cell activation. T cells and B cells are two main types of lymphocytes which carry specific receptors on their surface. To ensure the specificity of immune response, each T cell and B cell can bind only one antigenic determinant in an antigen molecule (epitope).

After foreign antigens enter a human body, they are taken up non-specifically by antigen presenting cells (APC) such as macrophages, monocytes, etc. The antigens are partially degraded into small peptides (Riley *et al*, 1991 and Harlow and Lane, 1988) and some peptides are presented on the cell surface in complex with a major histocompatibility complex (MHC) class II protein. Antigen presenting cells carry the processed antigens to the secondary lymphoid organs, such as spleen and lymph nodes, where the antigens are presented to the lymphocytes. The T cell activation process occurs when a T cell subpopulation called T helper cells, with a matched antigen receptor, bind to an antigen peptide-MHC class II complex on the surface of an APC leading to proliferation and differentiation.

For a B cell, the antigenic determinant region, a B cell epitope, on a foreign antigen is bound specifically to the B cell antigen receptor, an immunoglobulin. Similar to the APC, the antigen is taken up in the B cell and is processed into smaller peptides. Again, the processed peptides are also presented at the B cell surface in the antigen-MHC class II protein complex. This complex has to contain at least one T cell receptor binding site to create a strong immune response for T-cell-dependent antigen. Binding of a T helper cell to the antigen-MHC class II protein complex stimulates the B cell to undergo division and differentiation. The dividing B cell may differentiate into two types of cells, plasma cells and memory B cells. This B cell activation process also results in the class of secreted antibody shifting from IgM to IgG and higher affinity antibodies.

Plasma cells have an expanded cytoplasm and high rate of protein synthesis. They are the main sources of antibody production. Plasma cells are short lived cells, mostly seen in the lymphoid organs. To retain a specific immunological memory, some dividing B cells differentiate into memory cells. Memory B cells are long-lived cells which are ready to respond to the primary activated antigen. The secondary response caused by memory cells is therefore faster and more potent.

Some antigens can directly stimulate B cells to proliferate and secrete antibodies without a signal from T helper cells. These antigens are called T-cell-independent antigens. Most of these antigens are large polymeric molecules with repeated antigenic determinants and are resistant to degradation. Because the response by-passes the T helper cell interaction, there is no differentiation of B cells into memory B cells and so no secondary response in this case.

1.3.2. Immunity to malaria.

In endemic areas, human protective immune response against the malaria parasite has been naturally stimulated by prolonged infection since childhood (review by Ravetch *et al*, 1985). A baby, born to an immune mother, is protected from lethal malaria infection until the age of three months probably by transplacenta transfer of protective antibodies, para-amino benzoic acid deficiency in its milk diet and the nonpermissive environment of fetal erythrocytes compared with normal erythrocytes (Wyler, 1990). From this protective stage up to two years old, the child is at its most susceptible stage to lethal malaria infection. Through several infections, the protective immunity will build up gradually as the severity of clinical symptoms decreases. It should be noted, however, that natural stimulation rarely produces sterile immunity and that the immunity generated is species specific. People in endemic areas usually have low parasitaemias without showing any symptoms. Protective immunity is also vulnerable to the immunological and physiological changes in an individual. For example, immune people who stay outside endemic areas for a period of time and pregnant women in endemic areas once again become susceptible to lethal malaria infection.

1.3.3. Evasive factors in human.

The malaria parasite, like many other parasites, has to adapt in order to survive in the host and escape from the host immune system. Several evasive factors of the *Plasmodium* parasites play an important role for this purpose. Mainly, the parasites evade attack by the host immune response by (i) avoidance of the immune system (ii) antigen polymorphism (iii) alteration of the host immune response.

In both exoerythrocytic and erythrocytic stages, all of the parasites of the genus *Plasmodium* develop within the vertebrate host cells. Parasites come into close contact with the immune system for only a short period of time during invasion of new cells. Moreover, *P. falciparum* infected red blood cells are sequestered in the capillaries of internal organs. This mechanism is speculated to prevent infected cells from clearance and destruction (Ravetch *et al*, 1985).

During its complex life cycle, the malaria parasite expresses many proteins in each stage of development. Many proteins in each stage are immunologically distinct. Thus, an individual who has immunity against the exoerythrocytic stage may become infected if the erythrocytic stage of parasite is injected into his blood stream and *vice versa*. Two other mechanisms, which are believed to help the parasite to avoid immune recognition, are antigenic polymorphism and antigenic variation. The antigenic polymorphisms were identified among individual isolates of *P. falciparum* from the same and different geological sources using panels of monoclonal antibodies (McBride *et al*, 1982, McBride *et al*, 1985 and Schofield *et al*, 1982). Data from sequence analysis of the MSA-1 gene from different isolates and clones of *P. falciparum* suggested that this polymorphism may result from the recombination of a limited number of alleles (Tanabe *et al*, 1987, review by Day and Marsh, 1991). Antigenic variation is a mechanism by which a parasite clone 'switches' its antigen(s) by an undetermined mechanism to a new variant in order to avoid immune recognition when put under immune pressure (Miller *et al*, 1986). The only possibly example of

antigenic variation in *P. falciparum* is the existence of different forms of PfEMP-1 in prolonged culture of a genetically identical parasite clone (Leech, 1991). The possibility of antigenic variation in other malarial proteins has led to a postulation that repeated infections are needed to stimulate the immune response against a wide range of parasite variants before natural immunity against malaria infection is achieved (review by Mendis, 1991).

The malaria parasite is able to alter its host immune system in two ways. First, by unknown mechanism, the infected host immune system is suppressed (Hyde, 1990). This not only directly helps the parasite to survive in the host but also increases the susceptibility of the host to other pathogens. Second, it is thought that the malaria parasite is capable of creating a smoke-screen effect by producing highly immunogenic surface antigens with tandem repeats, which are shed after invasion into host cells, to distract the immune response from potentially protective antigens (Schofield, 1990). It is also believed the tandem repeat character of some parasite antigens causes polyclonal B-cell activation in a T-cell-independent manner. This prevents the host immune response from developing immunity against important targets which may induce protective immunity (Miller *et al*, 1986, Hyde, 1990).

1.3.3.1. Malaria vaccine strategies.

In 1961, Cohen and his colleagues shown that malaria immunity in children can be achieved by passive transfer of gamma-globulin from immune adults (Cohen *et al*, 1961). A similar experiment was also carried out in Thailand (Sabchareon *et al*, 1991). The latter experiment revealed that all Thai patients showed stage-specific but non-sterilizing immunity against *P. falciparum* and *P. vivax* after passive transfer of IgG from African adults in malaria endemic areas. This suggested that immunity to the malaria parasite can be achieved if an appropriate antigen(s) is found. However, the complexity of the malaria life cycle and the changes of antigen expression during

different stages of the life cycle make the identification and selection of such antigen(s) a major problem for malaria vaccine research.

The source of malaria antigens used in several malarial vaccine studies originally came from parasite cultures. Different stages of the *P. falciparum* can be successfully cultured *in vitro* and *in vivo* i.e. exoerythrocytic stage (Sacci *et al*, 1992, Mazier, 1984, Millet *et al*, 1991), erythrocytic stage (Tragger and Jensen, 1977) and gametocyte (Ifediba and Vanderberg, 1981). However, these antigens may be contaminated with some infectious agents or toxic components during culture or purification process and so can not be considered as human malaria vaccines. For this reason, it is important to identify protective antigens which can form the basis of recombinant protein vaccines.

Many proteins have been proposed to be vaccine candidates. These antigens mainly were identified by using either monoclonal antibodies or immune polyclonal human sera. The results of extensive screening has shown that these vaccine candidates may be divided into two broad groups; (i) surface antigens and (ii) other organelle proteins whose functions are important for parasite survival. Several surface antigens in different stages of the malaria parasite have been considered as vaccine candidates, for example, antigens on the surface of sporozoite, infected erythrocyte, gamete or gametocyte and merozoite. Many proteins in the latter group were also identified but their functions remain unclear. However, postulated functions of these proteins relate to host cell binding, invasion, sequestration, fertilization and other aspects of parasite development. Some of the vaccine candidates will be discussed in detail later.

For successful vaccination, some criteria are important to malarial vaccine design. These criteria are; (i) it must protect all or most immunized individuals i.e. it should not be genetically restricted (ii) it should contain both T and B cell epitopes for a maximized and prolonged immune response (iii) there should be no antigenic variation and little antigenic polymorphism in the gene which encodes for the protein(s) (iv) it must be safe, e.g. must not raise autoimmunity or cause

immunosuppression in immunized persons (v) ideally, it should stimulate the immune response equally well in both native and denatured forms so that the vaccine may be easily obtained using recombinant technology.

1.3.3.2. Malaria vaccine candidate.

Theoretically, immunity could be generated against several distinct stages of the parasite life cycle. Thus, many researchers attempt to find vaccine candidates in different stages of the parasite depending on their area of interest. Vaccines derived from different stages have their own advantages and disadvantages. A vaccine against the malaria sporozoite will protect an immunized individual from malaria infection but to achieve total protection, a high titer of antibody is probably needed because if a small number of sporozoites escape from the immune system, they may still lead to malaria infection. On the other hand, an erythrocytic stage vaccine may be able to stimulate a high titer of antibody due to the boosting effect of repeated infection of erythrocytes. Though a blood stage vaccine can not protect people from infection, the parasite may be cleared from the blood stream, or if only partial protection is achieved, the symptoms may be minimized. A transmission blocking vaccine which would be derived from gamete or gametocyte antigens acts differently to both the vaccines mentioned above. It does not benefit the vaccinated individual but would help to prevent the spreading of malaria disease.

1.3.3.2.1. Vaccine candidate against the sexual stage.

The aim of the sexual stage vaccines is to block malaria transmission and may be particularly valuable in low level transmission areas. The target stage for transmission blocking vaccines are the male and female gametes, the zygote and its motile product, the ookinete which develops in the mosquito midgut in 5-30 mins,

30-120 mins and 20-24 hours, respectively after mosquito feeding (Sinden, 1984). Candidate antigens in *P. falciparum* include a 230 kDa antigen and a 48/45 kDa doublet which are found on the macrogamete surface (Vermuelen, 1985). They contain immunodominant epitopes but only the 48/45 kDa protein can raise antibodies which interfere with the fertilization process of the macrogamete. However, the immune response to 48/45 kDa protein is MHC class II restricted (Good *et al*, 1988). Another vaccine candidate is a 25 kDa protein which is localized on the surface of zygote and ookinete. It contains an epidermal growth factor-like domains (Kaslow *et al*, 1988) and appears to be conserved (Kaslow *et al*, 1989). This protein is considered to be a good vaccine candidate because the immune response to this protein does not appear to be MHC class II restricted (Kaslow, 1989 and Good *et al*, 1988).

1.3.3.2.2. Vaccine candidate against the exoerythrocytic stage.

The potential for a vaccine against the exoerythrocytic stage of the malaria parasite was indicated when Mulligan and his colleagues found that immunization with ultraviolet irradiated sporozoites of the avian malaria can protect birds against sporozoite challenge (reviewed by Nussenzweig and Nussenzweig, 1985). In 1967, Nussenzweig also showed that irradiated sporozoites of *P. berghei* can protect mice from malaria infection (Nussenzweig *et al*, 1967).

Similar experiments have been carried out in humans. In one study immunization of human volunteers with sporozoites-infected mosquitoes which had been irradiated showed that the protection was induced after 6-8 immunizations by mosquito bites. The immunity, which is species and stage, but not strain, specific persisted only a few months. No boosting effect to the immunity was shown after the challenge (for review see Clyde, 1990 and Rieckman, 1990). In a more recent study, human volunteers were also immunized with irradiated sporozoite-infected

mosquitoes. Protection was induced after 19 sessions of immunization, consistent with the results of the former experiments (Herrington *et al*, 1991).

Monoclonal antibodies raised against the sporozoite stage can protect mice against malaria infection after passive transfer. These monoclonal antibodies identified an antigen on the sporozoite surface, the circumsporozoite protein, CS protein (see review by Nussenzweig, 1990). In 1984, the gene encoding the circumsporozoite protein of *P. falciparum* was cloned and sequenced (Dame *et al*, 1984). The sequence revealed that the middle of the gene contains an immunodominant domain of 37-41 tandem repeats of four amino acids, NANP and two to four repeats of a variant sequence, NVDP. The number of repeats varies among different isolates (reviewed by Nussenzweig, 1990).

As an immunodominant region of this potential vaccine candidate, the tandem repeats have been used as the basis of 4 *P. falciparum* vaccine trials in human. There is only a vaccine trial of which the protection against malaria infection was investigated while the other reports examined the possibility of immune response stimulation. A synthetic peptide, Acetyl-Cys-(NANP)₃, was used in an immunization. Only some immunized person responded to this vaccine but they produced a low titer of antibody to sporozoites, lower than those of people in the endemic areas. No strong boosting effect was shown (Etlinger *et al*, 1987). Recombinant protein containing the tandem repeats was also investigated. The protein used was a fusion protein of MDP(NANP)₁₅NVDP(NANP)₁₅NVDP and 32 amino acids from tetracycline resistance gene read out of frame. This protein fail to stimulate immune responses to the repeat region of the CS protein as high as those individuals in the endemic areas (Ballou *et al*, 1987). Recently, another clinical trial used recombinant protein of (NANP)₁₆ fused with hepatitis B virus surface antigen as a vaccine in a phase I trial. This vaccine can stimulate antibodies production in all volunteers and the antibodies persisted in most cases over ten months. However, the protection against malaria infection still has to be evaluated (Vreden *et al*, 1991). A

protection evaluation experiment has been carried out. The peptide (NANP)₃ conjugated to tetanus toxoid using aluminium hydroxide as an adjuvant was tested. The majority of vaccinees developed low titers of antibodies to the synthetic peptide (NANP)₅₀. Seven vaccinees who had high titers of antibodies were challenged with sporozoites by infected mosquitoes. One of these volunteers was protected and some of the other vaccinees showed a delay in their prepatent period (Herrington *et al*, 1987 and Herrington *et al*, 1990). All the vaccines tested were stated to be safe for human used, with no obvious side effects.

The failure of the tandem repeat peptides to induce protective immunity may due to the lack of an appropriate T cell epitope in the peptides used in vaccine trials (Sinigaglia and Pink, 1990, Egan *et al*, 1987). Moreover, the studies of animals immunized with irradiated sporozoites revealed that the protective immunity does not depend only on the antibodies, but also on the levels of γ -interferon and CD8⁺ cells (Schofield *et al*, 1987). A study in inbred congenial mice led to the conclusion that the T-cell immune response to the synthetic peptide vaccines and CS protein is genetically controlled through the major histocompatibility complex (Good *et al*, 1987). Recently, a nonpolymorphic T-cell epitope of CS protein which can be recognized by most mouse and human individual has been identified and may be included in a future malaria vaccine (Sinigaglia *et al*, 1988).

The CS protein is found to be a major component of the sporozoite surface. Its sizes vary from 30-60 kDa among different species (Nussenzweig and Nussenzweig, 1985). It accounts for 10-15% of the proteins synthesized by a mature sporozoite. The CS protein also found on the hepatocyte plasma membrane, parasitophorus vacuole membrane and flocculent material in the peripheral vacuole of infected hepatocytes (Millet *et al*, 1991).

It was suggested that the CS protein may play a crucial role in immune evasion and is not a target for protective immunity (Schofield, 1990). This was supported by the lack of correlation between the level of protection observed in an

immunization experiment, IFA titer(using glutaraldehyde-fixed *P. falciparum* sporozoite) and ELISA based on serum activity against the repeat region (Herrington *et al*, 1991). Recently, it was found that the CS protein of *P. yoelii* and *P. berghei* sporozoite bind specifically to sulfated glycoconjugates and the binding activities were inhibited by fucoidan and heparin (Pancake *et al*, 1992). The results of another experiment suggested that region II of the CS protein at the C terminal-domain has some homology to proteins bound specifically to sulfated glycoconjugates responsible for the binding to sulfatides [Gal(3-SO₄)β1-Cer] and cholesterol-3-sulfate (Cerami *et al*, 1992) which may be present on the hepatocyte surface. However, it should be noted that this experiment is based on the binding ability of the CS protein to a hepatocyte and not the invasion of the sporozoite. The results of this finding is very interesting but remain unclear because proteins which bind specifically to sulfated glycoconjugates are involved in widely diverse biological process (Holt, 1989).

It has also been suggested that the N1 region (flanking region at the N-terminus of the repeat in the CS protein) is responsible for the invasion of sporozoites into the human hepatoma cell line, HepG2-A16. Cross-linking studies show that the N1 region may bind to 55 and 35 kDa receptors on HepG2-A16 cells. These receptors are not found in primary human hepatocytes. The cross-linking studies between sporozoites and primary human hepatocytes revealed that 55 kDa (serologically distinct from that in HepG2-A16) and 20 kDa receptors interact with a 16 kDa non-CS protein, sporozoite hepatocytic binding antigen, termed SHEBA (Hollingdale, 1990)

Many researcher are trying to find a new vaccine candidate against the malaria sporozoite. Other sporozoite proteins are thought to be promising vaccine candidates. Khusmith and her colleagues have identified another surface sporozoite protein of *P. yoelii*, sporozoite surface protein 2 (SSP2). The SSP2 is a 140 kDa protein localized at the sporozoite surface. It is encoded by a gene distinct from CS protein but also contains six amino acids tandem repeats, NPNEPS, and some homology to the

region II of CS protein at the C-terminus. Mice immunized with a mixture of mastocytoma cells, which are transfected with the CS gene and a fragment of the SSP2 gene, are completely protected from malaria infection (Khusmith *et al*, 1991). By sequence comparison, it is now believed that the homologue of this protein in *P. falciparum* is thrombospondin related anonymous protein or TRAP (Rogers, *et al*, 1992). Other *P. falciparum* sporozoite vaccine candidates have been identified, such as CSP-2, a protein recognized by monoclonal antibody E12, namely LSA-1 (Hollingdale *et al*, 1990). The CSP-2 was identified as a protein which may be responsible for the induction of protective immunity in mice against *P. berghei*. Unlike CSP-2, LSA-1, recognized by monoclonal antibody E12, is a conserved protein in several species of *Plasmodium*, *Toxoplasma* and *Eimeria*. It also cross-reacts at the apical end of blood stage merozoites of *P. falciparum*, *P. yoelii* and the *P. berghei* exoerythrocytic stage merozoite. LSA-1 is also a sporozoite protein which cross-reacts with an antigen associated with parasitophorous vacuole. A similar antigen was also previously identified by Hope and his colleagues (Hope *et al*, 1984b).

1.3.3.2.3. Vaccine candidate against the erythrocytic stage.

This stage of malaria parasite is a possible target for a malaria vaccine. It was shown that *Aotus* monkeys were protected from lethal malaria infection after they had been immunized with mixture of merozoites and immature schizonts from culture using Freund's complete adjuvant (Siddiqui *et al*, 1977)

The target of immune sera was revealed by the study of the effect of immune *Aotus* serum (Chulay *et al*, 1981a). The serum of an *Aotus* monkey which was immunized with *P. falciparum*-infected *Aotus* monkey erythrocytes, aggregated the merozoites *in vitro* but there was no visible effect on intracellular parasites. From further study, the *in vitro* growth inhibition activity was shown to be serum dose-dependent and was associated with the IgG fraction from immune serum (Chulay

et al, 1981b). Human immune sera also caused aggregation of merozoites and the inhibitory effect appeared to be species specific (Vernes *et al*, 1984).

The protein(s) target responsible for the stimulation of this type of protective immunity has been seriously investigated. Several proteins have been identified and proposed as vaccine candidates. However, it is difficult to definitely state the best vaccine candidate because so little is known about the function of these proteins. The primate models allow an empirical study of the ability of interesting proteins to elicit protective immunity. Many proteins have been investigated in the primate trials. Some of these vaccine candidates will be described (see below).

Merozoite surface protein-1 (MSP-1) which is also known by several other names e.g., MSA-1 (merozoite surface antigen-1), PMMSA (precursor to the major merozoite surface antigen), Pf195 and p190 is a glycoprotein on the merozoite surface (see review in Hyde, 1990). It is initially synthesized as a 190-195 kDa protein and processed into smaller fragments during late schizogony. These processed polypeptides are thought to form a multi-subunit complex at the merozoite surface and most of them are shed during the invasion process (Blackman and Holder, 1992 and Blackman *et al*, 1991). Purified MSP-1 when immunized with Freund's complete adjuvant can elicit protective immunity in *Aotus* monkey (Siddiqui *et al*, 1987) and in *Saimiri* monkey (Hall *et al*, 1984). A synthetic peptide, covering the N-terminal of this protein coupled with tetanus toxoid, also gave partial protection in *Saimiri* monkeys (Cheung *et al*, 1986). Although, a study in different isolates of parasite showed that this protein is polymorphic (McBride *et al*, 1982), a more recent study suggested that this polymorphism is based on dimorphic genotypes (Tanabe *et al*, 1987). This suggested the MSP-1 may still be used as a vaccine candidate.

Another merozoite surface antigen has also been identified. Merozoite surface antigen-2 (MSA-2) is a surface protein which has a molecular weight about 45-51 kDa. This protein is recognized by a group of monoclonal antibodies (Epping

et al, 1988). This protein, however, also shows antigenic polymorphism in different isolates (Fenton, 1991).

The ring-infected erythrocyte surface antigen (RESA, Pf155) is a 155 kDa protein which is found associated with the dense granules of the developing merozoites (Brown *et al*, 1985). It is transferred from the merozoite to the host cell during the invasion and is found associated with the cytoskeleton at the surface of the infected red blood cells. The IgG fraction of some human immune sera which inhibit parasite invasion *in vitro*, mostly contain antibodies directed against this protein (Wählin *et al*, 1984). Rabbits immunized with the C-terminal octapeptide repeats induced antibodies which can react with the native protein (Berzins *et al*, 1986). The purified antibodies from these rabbits have an inhibitory effect in merozoite invasion assays. A significant result from this experiment was the finding of octapeptide-specific IgG in human immune sera and the discovery that these affinity purified IgGs also inhibit the parasite invasion *in vitro*. Protective immunity was elicited in *Aotus* monkey immunized with a fusion protein containing a fragment of the N-terminus repetitive sequence but not the C-terminus repeat sequence (Collins *et al*, 1986 and Collins *et al*, 1988).

A glycoprotein binding protein-130 (GBP-130) is also considered as a vaccine candidate. It was thought to act as a surface merozoite receptor of the glycoporphins on the erythrocyte surface because of its ability to bind to an acrylamide matrix coupled with glycoporphin (Perkins, 1984). Although, recent work revealed that this binding may be a non-specific reaction (Van Schravendijk *et al*, 1987), this protein may still be used as a vaccine based on the fact that it was able to stimulate the protective immune response in Saimiri monkeys (Dubois *et al*, 1984). However, an immunization study in *Aotus* monkeys using recombinant protein which contained a fragment of GBP-130 showed no protective immune response among the immunized animals (Aronson *et al*, 1991).

The most extensive studies on a malaria synthetic vaccine have been performed by Patarroyo and his colleagues (Moreno and Patarroyo, 1989, Patarroyo *et al*, 1988, Patarroyo *et al*, 1987). Purified proteins of different sizes from cultured parasite extracts, 155 kDa, 115 kDa, 105 kDa, 90 kDa, 83 kDa, 60 kDa, 55 kDa, 50 kDa, 40 kDa, 35 kDa, 30 kDa and 23 kDa, were used to immunize *Aotus* monkeys. After challenge of the immunized animals with *P. falciparum*, most of immunized monkeys showed no protection, except monkeys immunized with 35 kDa and 83 kDa proteins. The 155 kDa and 55 kDa also elicited partial protection. Sequencing the N-terminus sequences of these protective antigens revealed that the 83 kDa is a processed fragment of MSA-1 and the 155 kDa protein is the RESA protein. To identify the protective epitope of these antigens, several peptides were synthesized to cover the N-terminal regions of the 155 kDa, 83 kDa, 55 kDa and 35 kDa proteins. An immunization experiment, using these synthesized peptides, showed that *Aotus* monkeys immunized with peptides derived from 83 kDa, 55 kDa and 35 kDa were partially protected from *P. falciparum* challenge. Another experiment was carried out using a combination of these synthetic peptides. The result showed that 4 of 8 *Aotus* monkeys immunized with peptides which contain the N terminal sequence of 55 kDa and 35 kDa proteins self-cured after developing moderate parasitemia. These two peptides together with another peptide which has sequence corresponding to the 43rd-53rd amino acid of MSP-1 elicited protective immunity in 20 of 24 immunized monkeys.

Based on these experiment, two synthetic malaria vaccines were designed. One synthetic vaccine, SPf66, is composed of the N terminal sequence of 55 kDa and 35 kDa, 43rd-53rd amino acid of MSP-1 and 2 repetitive sequences of the CS protein. Another vaccine, SPf105, contains the 5' region repeat of RESA, the 277th-287th amino acid of MSP-1, a repetitive sequence of the CS protein and a T-cell epitope of CS protein. These two synthetic proteins were separately polymerized to form large synthetic protein molecules and used to immunized volunteers. The SPf105 only

elicited immune response which limited the infection for a short period in two of four immunized individuals. However, the SPf66 showed complete protection in some immunized individuals. There was no correlation between protection and antibody titer against the synthetic vaccine or stimulation index of peripheral blood mononuclear cells. A lack of correlation between the protection and the immunological parameter was also observed in a pilot-scale human immunization trial with SPf66 (Salcedo *et al*, 1991). Sera from 185 immunized individual exhibited *in vitro* growth inhibition activity but this did not correlate with antibody titer.

Recently, the analysis of the human sera immunized with the SPf66 revealed that the protection of malaria infection may correlate with antibodies recognizing a KEK-containing peptide (Molano *et al*, 1992). Polyclonal antibodies appear to bind to the amino terminal lysine (K) and glutamic acid (E). This KEK motif is also found in other malaria proteins involved in invasion process and is proposed to interact with the erythrocyte. This epitope may be responsible for the stimulation of malaria immunity in immunized volunteers.

An attempt to repeat the original monkey immunization experiment using SPf66 and a combination of those three synthetic peptides in another laboratory failed to achieve the same level of protection as originally observed by Patarroyo (Ruebush II, 1990). The reason of the difference in experiment results is not clear. The efficacy of the SPf66 vaccine is now being tested in a number of field trials.

1.3.3.2.4. Rhoptry antigens.

Because of the fact that the rhoptry contents may be crucial for the invasion process, a group of rhoptry antigens are also considered as vaccine candidates for an asexual stage vaccine. An early immunization experiment showed that a

235 kDa rhoptry protein from *P. yoelii* can protect mice from infection (Holder and Freeman, 1981 and Oka *et al*, 1984).

Several rhoptry antigens have been identified. Some of these antigens are found as non-covalent complexes of two or more proteins which are co-immunoprecipitated by certain monoclonal antibodies. In some cases, proteins are processed into smaller fragments during parasite growth.

Rhoptry proteins so far identified are a 80/65/42 kDa complex called RAP (rhoptry associated protein) complex (Ridley *et al*, 1991), a high molecular weight 240 kDa protein (Roger *et al*, 1988), a 150/135/105 kDa protein complex called the RhopH complex (Lustigman *et al*, 1988) and a 55 kDa protein (Smythe *et al*, 1988). The first of these complexes is the major subject in this theses and will be discussed later in section 3.3.2.5.

The 240 kDa protein was identified by a monoclonal antibody. The protein is synthesized in the schizont stage of the parasite and is processed into a 225 kDa protein. The 150/135/105 kDa protein components of another complex are named as RhopH1/RhopH2/RhopH3 respectively. They were also reported existing as a 140/130/105 kDa protein complex (Cooper *et al*, 1988). Many groups of researchers also identified similar protein complexes which are probably identical (Sam-Yellowe and Perkins, 1990, Campbell *et al*, 1984, Siddiqui *et al*, 1987 and Etzion *et al*, 1991). These complex components are synthesized in mid to late-trophozoite prior to formation of the rhoptry and the complex is formed soon after their synthesis (Lustigman *et al*, 1988, Sam-Yellowe *et al*, 1988, Cooper *et al*, 1988). The protein complex is shown to be recognized by immune sera from humans in endemic areas (Coppel *et al*, 1987). During the invasion, the protein complex transfers to the newly invaded erythrocyte where the RhopH3 is modified by an unclear mechanism. Some evidences suggested that this modification is not due to the glycosylation or phosphorylation (Lustigman *et al*, 1988). From immuno-gold electron microscopic study, Sam-Yellowe and her colleagues showed that the 110 kDa protein

is secreted into the parasitophorous vacuole during parasite invasion (Sam-Yellowe *et al*, 1988). The present evidence from both DNA and protein studies supports the existence and conservation of the RhopH3 gene in different isolates (Brown and Coppel, 1991). A DNA fragment encoding RhopH3 was cloned and expressed in *Escherichia coli* by Coppel and his colleagues (Coppel *et al*, 1987). The whole gene sequence was recently reported and revealed that the RhopH3 gene contains no repetitive sequence (Brown and Coppel, 1991). Although the function of this protein complex still unclear, it was suggested to act as an alternative ligand for binding with erythrocyte (Sam-Yellowe and Perkins, 1990). An immunization experiment in *Aotus* monkeys using a 143/132/102 kDa rhoptry complex gave partial protection (Siddiqui *et al*, 1987).

A low molecular weight rhoptry antigen was found as a 55 kDa protein (Smythe *et al*, 1988). Very little is known about the 55 kDa protein. It is thought to be anchored in a membrane *via* a glycosylphosphatidylinositol moiety because it was biosynthetically labelled with [³H]glucosamine and [³H]myristic acid.

Another identified protein is a 80 kDa protein, called apical complex antigen-1 or AMA-1 (Peterson *et al*, 1989). It is localized at apical complex, possibly in rhoptries, and on the surface of the merozoite after erythrocyte rupture. There is no evidence of the presence of this protein in a newly invaded ring. The AMA-1 is first synthesized in the trophozoite stage and is rapidly processed to a 62 kDa peptide (Crewther *et al*, 1990). Like all known rhoptry protein encoded genes, the *ama-1* gene contains no repetitive repeat and is conserved among different isolate of *P. falciparum* (Thomas *et al*, 1990).

1.3.3.2.5. Rhoptry associated protein-1 (RAP-1).

The RAP complex is able to be immunoprecipitated by many monoclonal antibodies, which recognized 80 kDa peptide, as 80/65/42/40 kDa major peptides and

77/70 kDa minor bands (Ridley *et al*, 1990a). The 77/70 kDa bands are suggested to be degradative products of the RAP-1 protein during preparation. Similar molecular weight proteins were also identified by other researchers (Campbell *et al*, 1984, Howard *et al*, 1984, Schofield *et al*, 1986, Clark *et al*, 1987, Bushell *et al*, 1988 and Howard, 1990). Western blot study of the RAP complex suggested that the RAP complex is composed of, at least, two gene products, a 80 kDa protein which was processed to a 65 kDa protein after translation, so called RAP-1(Ridley *et al*, 1990a) and a 42 kDa protein, called RAP-2 (Saul *et al*, 1992). As it forms the major interest of this thesis, the RAP-1 protein will be discussed in more detail.

The rhoptry associated protein-1 was localized in the rhoptry organelle of the *P. falciparum* by using immunofluorescence assay (IFA) and electron microscopy. The IFA gave an punctate pattern which corresponds to the rhoptry organelle of the parasite. The localization of this protein also confirmed by immuno-gold labelling technique. Most of the RAP-1 protein was detected in the body of the rhoptry organelle and less dense at the neck of this organelle (Bannister, unpublished results).

The RAP-1 is first synthesized as a 84 kDa protein precursor at mid-trophozoite stage before it is processed into a 80 kDa peptide (Bushell *et al*, 1988). This processing may be required for its complex formation with the RAP-2, which is synthesized at the same time from a different gene (Ridley *et al*, 1991 and Saul *et al*, 1992). After complex formation, the 80 kDa protein is further processed into a 65 kDa (or 66 kDa) peptide at around the time of schizont rupture (Schofield *et al*, 1986, Clark *et al*, 1987, Ridley *et al*, 1990a).

Of the monoclonal antibodies against RAP-1 protein so far described, most of them bind to conserved epitopes among different isolates and some have shown inhibitory effects on parasite growth *in vitro* (Schofield *et al*, 1986, Clark *et al*, 1987 and Hope, 1984a). The inhibitory effect was proposed to be the result of merozoite invasion blocking . It was suggested that rhoptry proteins may be externally accessible during release and reinvasion (Sam-Yellowe and Perkins, 1990). The RAP-1 protein is

not only found in rhoptries (punctate pattern by IFA and immuno-labelling electron microscope) but also found associated with membrane-like material protruding from rhoptries (Bushell *et al*, 1988). After invasion, only the 80 kDa protein can be detected in the newly infected red blood cell but not the 65 kDa protein (Clark *et al*, 1987, Howard and Reese, 1984). This evidence suggests that the RAP-1 protein may be functionally important for merozoite release and/or the reinvasion of erythrocytes and may responsible for the induction of protective immunity.

Indeed, an immunization experiment in *Saimiri* monkeys showed that the RAP complex can stimulate protective immunity in immunized animals (Ridley *et al*, 1990b). Importantly, the serum from protected monkeys only recognized the RAP complex in the total parasite extract on a western blot. This immunization experiment proved that the RAP complex alone can elicit protective immune responses against malaria infection and that the RAP complex is a good malaria vaccine candidate.

The gene encoding RAP-1 protein was first studied in detail by Ridley and his colleagues (Ridley *et al*, 1990a). A sequencing study revealed a single reading frame of 2349 base pairs encoding 813 amino acids. The RAP-1 protein contains no repetitive sequence but has a serine-rich region with KSSSPS motifs at the N-terminus half of the peptide. A sequence at the end of N-terminus was also proposed as a signal peptide. All of the cysteine residues, some of which are known to take part in disulfide bridge formations, are located at the C-terminal half of the molecule. These cysteine amino acids are flanked by amino acid sequences which have potential for amphiphilic helix formation, a structure found in many membrane interacting proteins. A cleavage site for processing of 83 kDa into 65 kDa has also been located between A₁₉₀ and D₁₉₁ (Ridley *et al*, 1991).

The sequence comparison of this gene in Honduras I/CDC isolate (Howard *et al*, 1992) and K1 isolate (Ridley *et al*, 1990a) revealed that RAP-1 gene is highly conserved in these two isolates. Up till now, the data about the RAP-1 protein has led to the conclusion that this protein is a good *P. falciparum* vaccine candidate.

However, more studies are needed to gain a better understanding of both the immunology and function of the RAP-1 protein.

1.4. Scope of the thesis.

The importance of the RAP-1 protein as a malaria vaccine candidate has been progressively solidified in the past few years. Like many other malaria vaccine candidates, the function and immunological aspects of the RAP-1 protein are still unclear. Obviously, these informations are crucial for vaccine design and development.

Evaluation of inhibitory experiments shows that several monoclonal antibodies which directly recognized the RAP-1 protein can inhibit parasite growth *in vitro*. This suggested that the binding of these monoclonal antibodies at specific sites may affect the function of the RAP-1 protein and lead to the growth inhibition of the parasite. Thus, it is of great interest to identify those antibody binding sites on the RAP-1 protein.

The aim of this thesis is to map the epitopes of the inhibitory monoclonal antibodies in order to identify functional and immunologically significant regions of the RAP-1 protein for future malaria vaccine development and to determine possible inhibition mechanisms (Chapter 1-5). Another part of the thesis is devoted to epitope mapping of the monoclonal antibodies generated by a recombinant RAP-1 protein to compare immunogenicity different between the recombinant protein and the native RAP-1 protein (Chapter 6). To emphasize the degree of conservation in the *rap-1* gene, sequences of the *rap-1* gene from different isolates of the *P. falciparum* were determined and compared (Chapter 7).

Chapter 2

Materials and methods

2.1. Materials.

2.1.1. Bacterial strain and recombinant bacteriophage.

The important genotypes and phenotypes of a *E. coli* strain, recombinant bacteriophage and expression plasmids used in this study are described below.

2.1.1.1. Bacterial strain.

<i>E. coli</i> strain	Genotypes and reference
NM522	<i>supE, thi, Δ(lac-proAB), Δhsd5(r⁻, m⁻), [F', proAB, lacI^qZΔM15].</i> (Gough and Murray,1983)

This strain of *E. coli* grows well in LB medium or minimal medium (see section 2.1.7.2.) supplemented with thiamine (vitamin B1) because it cannot synthesize thiamine (*thi*). The *supE* genotype allows suppression of an amber (UAG) mutation during translation of mRNA. Because the α -glutamyl phosphate reductase (*proA*) and the α -glutamyl kinase (*proB*) encoded genes on the genomic DNA were deleted, the bacteria has to maintain its F' episome which carries both genes if it is grown on the minimal medium supplement with thiamine. The Δ hsd5 (*r⁻, m⁻*) genotype

of this strain will prevent the cleavage of DNA by endogenous restriction endonuclease and also modification process by methylation.

2.1.1.2. Recombinant bacteriophage.

The RAP-1 gene insert was originally obtained from the λ rap1.1 (Ridley *et al*, 1990a). The λ rap1.1 is the recombinant λ gt11 which contains a part of *rap-1* gene from nucleotide 182 to 1953 (amino acid D₆₂ to H₆₅₁) The *rap-1* gene insert is flanked by an *EcoRI* linker at both ends (see detail in chapter 3).

2.1.2. Plasmids used in this experiment.

Expression plasmids	References
pUR278	Rüther and Müller-Hill, 1983
pMS1S	Scherf et al,1990

The pUR278 and pMS1S are expression plasmids derived from pBR322. Both plasmids carry β -lactamase genes which lead to the ampicillin resistance phenotype in the transformant cell. The interested gene or fragment may be subcloned into the linker region at the C-terminus of the β -galactosidase gene which is regulated by lac promoter (see more detail in chapter 3).

2.1.3. Genomic DNA of the *P. falciparum* .

Genomic DNAs from different strains of the *P. falciparum* were used in the PCR experiment (see chapter 7). The genomic DNAs of the strain GF881, K29, T9/94, T9/96 were kindly provided by Dr. Jana McBride. The HB3 and 3D7 genomic DNAs

were gratefully received from Dr. Shiu-wan Chan. The K1 genomic DNA was kindly prepared by Dr. Robert Ridley. The MAD20 genomic DNA was prepared by mini-preparation method as described in the method section (see method 2.2.2.3.).

2.1.4. Enzymes and buffers.

Most enzymes were purchased from commercially available sources for DNA manipulation. The buffers for these enzyme reactions were usually provided by the manufacturer but some buffers were prepared in the laboratory. The table below shows the sources from where the enzymes and the buffers were ordered. It also includes the components of the buffers used which were prepared in the lab from sterile reagents.

Enzymes and buffers	Sources or components
All restriction endonuclease enzymes and their buffers	Boehringer Mannheim
T4 DNA ligase	Pharmacia
Klenow fragment of DNA polymerase	Northumbria Biological Limited
Thermalase	IBI
10×One-phor-All buffer	Pharmacia
10×ligase buffer	200 mM Tris-Cl (pH 7.6) 50 mM MgCl ₂ 50 mM dithiothreitol
10×Thermalase buffer	IBI

2.1.5. Commercial kits.

In this study, several commercially available kits were used. Their manufacturer sources of the kits are listed here.

Kits and their sources	Manufacturers
PRIME IT™ RANDOM PRIMER KIT	Stratagene
Erase-a-Base system	Promega
Gene clean II kit	BIO 101 Inc.
Qiagen plasmid preparation kit	Hybaid
Nick column	Pharmacia
Protoplot System	Promega
Pepscan kit	Cambridge Research Chemicals

2.1.6. Antibodies.

Antibodies and monoclonal antibodies were used for localization of their epitopes in this study. The characters of each monoclonal antibodies and polyclonal antibodies will be described in Table 3.1 (see chapter 3) and Table 6.1 (see chapter 6). Other antibodies were also used as second antibody for antigen detection in western blot analysis and the Pepscan system, an overlapping peptide synthesis kit. The antibodies used on the western blot are alkaline phosphate conjugated but the antibodies used in the Pepscan system are horseradish peroxidase conjugated. Both types of the antibodies are listed below with the commercial sources.

Second antibodies	Sources
Goat anti-rabbit IgG alkaline phosphatase conjugated	Promega
Anti-mouse IgG alkaline phosphatase conjugated	Promega
Anti-human IgG alkaline phosphatase conjugated	Promega
Swine anti-rabbit IgG peroxidase conjugated	Cambridge Research Chemicals
Rabbit anti-mouse IgG peroxidase conjugated	Cambridge Research Chemicals
Goat anti-human IgG peroxidase conjugated	Cambridge Research Chemicals

2.1.7. Important reagents and chemicals.

Most chemicals used in this study were analytical grade chemicals which are commercially available. In Pepscan peptide synthesis experiment, high grade chemicals are required for reliability of the peptide synthesis. These high grade chemicals can be obtained only from special suppliers. The lists below show the suppliers and the high grade chemicals used for this study.

2.1.7.1. Chemicals for Pepscan experiment.

Suppliers	Chemicals	Grades
Aldrich	Piperidine	99% grade
	Ethanedithiol	
	Diisopropylethylamine	

Suppliers	Chemicals	Grades
BDH	Methanol	AnalaR
	Dichloromethane	AnalaR
	Sodium lauryl sulfate	AnalaR
	2-mercaptoethanol	AnalaR
	Sodium dihydrogen orthophosphate	AnalaR
	Citric acid	AnalaR
	Sodium hydroxide	AnalaR
	Hydrogen peroxide(60% hydrogen)	AnalaR
	Triethylamine	AnalaR
	Phenol	AnalaR
	Acetic anhydride	AnalaR
Sigma	Trifluoroacetic acid	protein sequencing grade
	Bovine serum albumin	IgG free
	Ovalbumin	grade II
	Tween20 (polyoxyethylene sorbitan monolaurate)	
Rathburn	NN-Dimethylformamide	protein synthesis grade

2.1.7.2. Media and reagents.

The reagents and their components used in this study are listed below. All reagents were sterilized by either autoclaving or filtration, except when stated otherwise.

-Bacteriological media.

Media	Compositions
LB medium.	per liter; Bacto-tryptone 10 g Bacto-yeast extract 5 g NaCl 10 g adjusted pH to 7.0 with 5 N NaOH
LB Agar.	as LB broth; Bacto agar 15 g
5×Spizizen salts.	per liter; (NH ₄) ₂ SO ₄ 2 g K ₂ HPO ₄ ·3H ₂ O 18.3 g KH ₂ PO ₄ 6 g Trisodium citrate·2H ₂ O 1 g MgSO ₄ ·7H ₂ O 0.2 g adjusted pH to 7.2
Minimal medium.	per 400 ml; 20% Glucose 4 ml Spitzizen salts 80 ml Thiamine HCl (100 mg/ml) 200 µl Ampicillin (100 mg/ml) 200 µl Bacto agar 6 g

-Competent cell preparation reagents.

Reagents	Compositions
STE buffer.	0.1 M NaCl 10 mM Tris-Cl, pH 8.0 1 mM EDTA
TSB buffer	10% PEG 5% DMSO 10 mM MgCl ₂ 10 mM MgSO ₄ . in LB broth

-Bacteriophage DNA preparation reagents.

Buffers	Compositions
SM buffer	per liter NaCl 5.8 g MgSO ₄ ·7H ₂ O 2 g 1 M Tris-Cl, pH 7.5 50 ml 2% gelatin solution 5 ml
Adsorption buffer.	10 mM MgCl ₂ 10 mM CaCl ₂

-DNA preparation reagents.

Reagents	Compositions
Solution 1	50 mM glucose 25 mM Tris-Cl, pH 8.0 1 mM EDTA, pH 8.0
Lysozyme solution.	10 mg/ml Lysozyme 10 mM Tris-Cl, pH 8.0
Solution 2.	0.2 N NaOH 1% SDS
Solution 3.	5 M Potassium acetate 60 ml Glacial acetic acid 11.5 ml Sterile distilled water 28.5 ml
Solution 4.	1.6 M NaCl 13% w/v PEG 8,000.
Phenol:chloroform.	50% Phenol 48% Chloroform 2% Isoamyl alcohol
10×TE	100 mM Tris-Cl, pH 8.0 10 mM EDTA, pH 8.0
bufferI for genomic DNA mini-preparation	150 mM NaCl 25 mM EDTA, pH 8.0

-Enzyme buffers.

Buffers	Compositions
10×Klenow buffer	0.5 M Tris-Cl, pH 7.6 0.1 M MgCl ₂
dNTP mix	0.125 mM dATP 0.125 mM dTTP 0.125 mM dGTP 0.125 mM dCTP
10×Bacteriophage T4 polynucleotide kinase	0.5 M Tris-Cl, pH 7.6 0.1 M MgCl ₂ 50 mM Dithiothreitol 1 mM Spermidine HCl 1 mM EDTA, pH 8.0
10×S1 nuclease buffer	2 M NaCl 0.5 M Sodium acetate (pH 4.5) 10 mM ZnSO ₄ 5% Glycerol

-Electrophoresis reagents.

Reagents	Compositions
Loading buffer	0.25% Bromophenol blue 0.25% Xylene cyanol FF 30% Glycerol in water

Reagents	Compositions
10×TBE	890 mM Tris-borate 20 mM EDTA
10×TAE	0.4 M Tris-acetate 0.01 M EDTA

-Nick column reagents.

Reagent	Compositions
Buffer solution	10 mM Tris-Cl, pH7.5 1 mM EDTA

-Hybridization reagents.

Reagents	Compositions
Denaturing solution	1.5 M NaCl 0.5 M NaOH
Neutralization solution	1.5 M NaCl 0.5 M Tris-Cl, pH 7.2 0.001 M EDTA, pH 8.0
Prehybridization solution	6×SSC 0.4 mM EDTA, pH 8.0 0.1% Tetrasodium pyrophosphate 0.2% SDS 50 mg/L Heparin

Reagents	Compositions
20×SSC	3 M NaCl 0.3 M Trisodium citrate adjust to pH 7.0 with 10 N NaOH
Washing solution (for probe removal)	0.1×SSC 0.1% SDS 0.2 M Tris-Cl, pH 7.5

-ExonucleaseIII digestion reagents.

Reagents	Compositions
S1 nuclease mix	7.4X S1 buffer 27 µl (supplied with the kit) S1 nuclease 60 units Distilled water 172 µl
Klenow mix	10× Klenow buffer 30 µl Klenow DNA polymerase 3 units
dNTP mix	0.125 mM dATP 0.125 mM dTTP 0.125 mM dGTP 0.125 mM dCTP
Ligase mix	10× Ligase buffer 100 µl 50% PEG 100 µl 100 mM DTT 10 µl T4 DNA ligase 5 units Distilled water 790 µl

-Sequencing reagents.

Reagents	Compositions
10×TM buffer	70 mM Tris-Cl, pH 7.5 70 mM MgCl ₂ 300 mM NaCl 100 mM Dithiothreitol 1 mM EDTA
T0.1E	10 mM Tris-Cl, pH 7.5 0.1 mM EDTA
chase mix	0.5 mM dATP 0.5 mM dCTP 0.5 mM dTTP 0.5 mM dGTP
Fixing solution	10% Methanol 10% Acetic acid
Formamide dye mix	98% Formamide 0.025% Xylene cyanol FF 0.025% Bromophenol blue 10 mM EDTA, pH 8.0

For ddNTP solutions were prepared as follow:

Stock solution	A(μ l)	C(μ l)	G(μ l)	T(μ l)
0.5 mM dCTP	500	25	500	500
0.5 mM dGTP	500	500	25	500
0.5 mM dTTP	500	500	500	25
10 mM ddATP	1	-	-	-
10 mM ddCTP	-	8	-	-
10 mM ddGTP	-	-	16	-
10 mM ddTTP	-	-	-	50
T0.1E buffer	500	1000	1000	1000

-SDS-PAGE gel electrophoresis reagents.

Reagents	Compositions
30% Acrylamide solution	29.2% Acrylamide 0.2% N, N'-methylenebisacrylamide
2 $\times$ Sample buffer	0.125 M Tris-Cl, pH 6.8 4% SDS 20% Glycerol 10% 2-mercaptoethanol 0.05% Bromophenol blue
Tank buffer	0.025 M Tris base 0.192 M Glycine 0.1% SDS

Reagents	Compositions
Stain solution	0.125% Coomassie blue R-250 50% Methanol 10% Acetic acid
Destaining solution	50% Methanol 10% Acetic acid

-Western blot reagents.

Reagents	Compositions
PBS	0.9% NaCl 10 mM Tris-Cl, pH 7.4
Blocking solution	5% Fat-free milk powder in PBS
Transfer buffer	25 mM Tris base 192 mM Glycine 20% Methanol 0.1% SDS
Alkaline phosphatase buffer	100 mM Tris-HCl, pH 9.5 100 mM NaCl 5 mM MgCl ₂



-Pepscan reagents.

Reagents	Compositions	
Acetylation solution	DMF	5 parts
	Acetic anhydride	2 parts
	Triethylamine	1 part
Cleavage solution (for deprotection)	95%	Trifluoroacetic acid
	2.5%	Phenol (w/v)
	2.5%	Ethanedithiol
Disruption buffer	1%	Sodium dodecyl sulphate
	0.1 M	Sodium dihydrogen orthophosphate
	adjust pH to 7.2 with 50% NaOH	
Supercocktail	1%	Ovalbumin
	1%	Bovine serum albumin
	0.1%	Tween 20 in PBS
10×PBS	per liter;	
	NaCl	85 g
	Disodium hydrogen orthophosphate	10.7 g
	Sodium dihydrogen orthophosphate	3.9 g
10×PBS/Tween 20	as 10×PBS	
	Tween 20	5 ml
ABTS buffer	per 2 liter;	
	1 M disodium hydrogen orthophosphate	200 ml
	1 M citric acid	160 ml
	adjust pH to 4.0 with citric acid or NaOH	

Reagents	Compositions
Substrate solution	per 100 ml of ABTS buffer; azino-di-3-ethyl- benzthiazodinsulphonate 50 mg 60% hydrogen peroxide 60 μ l

2.1.8. Computer analysis software.

In the Pepscan experiment, the peptide synthesis schedules were produced by a software provided with the Pepscan kit. Although, the software is also capable of analysis the data directly from the ELISA plate reader, the software was not used for data analysis because the ELISA plate reader used in this study has not been connected to the IBM-compatible computer. Instead, the data was manually typed into the mainframe computer, the Castle, at the Edinburgh University and histogram graphs were produced by a programme called Easygraph (as shown in chapter 6 and 7).

The pictures of gel electrophoresis, autoradiograph and western blot detection illustrated in this thesis were reproduced by a scanner (256 greyscales from Logitech) and fototouch programme (window version) supplied with the scanner. The scanned pictures were rescaled to fit A4 pages without further alteration.

2.2. Methods.

2.2.1. Microbiological techniques.

2.2.1.1. Bacterial culture.

Three techniques were used for bacterial culture in this study, namely streak plate, spread plate and liquid culture. The purpose of streak plate technique was to purify a single colony from liquid culture or a mixed colony. To receive bacteria colonies from liquid culture, the spread plate technique must be applied. The liquid culture technique is used to multiply a large amount of bacteria and for fusion protein production. To obtain exponential phase culture, the overnight grown culture had to be diluted with prewarmed medium in a ratio 1:10 and was further grown for 1 hour.

2.2.1.2. Bacterial storage.

Bacteria can be stored on an agar plate for short term storage or as a stab for long term storage. The short term storage is stored as single colonies which are ready for further manipulation. The long term storage, nevertheless, must be re-streaked before single colony can be obtained.

-Short term storage.

The aim for the short term storage of the bacteria is to maintain the clone stock during the study. The *E. coli* is usually streaked on solid medium and allowed to grow overnight in 37°C before the plate is stored at 4°C up to 1-2 month. However, different clones of *E. coli* need different media during storage. The *E. coli* strain NM522 can be stored on the minimum medium. The *E. coli* clones with recombinant plasmids which carry the β -lactamase gene have to be stored on the minimum medium supplement with ampicillin to sustain their plasmids.

-Long term storage.

A well separated colony was picked by a flame sterile inoculating needle. The needle was stabbed through the LB agar in a small glass vial. The cap of the vial was tightened and the vial was stored in the dark at room temperature.

2.2.1.3. Competent cell preparation.

For convenience, the competent cells were prepared on a large scale by a rapid method modified from a method described by Chung and Miller (Chung and Miller, 1988). A single colony of NM522 was grown in 250 ml flask containing 100 ml LB broth overnight. 10 ml of the culture was diluted with 90 ml of pre-warmed LB broth and was grown further for 1 hour to achieve early log phase growth. The cells were collected by centrifugation (2,000 rpm for 15 mins). The cell pellet was resuspended in 10 ml of ice-cold TSB buffer and incubated on ice for approximately 10 mins. The treated cells were either used immediately or they could be frozen with liquid nitrogen and stored at -70°C as 0.1 ml aliquots in microfuge tubes.

2.2.1.4. Induction of fusion proteins.

To obtain recombinant fusion proteins from a recombinant clone, a single colony of bacteria was grown in 5 ml bottle containing 1 ml LB broth and 50 µg/ml of ampicillin overnight at 37°C with 200 rpm shaking. The culture was diluted 1:10 in 1 ml pre-warmed LB broth and grown for 2 hours at the same condition. 2 mM of IPTG was then added before the culture was grown further for 90 mins. In one experiment, the culture was induced overnight after the dilution of the bacterial culture.

2.2.2. DNA manipulation techniques.

Many molecular biology techniques related to DNA were used in this study. These DNA manipulation techniques are described below.

2.2.2.1. Phage DNA preparation.

The phage miniprep was modified from the method described by Grossberger (Grossberger, 1987). The original plate of λ rap1.1 was kindly prepared by Dr. Rob Ridley. A single plaque was transferred into a 0.3 ml adsorption buffer (see 2.1.7.2.) in 20 ml glass tube with a sterile 200 μ l Eppendorf pipette tip. To the tube was added 0.2 ml of an exponential culture of bacteria which was previously grown in LB broth containing 0.4% maltose (see 2.2.1.1.). The culture was incubated at 37°C for 10 mins to allow adsorption of the phage before 10 ml of LB broth supplement with 10 mM $MgCl_2$ and 0.1% glucose was added. The tube was then incubated at 37°C overnight. The bacterial debris was separated by centrifugation (3,000 rpm for 10 mins). DNase free RNase was added to digest contaminating RNA. The supernatant was centrifuged at 26,000 rpm for 2 hours to collect the phage particle. The pellet was resuspended in 200 μ l of SM buffer, and freshly made 200 μ l of SM buffer containing 1 mg/ml proteinase K was added before the mixture was incubated for 2 hours at 37°C.

The mixture was extracted once with phenol:chloroform to extract the phage particles. To precipitate the phage DNA, 100 μ l of 7.5 M ammonium acetate and 1 ml 100% ethanol were added. The DNA precipitate was centrifuged (12,000g for 15 mins) and washed with 0.5 ml 100% ethanol, air dried and dissolved in 50 μ l of TE.

2.2.2.2. Plasmid DNA preparation.

-Medium scale plasmid preparation by PEG.

A single colony of bacteria was grown in LB broth in the presence of 50 µg/ml ampicillin overnight (see 2.2.1.1.). The cells were collected by centrifugation (2000g for 15 mins) at 4°C. The pellet was washed once in 100 ml ice-cold STE and was resuspended in 18 ml of ice-cold Solution1 (see 2.1.7.2.). The bacteria suspension was mixed with 2 ml of lysozyme solution (see 2.1.7.2.). The cells were lysed with 40 ml freshly prepared Solution 2 (see 2.1.7.2.). The mixture was inverted 2-3 times and incubated on ice for 10 mins. After adding 20 ml of ice-cold Solution 3 (see 2.1.7.2.), the mixture was shaken and further incubated for 10 mins. The bacterial genomic DNA and other cell debris were pelleted by centrifugation at 16,000g for 15 mins at 4°C without a break. 0.6 volume of isopropanol was added to the supernatant, mixed and incubated at room temperature for 10 mins to allow the precipitation of plasmid DNA. The centrifugation (4000g for 15 mins at room temperature) brought down the plasmid precipitate from the solution. The pellet was washed with 70% ethanol. The plasmid pellet was resuspended in 6 ml of TE. After adding 6 ml of ice-cold 5 M LiCl, the high molecular weight RNA can be separated from the plasmid mixture by centrifugation (12,000g for 10 mins at 4°C). The plasmid was then precipitated from the solution by adding an equal volume of isopropanol and leaving on ice for 5 mins before it was pelleted by 12,000g centrifugation at room temperature for 10 mins. The pellet was again washed with 70% ethanol.

To purify the plasmid DNA, the PEG precipitation technique was used. The plasmid resuspended in 500 µl TE with 20 µg/ml DNase-free RNase and incubated at room temperature for 30 mins. 500 µl of Solution4 (see 2.1.7.2.) was added. The mixture was centrifuged 12,000g for 5 mins at room temperature. The obtained pellet was washed with 70% ethanol and dried. The pellet then was dissolved in 400 µl of TE

before being extracted with equal parts of phenol, phenol: chloroform and chloroform.

-Ethanol precipitation of DNA.

The purified plasmid was precipitated by adding 3 M sodium acetate about 1 in 10 volume of the DNA solution and 2 volumes of ethanol and leaving at room temperature for 10 mins. The tube was centrifuged 12,000g for 5 mins. The pellet was washed with 200 µl of 70% ethanol.

-DNA storage.

The pellet of plasmid was dissolved in 500 µl of TE and dispensed in 50 µl aliquots into microfuge tubes. One tube of plasmid DNA was stored at -20°C for future use and the others stored at -70°C as stock plasmid.

-Qiagen column method.

The use of a Qiagen column allows the DNA preparation to be achieved by simple step operation. The quality of plasmid DNA is high enough for double stranded sequencing. The method described below is recommended by the manufacturer.

A single bacterial colony was grown overnight in 100 ml LB broth supplement with 50 µg/ml ampicillin (see 2.2.1.1.). The cells were pelleted and resuspended in 4 ml of buffer P1 (see 2.1.5.). 4 ml of buffer P2 (see 2.1.5.) was added, mixed and incubated at room temperature for 5 mins. Then 4 ml of buffer P3 (see 2.1.5.) was added. The mixture was centrifuged 20,000 rpm at 4°C for 30 mins. The supernatant was again centrifuged for 10 mins to obtain a clear lysate. The solution was passed through qiagen-tip100 which had been equilibrated with 3 ml of buffer QBT (see 2.1.5.). The column was washed with 10 ml of buffer QC (see 2.1.5.) before the column was eluted with 5 ml of buffer QF (see 2.1.5.). The plasmid was precipitated with 0.7 volume of isopropanol and centrifuged. The pellet was washed with 70% ethanol and was dissolved in 100 µl of TE.

-Quantitation of DNA concentration.

The amount of DNA can be measured by spectrophotometer. The DNA solution was diluted between 1/100 and 1/1000 with TE and the optical density of the solution was read at a wavelength of 260 nm. The concentration of DNA can be calculated from the standard:

At approximately 50 µg/ml of double-stranded DNA; $OD_{260} = 1$

2.2.2.3. Parasite DNA mini-preparation.

The mini-preparation for the genomic DNA of the *P. falciparum* used in this study was developed by Dr. Shiu-wan Chan (Chan, 1991). The genomic DNA obtained from this method provided genomic DNA suitable as a template for the PCR reaction. The *P. falciparum* strain MAD20-infected human red blood cells were received from the parasite culture which has been maintained in the laboratory.

A 100 µl aliquot of *P. falciparum*-infected red blood cells were washed three times with 0.5 ml of buffer I (see 2.1.7.2.). The parasitized red blood cells were resuspended in 0.4 ml of the same buffer with 50 µg of proteinase K, and 5 µl of 10% SDS was added. The mixture was incubated overnight at 37°C. The lysed infected red blood cell was extracted with phenol once. The genomic DNA was obtained after the ethanol precipitation (see 2.2.2.2.). The DNA was resuspended in 5 µl of TE.

2.2.2.4. Digestion of DNA by restriction enzymes.

DNA was digested by mixing the desired amount of DNA with 1 µl (or more) of diluted restriction enzyme (allow at least 1 unit per 1 µg of DNA) and 1 µl of 10×One-phor-All buffer or another appropriate restriction enzyme buffer. The reaction was made up to 10 µl with sterile distilled water and incubated at 37°C from 1 hour to

overnight. In case of a double enzyme digestion, the total volume of the reaction was increased to 20 μ l and the same amount of another restriction enzyme and buffer was added at the same time. After the digestion was completed, the restriction enzyme was inactivated at 65°C.

For partial digestion, 0.5 units of restriction enzyme per 1 μ g of DNA were used. The reaction was incubated only 1 hour at 37°C before the enzyme was inactivated by heat as described above.

2.2.2.5. End-filled reaction by Klenow fragment of DNA polymerase.

DNA with sticky-end was mixed with 1 μ l of Klenow fragment (2 units/ μ l), 1 μ l of dNTP mix (see 2.1.7.2.) and 1 μ l of 10 $\times$ Klenow buffer (see 2.1.7.2.). The reaction mixture was made up to 10 μ l with sterile distilled water and incubated at 37°C for 30 mins. The mixture was removed from the 37°C incubator and the enzyme was inactivated at 65°C for 20 min.

2.2.2.6. Digestion of the sticky-end of DNA by S1 nuclease.

DNA with sticky-end was digested by mixing a desired amount of DNA with 1 μ l of S1 nuclease enzyme (1 unit/ μ l) and 1 μ l of 10 $\times$ S1 nuclease buffer. The mixture was made up to 10 μ l with sterile distilled water and incubated at 37°C for 30 mins. The enzyme was then denatured by incubation at 65°C for 20 mins.

2.2.2.7. Ligation of DNA.

Linearized plasmid DNA was mixed in total of 10 μ l with a DNA fragment with compatible ends to the plasmid, 1 μ l of 10 $\times$ T4 DNA ligase buffer and 1 μ l of T4

DNA ligase(1 unit/ μ l) The mixture was incubated at 15°C overnight. The enzyme was inactivated at 65°C for 20 mins.

The ligation mixture must contain equal or higher concentration of DNA fragment molecules than the plasmid to optimize the ligation reaction.

2.2.2.8. Transformation.

An aliquot (0.1 μ l) of the competent cells (see 2.2.1.3.) was mixed with 100 pg of plasmid DNA in an ice-cold microfuge tube. The tube was incubated on ice for 30 mins and 0.9 ml of LB broth containing 20 mM glucose was added. The microfuge tube was rotated slowly at 37°C for 1 hour. The culture was spreaded onto LB agar plate supplement with 50 μ g/ml ampicillin (see 2.2.1.1.) for selection of transformants.

2.2.2.9. Agarose gel electrophoresis.

Agarose gel electrophoresis is a technique for identification of DNA fragments, for example, following the digestion of DNA fragment by restriction enzymes and for their separation. Large 'maxi' gels were used for purification of a digested DNA fragment.

-Sample preparation.

The DNA sample for gel electrophoresis was mixed with 10×loading buffer approximately 1 in 10 of the sample total volume. The volume of the sample was made up to multiple of 10 μ l.

-Mini gel electrophoresis.

To prepare a minigel, 0.8 g of agarose was melted in 100 ml of 0.5×TBE buffer (prepared as 10×TBE, see 2.1.7.2.). The gel was cast on a plastic base (about 5 cm×10 cm in size) and a comb was used to form sample wells. The gel was submerged in 0.5×TBE buffer before samples were loaded into each of the wells. The

gel was run at 100 volts for 1.30 hours or until DNA bands could be individually distinguished.

-Maxi gel electrophoresis.

Maxi gel was prepared as 0.8% of agarose in 1×TAE buffer (prepared as 10×TAE, see 2.1.7.2.) . Ethidium bromide was added to agarose gel and buffer. Both ends of the gel were placed on buffer tanks. Wick paper was used to draw buffer from the tanks into the gel. The agarose gel was covered with cling film to prevent evaporation . A salt bridge linked between the tanks to circulate buffer in both tanks. The gel was run at 80 volts overnight with 1×TAE buffer. The electrophoresis time was extended until the DNA bands were well separated.

-Agarose gel staining.

The gel was removed from the casting base and soaked in 0.5 µg/ml ethidium bromide with slow shaking. The gel was left in the solution for at least 15 mins. The DNA bands were detected with a UV transilluminator which transmits ultraviolet light at 302 nm. The double strand DNA was visible as red-orange bands when the intercalating ethidium bromide emitted energy as visible light at 590 nm.

2.2.2.10. DNA fragment purification from agarose gel.

DNA fragments separated by Maxi gel electrophoresis can be purified from agarose by one of the two methods described below.

-Electroelution of DNA fragment from Maxi gel electrophoresis.

A narrow block of agarose gel was removed in front of the desired DNA band to form a square well which was about 2-3 cm. wider than the DNA band on both size. A dialysis membrane was placed at the far side wall of the well to prevent the DNA from moving out of the well during electroelution. The well was filled with 1×TAE buffer and 0.5 µg/ml ethidium bromide. The eletrophoresis was continued at 300 volts until the DNA attached to the membrane (the DNA was located by UV

transilluminator, see 2.2.2.9.). The current direction was then reversed for 30 sec causing the DNA to move back into the well. The buffer in the well was collected and the ethidium bromide was extracted 3-4 times with an equal volume of 1×TAE-equilibrated butanol. The solution was extracted once with phenol:chloroform and once with chloroform. The DNA was precipitated by ethanol precipitation (see 2.2.2.2.).

-GENECLEAN II Kit.

A band of DNA as detected by ethidium bromide was cut from agarose gel run in 1×TAE and transferred to a microfuge tube. The piece of agarose was melted at 55°C after approximately 3 volume of NaI stock solution (supplied with the kit) had been added. If TBE had been used for agarose preparation, 1/2 volume of TBE modifier (see 2.1.5.) and 4.5 volumes of NaI must be used instead of the NaI alone. When the agarose was completely melted, GLASSMILK suspension was added. At least 5 µl of GLASSMILK was needed for a sample containing 5 µg of DNA. The microfuge tube was centrifuged at 12,000g for 5 seconds to pellet the GLASSMILK/DNA complex. The supernatant was removed and the pellet was washed 3 times with NEW WASH (supplied with the kit). The DNA was eluted with 10 µl of 1×TE buffer.

2.2.2.11. Radioactive labelling of DNA.

To detect a specific sequence in a DNA fragment, a radioactively labelled probe was used. The probe was obtained from the labelling of an oligonucleotide or a DNA fragment having a complementary sequence

-End labelling.

The end labelling method was used for labelling a short synthesized oligonucleotide. Bacteriophage T4 polynucleotide kinase in the reaction catalysed the transfer of a γ -³²P of ATP to the 5' terminus of unphosphorylated oligonucleotide

(forward reaction). The reaction was set up in a total volume of 20 μl . The reaction mixture includes 1 μl of oligonucleotide (3 ng/ μl), 2 μl of 10 $\times$ polynucleotide kinase buffer, 2 μl of [γ - ^{32}P]dATP (10 $\mu\text{Ci}/\mu\text{l}$), 2 μl of bacteriophage T4 polynucleotide kinase (4 units/ μl) and 13 μl of sterile distilled water. The reaction was incubated at 37°C for 1 hour. The labelled oligonucleotide can be used without further purification.

-Random-primed labelling.

A new technique had been developed using T7 DNA polymerase enzyme to synthesize a radioactive probe complementary to a DNA template. A labelling kit (Stratagene) based on this technique was used in this study. The target DNA fragment was denatured in a microfuge tube by mixing 25 ng of target DNA and 10 μl of random oligonucleotide primers. The mixture was made up to 34 μl with sterile distilled water.

The DNA was incubated in a boiling bath for 5 mins to separate double strand target DNA. The tube was centrifuged briefly at room temperature to collect the mixture at the side of the tube. The following components were added to the denatured DNA; 10 μl of 5 $\times$ Primer buffer, 5 μl of [α - ^{32}P]dATP (10 $\mu\text{Ci}/\mu\text{l}$) and 1 μl of T7 DNA polymerase. The reaction was incubated at 37°C for 10 mins to allow the complementary strand probe to be synthesized. 2 μl of stop mix was added to end the reaction. The probe was further purified on a Nick column.

-Nick column.

The nick column is a ready prepared Sephadex G-50 column for the separation of DNA fragments from unincorporated ^{32}P -labelled nucleotides.

The column was equilibrated with 3 ml of buffer solution (see 2.1.7.2.) before the mixture of labelled DNA probe was added. The column was then washed with 400 μl of buffer solution. The purified DNA probe was eluted from the column by adding 400 μl of buffer solution into the column.

2.2.2.12. Hybridization.

The presence of a target sequence in a DNA fragment can be confirmed by the use of the hybridization technique. This technique required the transferring of DNA molecule onto a Nylon membrane (Southern blotting). The target sequence was then identified using a radioactive labelled probe. Hybridization was also used for detection of recombinant clones after transformation of recombinant plasmid (Colonies screening). Both methods are described below.

-Southern blotting.

After agarose gel electrophoresis (see 2.2.2.9.), the gel was submerged in denaturing solution (see 2.1.7.2.) for 15 mins with slow rotation in order to denature double strand DNA into single strand DNA. This step was repeated for a further 30 mins. To neutralize the pH of the gel, the denaturing solution was replaced with neutralizing solution (see 2.1.7.2.) with slow rotation for 30 mins. The neutralization step was repeated twice. The gel was placed on a bridge link between two reservoirs filled with 20×SSC (see 2.1.7.2.). A pre-wet gel size Nylon membrane (Hybond-N membrane from Amersham) was carefully placed on the top of the gel so that no air was trapped. Three sheets of pre-wet gel size blotting paper were then laid over the Nylon membrane. To create a capillary effect, many gel size absorbent papers were stacked on top of the blotting papers and the top of the absorbent paper stack was compressed by a glass plate and a 1 kg weight. The DNA transfer was continued for one night. After blotting, the membrane was washed with 2×SSC to remove adhering gel and allowed to air dry at 37°C. The transferred DNA was fixed onto the Nylon membrane by exposing the membrane, DNA side down, on a UV transilluminator for 5 mins. The membrane was then prehybridized and hybridized as described below.

-Colony screening.

Grids were drawn on a sheet of Nylon membrane which had been cut to fit an agar plate. The grid membrane was placed in a plate on a LB medium supplement

with 50 µg/ml of ampicillin. In a plate, about one hundred colonies of transformants obtained from method 2.2.2.8. were patched on each grid. The replicas of the same colonies were also maintained on a LB plate containing 50 µg/ml ampicillin for use as a master plate. Both plates were incubated up side down at 37°C overnight. The membrane was then removed and laid colony side up on a sheet of absorbent paper previously soaked in denaturing solution (see 2.1.7.2.) for 9 mins. The denatured membrane was again placed colony side up on a sheet of absorbent paper which was previously soaked with neutralizing solution (see 2.1.7.2.) for 4 mins twice. The membrane was washed with 2×SSC and air dried with the colony side up. The bacterial DNA was bound to the membrane by UV irradiation for 5 mins. To detect target sequence, the membrane was prehybridized and hybridized as the method described below.

-Hybridization.

The membrane containing denatured DNA was prehybridized in 10 ml of prehybridization solution (see 2.1.7.2.) for at least 1 hour using the Hybridization oven (Hybridizer HB-1, Techne). Radio-labelled probe (see 2.2.2.11.) was added into the prehybridization solution and the membrane was hybridized overnight. The hybridization solution was then removed and the membrane was washed and autoradiographed (see 2.2.2.14.). The condition of the hybridization and washing varied depending on the experiment.

-Removal of probe from a Nylon membrane.

The radioactive Nylon membrane was incubated in 0.4 M NaOH at 45°C for 30 mins and transferred to washing solution (see 2.1.7.2.) at 45°C for 30 mins. It was then allowed to air dry.

2.2.2.13. Erase-a-Base system for generating nested deletion constructs.

-ExonucleaseIII digestion.

Linearized plasmid was progressively digested by using Erase-a-Base system (Promega Corporation). The procedure and conditions of digestion were similar to that described in the manual supplied together with the system. In short, 5 µg of PEG-preparation plasmid DNA was linearized with a restriction enzyme. The digested plasmid was then phenol-extracted and precipitated with ethanol. The plasmid was dissolved in the 60 µl of one time exonuclease III buffer (see 2.1.5.). The mixture was incubated at 25°C for 2 mins before 500 units of the exonuclease III was added. The plasmid-exonuclease III mixture was incubated for 30 second for lag period and further 2 mins to allow the digestion to proceed near to the region of interest (the *SspI* site of the *rap-1* gene). Aliquots of 2.5 µl of the reaction mixture were taken every 20 seconds intervals and added into ice-cold 7.5 µl S1 nuclease mix (see 2.1.7.2.). The digested plasmid in the S1 nuclease mix was kept on ice until 10 samples were taken. All the ice-cold samples were then incubated at room temperature for 30 mins before the 1 µl of S1 stop buffer (see 2.1.5.) was added. The reactions were heated up to 70°C to inactivate the S1 nuclease. The samples were prewarmed at 37°C prior to 1 µl of the Klenow mix (see 2.1.7.2.) being added in all samples. The samples were incubated for another 3 minutes and 1 µl of the dNTP mix (see 2.1.7.2.) was added. After 5 minutes incubation, the reaction was completed.

-Ligation and transformation.

The linear DNA samples obtained after Klenow DNA polymerase treatment were circularized by the T4 DNA ligase. A 40 µl of ligase mix (see 2.1.7.2.) was added to each samples. The reactions were incubated at room temperature for 1 hour. Aliquots of 10 µl per samples were used in the transformation (see method 2.2.2.8.)

2.2.2.14. Polymerase chain reaction.

To amplify genomic DNA from *P. falciparum*, the following components were mixed up in a 0.5 ml microfuge tube; 10 µl of 10×Thermalase buffer, 16 µl of the mixture of dNTP (1.25 mM each), 10 µl of 5' end oligonucleotide (7.5 ng/µl), 10 µl of 3' end oligonucleotide (7.5 ng/µl), 1 µl of parasite genomic DNA (140 ng/µl), 0.5 µl of Thermalase (IBI) and 53 µl of sterile distilled water.

The surface of the aqueous phase was laid with 50 µl of light mineral oil to prevent vaporization. After the lid was pierced, the tube was fitted in thermo-block of the Thermocycler [PHC-2 Dri-Block, Techne (Cambridge) Ltd.]. The temperature of the reaction was controlled as follow:

Cycles	Denature	Anneal	Polymerization
1	5 mins 95°C	1 mins 40°C	2 mins 70°C
2	3 mins 93°C	1 mins 40°C	2 mins 72°C
3	1 mins 93°C	1 mins 40°C	2 mins 72°C
4-31	30 secs 93°C	30 secs 40°C	2 mins 72°C
32			5 mins 70°C

Ramp rate = 4

The amplified product was extracted twice with 100 µl of chloroform. The amplified DNA fragment was then purified from aqueous phase of the extracted mixture by GENECLAN II kit. (see 2.2.2.10.).

2.2.2.15. Double strand DNA sequencing.

The method of double-stranded sequencing used in this experiment was modified from the chain-terminating method (Sanger *et al*, 1977).

-DNA denaturation.

For 2 sequencing reaction, 5 µg of polyethylene glycol purified, or Qiagen purified DNA was dissolved in total volume of 20 µl TE. 5 µl of 1 N NaOH, 1 mM EDTA was added for DNA denaturation. The mixture was left at room temperature for 5 mins. The denatured DNA was purified by the spin-dialysis procedure (see below).

-Spin-dialysis.

Sepharose-6CBL was equilibrated with T0.1E (see 2.1.7.2.). A 0.5 ml microfuge was pierced through the bottom with the tip of a small syringe needle. The tube was then placed inside a 1.5 microfuge tube. About 20 µl of acid-washed glass beads, 212-300 microns(Sigma) in T0.1E, was added to the 0.5 ml microfuge tube to act as a supporter and 0.3 ml of Sepharose slurry was added at the top of the glass bead layer. The tubes were placed in a centrifuge tube and were spun at 200g with a swing-out rotor until all liquid was removed. A new 1.5 ml microfuge tube was replaced. A denatured DNA sample was carefully pipetted onto the top of the Sepharose surface. The tube was again centrifuged at the same speed. The purified denatured DNA solution obtained from the column was collected for subsequent use.

-Sequencing.

The purified single stranded DNA was used as a sequencing template. For one primer, the spin-dialysate was mixed with 8.5 µl of spin-dialysate, 1 µl of 10×TM buffer (see 2.1.7.2.) and 1 µl of primer (10 µg/ml). The mixture was incubated at 37°C for 15 mins to allow the primer to prime to the DNA template. Immediately, 2.5 µl of the primed DNA was dispensed into 4 tubes each of which contains 2 µl of one of dATP, dTTP, dGTP, dCTP mix. The tubes were incubated at 42°C and 2 µl of the following mixture was added into each tube; 1 µl of 10×TM, 67 µl of sterile distilled water, 0.5 µl of Klenow fragment (5 units/µl) and 1 µl of [α -³⁵S]dATP (10 mCi/ml). The reaction was incubated at 42°C for 10 mins and then 2 µl of chase-mix was added into each tube. The reaction was further incubated for 5 mins before 4 µl of formamide

dye mix was added to stop the reaction in each tube. The reaction was boiled for 2 mins to denature double strand DNA immediately before the sample was loaded.

-Polyacrylamide gel electrophoresis.

To prepare a polyacrylamide gel for sequencing analysis, the following components were mixed; 28.8 g of urea, 0.18 g of N, N'-methylenebisacrylamide, 3.6 g of acrylamide, 6 g of 10×TBE buffer and 28 ml of sterile distilled water. The mixture was stirred for 30 mins and filtered with No 1 Whatman paper. Adding with 360 µl of 10% ammonium persulfate and 50 µl of N, N, N', N'-tetramethylethylenediamine (TEMED), the solution was poured in between two glass plate to form a 0.5 mm thick 6% polyacrylamide gel. The gel were left for at least 1 hour to allow polymerization of the acrylamide. 2 µl of sample from each tube was loaded on 6% polyacrylamide gel and the gel was run at 40 watts in 1×TBE buffer.

-Fixing and drying of sequencing gel.

After running, the gel was submerged in fixing solution (see 2.1.7.2.) for 15-20 mins. The gel was laid on a chromatography paper and covered with a piece of Syran wrap (Dow Chemical Company). The edge of the chromatography paper was cut out to the gel-size. The gel was dried in vacuum at 80°C for at least 1 hour (Slab gel dryer SE1160, Hoefer Scientific Instruments) and subjected to autoradiography (see 2.2.2.14.).

2.2.2.16. Autoradiography.

Autoradiography of the hybridized membrane was carried out by placing the membrane on a preflashed X-Ray film(Du Pont) which was laid on an intensifying screen in an autoradiograph cassette. The film was exposed at -70°C for overnight and was developed with Automatic X-Ray film developer(X1,X-OGRAPH).

For a sequencing gel, the cling film was removed from the dry gel and the gel was exposed to a X-Ray film(Du Pont) without preflashing. The exposure was

carried out at room temperature for overnight before being developed with Automatic X-Ray film developer(X1,X-OGRAPH).

2.3. Protein techniques.

2.3.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-polyacrylamide gel electrophoresis is the protein separation method. The method used in this study was modified from the method described by Laemmli (Laemmli, 1970). Most samples used in this study were bacterial cell extracts expressing recombinant protein.

-Sample preparation.

The induced cells were pelleted in a microfuge tube by centrifugation (12,000g for 1 mins) and washed once with 1 ml ice-cold STE solution. The cells were resuspended in 0.1 ml of sterile distilled water. To obtain a SDS-PAGE sample, the cell solution was mixed with 0.1 ml of 2 times loading buffer. The mixture was boiled for 5 mins in a boiling bath. After centrifugation (12,000g for 5 mins), the sample was kept on ice until it was used or stored at -20°C.

-SDS-PAGE gel electrophoresis.

The SDS-PAGE gel apparatus used in this experiment is an SDS-PAGE gel electrophoresis Unit (SE250 Mighty Small II Slab Gel Electrophoresis Unit, Hoefer Scientific Instruments). The gel casting method used in this experiment was followed according to the manufacture instruction. Ten 0.75 thick gels were cast at a time to ensure homogeneity of the gels. The gels were made up by using discontinuous buffer system which is composed of stacking gel and resolving gel. The stacking gel forms wells for sample loading and improves the gel resolution. The resolving gel is responsible for protein band separation. For this study, the resolving gel was cast as a 7.5% polyacrylamide gel. The components of these gel are as follow:

	resolving gel	stacking gel
30% acrylamide	15 ml	4.02 ml
Tris·Cl (pH 8.8)	15 ml	-
Tris·Cl (pH 6.8)	-	7.5 ml
10% SDS	600 μ l	300 μ l
Sterile distilled water	30 ml	18 ml

Prior to pouring of the gel, ammonium persulfate and N, N, N', N'-tetramethylethylenediamine (TEMED) were added as the following:

	resolving gel	stacking gel
10% Ammonium persulfate	300 μ l	150 μ l
TEMED	30 μ l	12 μ l

The acrylamide solution for the resolving gel was poured into a casting chamber. The gels were left for polymerization at least 1 hour before the stacking gel was poured. Plastic combs were inserted into the stacking gel to form sample wells and the gels were polymerized for 1 hour. Two gels were assembled to the upper buffer chamber pod. Tank buffer (see 2.1.7.2.) was filled into the upper buffer and lower buffer chambers. 10 μ l of the boiled samples were loaded into wells and the gels were run at 40 mA for about 1 hour or until the dye front reached about 0.5 cm from the end of the gel. During electrophoresis the gels were cooled with tap water. The gels were removed from the gel plate and either stained using coomassie blue or used for western blot analysis.

-Staining and drying of a polyacrylamide gel.

After electrophoresis, the protein bands can be stained with coomassie blue dye. The gel was submerged into stain solution (see 2.1.7.2.) and incubated at 37°C for

at least 3 hours or until the gel was dark blue. The background dye can be washed off by incubating the gel in destaining solution at 37°C. The destaining process was repeated several times until the background dye was removed. Only 1 µg of protein in a band can be detected by this method.

The gel can be stored dry at room temperature for unlimited period. The drying method similar to that described in section 2.2.2.13.

-Protein molecular weight markers.

Protein markers for molecular weight determination used in this study were Prestained SDS molecular weight markers (Sigma) and RainbowTM protein molecular weight markers (Amersham).

2.3.2. Antigen detection.

Several methods were developed for the presence of an antigen which can be detected by the use of related antibody. In this study, two methods of antigen detection were used in different situations. Western blotting was a technique for the detection an antigen in electrophorized samples. Another technique was used for identification of a clone which expresses the antigen of interest for colony screening purposes.

2.3.2.1. Western blotting.

-Blotting procedure.

To allow localization an antigen which was run on SDS-PAGE gel, the electrophorized sample must be electrically transferred onto a solid support so that the sample can be easily manipulated. The solid support used in this study was Hybond-C (Amersham).

A western blotting apparatus(Trans-Blot™ CELL, Bio-Rad) was used for transferring electrophorized samples. For efficient transferring, all filter papers and Hybond-C were cut into gel-size and were pre-wetted with transfer buffer before use. A pre-wet sponge pad was laid flat on one side of a transferred cassette. Three sheets of filter paper were placed on the sponge pad. A SDS-PAGE gel was put on the filter paper and a Hybond-C membrane was carefully laid on the gel. Another three sheets of filter paper were put on the top of the Hybond-C membrane. Each time the filter papers, gel and Hybond-C membrane were laid, precaution must be taken to avoid trapping air bubbles. Up to 4 SDS-PAGE mini-gels can be fitted in one cassette. Another pre-wet sponge pad was placed on the top before two sides of the cassette were then assembled. The cassette was submerged in transfer tank containing transfer buffer. The gel was put at the cathode side and the Hybond-C membrane at the anode side of the transfer apparatus. The transferring was operated at 30 volts overnight with tap water cooling. After the transfer, the Hybond-C membrane was ready for antigen detection. The completion of the transfer can be checked by the transfer of the prestained or rainbow markers which were loaded into the same gel.

-Antigen detection.

The blotted membrane was immediately submerged in blocking buffer for 1 hour at room temperature on a Rocking Table (LUCKHAM). The blocking buffer helps to prevent non-specific binding of antibody to the Hybond-C. The antibody raised against the antigen of interest was added. The final dilution of the antibody depends on the origin of the antibody (see table below).

Sources of antibody	Final dilution
Mouse ascites fluid	1:1000
Mouse cell culture supernatant	1:2
Rabbit serum	1:1000
Human serum	1:2

The membrane was incubated in antibody solution for 1 hour at the same condition as described above. The membrane was then washed with 10 ml of washing solution as follow:

Washing solution	Time lengths
Tris-saline	10 mins
Tris-saline with 0.05% NP40	2×10 mins
Tris-saline	10 mins

The washing solution was removed before appropriate secondary antibody conjugated with alkaline phosphatase (1/7500) was added and incubated for 1 hour. The membrane was again washed as above. The antigen band can be visualized by submerging the membrane in alkaline phosphatase buffer containing 330 µg/ml of NBT and 165 µg/ml of BCIP as substrate of alkaline phosphatase. The membrane was washed with distilled water several times to prevent developing of the background. The membrane was then air-dried before it was stored.

2.3.2.2. Colony screening.

In order to facilitate screening of expression clones containing ExonucleaseIII digested plasmid a colony screening method was used. Bacterial

colonies obtained from transformation after Exonuclease III digestion were streaked by sterile toothpicks on a grid Hybond-C membrane which was laid on LB broth agar supplement with 50 µg/ml ampicillin. The plate was incubated upside down at 37°C overnight. The membrane was then removed and laid, colony side up, on another LB plate containing 50 µg/ml ampicillin which had been previously spread with 100 µl of 8 mg/ml IPTG (isopropylthio-β-D-galactoside). The plate was incubated for 2 hours at 37°C upside down. To lyse the bacterial colonies on the membrane, a Whatman 3MM paper was soaked with 1% SDS and placed in a lid covered plastic box. The box was incubated in 70°C incubator at least 20 mins. The membrane containing bacteria was laid on the soaked 3MM paper. The lid was tightly closed and the box was immediately returned to 70°C. The membrane was incubated at 70°C for 30 mins. The membrane was then washed several times with Tris-saline and the antigen of interest was detected as described in western blot procedure (see 2.3.2.1).

2.3.3. Pepscan kit.

The peptide synthesis in this study was performed using Pepscan kit purchased from Chembridge Research Chemicals. The method used based on method described by Geysen and his colleagues (Geysen *et al*, 1987). The synthesis procedure composes of two steps, Fmoc-group deblocking and addition of Fmoc-amino acid active esters to pins which are assemble to a plastic block. After the synthesis, the synthesized peptides were then acetylated to remove the unnatural charge at the N-terminus. Finally, protecting groups were removed. All the reactions can be easily operated in polypropylene sandwich boxes. Because of the hazardous nature of the chemicals used for peptide synthesis, the reaction must be done in fume hood. The peptides which contain epitopes of antibody of interest were detected by an ELISA based technique. To reprobe the synthesized peptide, bound antibody can be removed by sonication described below.

-Fmoc-group deblocking and washing.

The Fmoc-group can be easily removed by piperidine in DMF. Pins assembled to a plastic block were submerged at half their height into 20% (v/v) piperidine in DMF for 30 mins at room temperature. The block was removed and shaken off to remove the excess liquid. The pins were washed in solution as follow:

Solution	Times × treated times (mins)
DMF	1 × 5
methanol	4 × 2

The block was air-dried for at least 10 mins and again washed with fresh DMF for 5 mins. The block was removed and the excess liquid was blotted with clean tissue.

-Addition of Fmoc-amino acid active esters.

The synthesis schedule was created by a software (DOS version) supplied with the kit. Fmoc-amino acid active esters and 1-hydroxybenzotriazole were dissolved in DMF according to the schedule. 100 µl of each solution was carefully dispensed in each wells of reaction tray supplied with the kit. The deblocked pins were submerged into wells of the reaction tray as designed by the schedule. The pins were incubated in the amino acid ester solution for 18 hours at 30°C in a black plastic box. After the synthesis was completed, the block of pins was removed and washed with:

Solutions	Times × treated times (mins)
DMF	1 × 2
Methanol	4 × 2
DMF	1 × 2

The Fmoc-group of coupled amino acid was then deblocked, washing as described before. The coupling procedure was again repeated until the target size peptides were accomplished. After the final deblocking and washing, the pins were processed as below.

-Acetylation of terminal amino groups.

100 μ l of acetylation solution (see 2.1.7.2.) was pipetted in to each well of fresh reaction tray. The synthesized pins were lowered into the wells. The pins were incubated for 90 mins at room temperature in a plastic bag. The pins were then washed with:

Solutions	Times $\times$ treated times (mins)
DMF	1 $\times$ 2
Methanol	4 $\times$ 2

The pins were allowed to air-dry at least 10 mins before the next step was operated.

-Deprotection and neutralization.

The side chains of synthesized peptides were removed by deprotection and neutralization step. The pins were incubated in cleavage solution (2.1.7.2.) for 4 hours at room temperature. The pins were then finally washed as follow:

Solutions and treatment	Times × treated times (mins)
dichloromethane	2 × 2
5% diisopropylethylamine in dichloromethane	2 × 5
dichloromethane	1 × 5
air-dry	at least 10 mins
water	1 × 2
methanol	1 × 18 (hours)

The block of pins then was dried in vacuum in the presence of silica gel for 18 hours and could be stored in this condition. The pins can be used for epitope mapping after disruption process described below.

-Pin disruption.

Disruption buffer (see 2.1.7.2.) was prewarmed to 60°C and poured into a sonication bath. The block of pins was submerged into the disruption buffer. The sonication was operated for 30 mins. The block was removed and submerged into prewarmed water (60°C). This step was repeated 3 times with fresh prewarmed water. The block was finally immersed in boiling methanol for 2 mins and then air dried. The pins then ready to be reprobed with an antibody or stored for further used.

-epitope detection.

A 200 µl of supercocktail blocking solution (see 2.1.7.2.) was dispensed into each well of a U-bottom MicrotestIII™ Assay Plate (Becton Dickinson Labware). The pins were submerged into supercocktail solution in order to block non-specific binding of antibody to the pins. The pins were incubated for 1 hour at room temperature. After the blocking process was completed, the pins were incubated in a microtitre plate which contained 175 µl of supercocktail diluted antibody in each

well using the dilutions described (see 2.3.2.1.). The plate was left at 4°C overnight. To remove non-specific binding antibody, pins were washed 4 times in PBS/Tween 20 at room temperature for 10 mins each with agitation. The antibody bound peptides were detected by appropriate second antibody conjugated with horseradish peroxidase (1/2000) in the same condition as first antibody. Again, the pins were thoroughly washed as above. The epitope of first antibody was revealed after each pins were incubated in 150 µl of substrate solution in a sterile flat-bottom multi-well plate (Flow Laboratories, Inc.) at room temperature in the dark with agitation for approximately 40 mins or until sufficient color was developed. The block of pins was then removed and the color product was monitored by a plate reader at 410 nm. The pins were finally cleaned by sonication and stored for re-use.

Chapter 3

Expression of RAP-1 recombinant proteins

3.1. Introduction.

3.1.1. Expressed protein as studied material.

One technical problem for the study of *P. falciparum* protein is the availability of the purified protein which was difficult to prepare. The purified protein also tends to be contaminated by other components. To avoid such a problem, the epitope mapping of the inhibitory monoclonal antibodies in this study was achieved by using fusion proteins between the RAP-1 protein and β -galactosidase. The fusion protein was generated by recombinant techniques.

The development of recombinant DNA techniques allows the subcloning of a defined DNA fragment from an organism into a prokaryotic cell, *Escherichia coli*, by the use of plasmids or bacteriophages. Hence, a gene or a part of a gene can be subcloned in frame with the host bacterial gene, for example *lacZ* gene, in a plasmid or bacteriophage and expressed as a hybrid product under the control of the host gene operating system. Large amounts of a fusion protein can be produced by induction of the host bacterial gene. Many foreign proteins have been successfully expressed in *E. coli* e.g., the expressed capsid proteins of foot and mouth disease virus which can elicit neutralizing antibodies (Kleid *et al*, 1981).

This introduction will be divided into two parts, the first part will outline the expression system used in this experiment and the second part will describe monoclonal antibodies used in this study and their inhibitory effect on parasite growth *in vitro*.

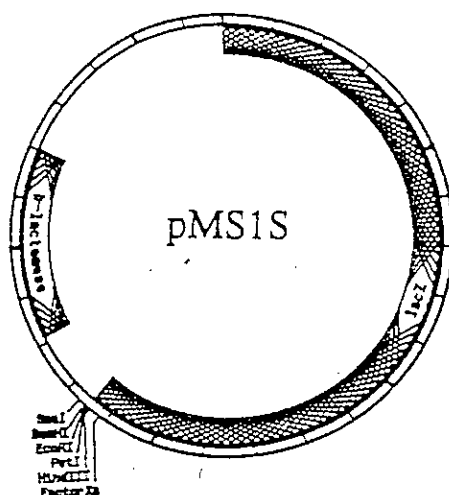
3.1.2. Expression system used in this experiment.

Several plasmids have been engineered for expression work in *E. coli* cell utilizing the *lac* operon. Among those are the pUR series plasmids (Rüther and Müller-Hill, 1983) and pMS series plasmids (Scherf *et al*, 1990). Both sets of plasmids contain an ampicillin resistance gene as shown in Fig. 3.1. Thus, the *E. coli* clone which possesses one of these plasmids may be selected and maintained on medium supplemented with ampicillin. Each plasmid series was designed to allow a foreign DNA fragment to be subcloned into an appropriate restriction site at the C-terminus of the β -galactosidase encoded gene (*lacZ* gene) in three different reading frames. The insert fragment in frame with the *lacZ* gene will be translated as a β -galactosidase fusion protein. The advantage of pMS plasmids over the pUR plasmids is that the β -galactosidase fusion protein from this plasmid can be cleaved with a protease, blood coagulation factor Xa, at a specific site between the β -galactosidase protein and the insert encoded protein. This allows the insert encoded protein to be separated and purified from the fusion protein.

The fusion proteins from both plasmids are under the control of the promoter and operator of the β -galactosidase gene. To suppress the expression of the fusion protein in normal conditions, the recombinant plasmid is transformed into a specific *E. coli* strain, such as NM522 (Gough and Murray, 1983). This strain of *E. coli* possesses an episome which overexpresses a repressor of the *lac* operon from a mutant repressor gene, the *lacI^q* gene as shown in Fig. 3.2a.. The expression of the fusion protein can be induced by adding an inducer, isopropyl β -D-thiogalactoside, into the medium in order to inhibit the activity of the repressor (Fig. 3.2b).

Because plasmid is considered to be a metabolic burden to the bacteria cell, a cell which loses its plasmid will grow rapidly and finally become the dominant population in the culture. The stability of the plasmids and the recombinant plasmids is increased by maintaining the transformants on a medium supplemented with ampicillin

A)



B)

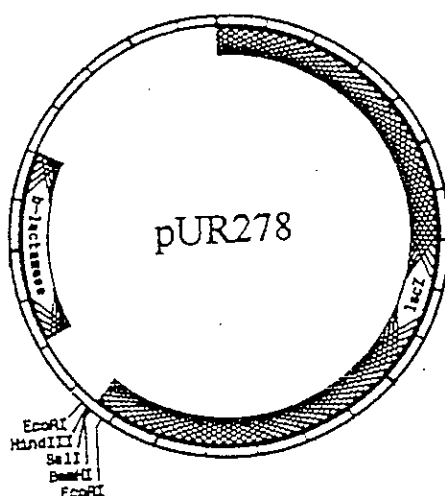


Figure 3.1. The schematic drawing of the pMS (A) and pUR (B) series plasmids. The restriction sites used in this experiment are also shown (not to scale). An insert fragment can be subcloned into the restriction sites in the appropriate frame by using a suitable plasmid in each series. However, the pMS series plasmids can only be subcloned into three different frames at the *EcoRI* site.

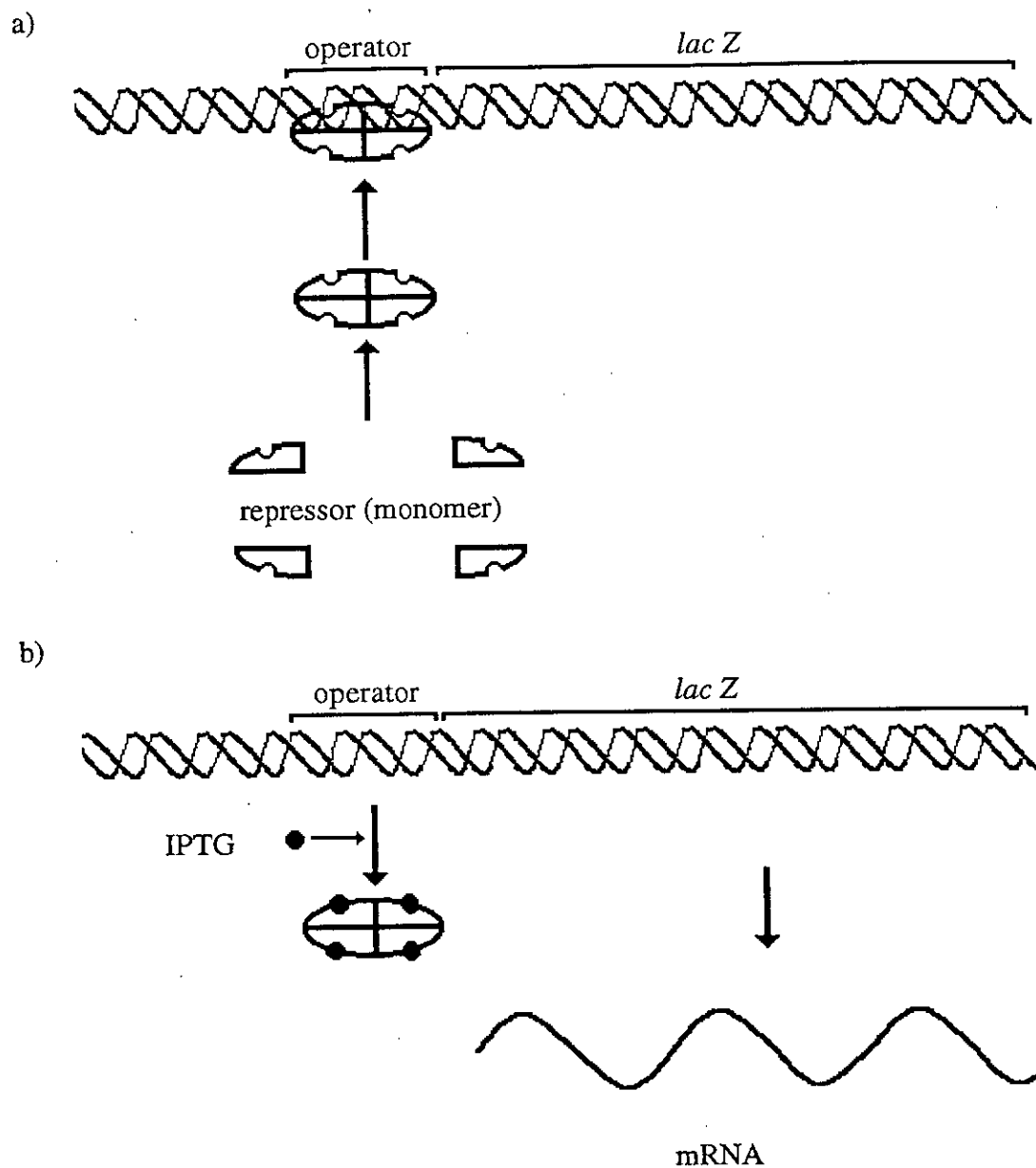


Figure. 3.2. The Schematic diagrams show the induction phenomenon of the *lacZ* gene. At normal circumstance (a), a repressor gene on an episome produces repressor monomers which formed into tetramer molecules. A tetramer repressor is then able to bind to operator region of the *lacZ* gene. As a result, the *lacZ* gene is suppressed. In the presence of IPTG (b), the repressor is inactivated and dissociated from the operator so the *lacZ* gene can be transcribed to mRNA.

to inhibit the growth of those cells which lose their plasmids. The same principle also applies to the episome in a host cell such as *E. coli* NM522. The episome in this strain carries a *pro AB* gene, a gene needed for the synthesis of proline, which is absent from the genomic DNA of the *E. coli* NM522. This strain of *E. coli* needs to be maintained in minimal medium which lacks the amino acid in order to stabilize the episome in the cell.

It should be noted that expression of foreign proteins in *E. coli* is not always successful. In highly expressed prokaryotic genes, the codon usage exhibits a preference for the abundant tRNA species in the cell (Scaife, 1988). Expression of a foreign gene which contains a different codon bias may result in early termination of translation. This particular problem should be especially considered in the expression of *P. falciparum* proteins in *E. coli* because the parasite has a different codon usage from those in *E. coli*. The AT-rich nature of the *P. falciparum* gene may also cause difficulties in transcription process.

Other problems related to the expression of foreign proteins in *E. coli* are degradation of expression protein, the lack of a post-translational modification system and non-native protein folding. Nevertheless fragments of several *P. falciparum* genes have been successfully subcloned and expressed in *E. coli* as non-native forms, for example the CS protein (Dame *et al*, 1984), blood stage antigens (Kemp, *et al*, 1983), the MSP-1 protein (Hall *et al*, 1984) and Ag5.1 protein (Hope *et al*, 1985).

3.1.3. Analysis of inhibitory monoclonal antibodies which recognized the RAP-1 protein.

Previous works on production of monoclonal antibodies against whole *P. falciparum* cells had identified seven monoclonal antibodies which can immunoprecipitate the RAP complex from total cell parasite extracts (Hall *et al*, 1983, Clark *et al*, 1987 and Ridley *et al*, 1990a). Five of these monoclonal antibodies, 2.13,

2.15, 2.29, 7.12, 8.1, were shown to recognize nonconformational or continuous epitopes on the RAP-1 protein while other monoclonal antibodies, 11.4 and 2.22 were thought to recognized conformational or discontinuous epitopes in the RAP protein complex.

In vitro inhibition assay data of these monoclonal antibodies were produced by Dr. Jana McBride (McBride, personal comm.). The IgGs of mAbs 2.15, 2.22, 2.29, 7.12 and 11.4 were purified and their inhibition effects were evaluated. The analysis of these data showed that all of these monoclonal antibodies have a similar level of inhibition on the parasite growth *in vitro* as shown in Fig. 3.3. This result raises several questions about the immunogenicity and the function of the RAP-1 protein; (i) what is the inhibitory mechanism? (ii) where are the epitopes of these monoclonal antibodies in the RAP-1 molecule? (iii) Do these monoclonal antibodies bind to the regions of functional significance in RAP-1? It is obvious that most of these questions may be answered after the epitopes of these inhibitory monoclonal antibodies have been mapped.

This chapter describes an attempt to express fragments of *rap-1* gene which encode for the epitopes of inhibitory monoclonal antibodies as a fusion protein forms in *E. coli* for a subsequent study of the epitope mapping. The monoclonal antibodies investigated in this study are listed in Table 3.1.

3.2. Results.

In order to express fragments of the *rap-1* gene which contain the epitopes of inhibitory monoclonal antibodies in *E. coli*, the *EcoRI-SalI* fragment *rap-1* gene was initially subcloned from the λ rap1.1 (Ridley *et al*, 1990a, see Fig. 3.4.) into the pUR278 and pMS1S. This λ gt11 clone contains a middle part of the *rap-1* gene flanked by an *EcoRI* linker on both ends. The *rap-1* fragment was inserted in frame with the β -galactosidase gene. The fusion protein of this *rap-1* gene fragment was

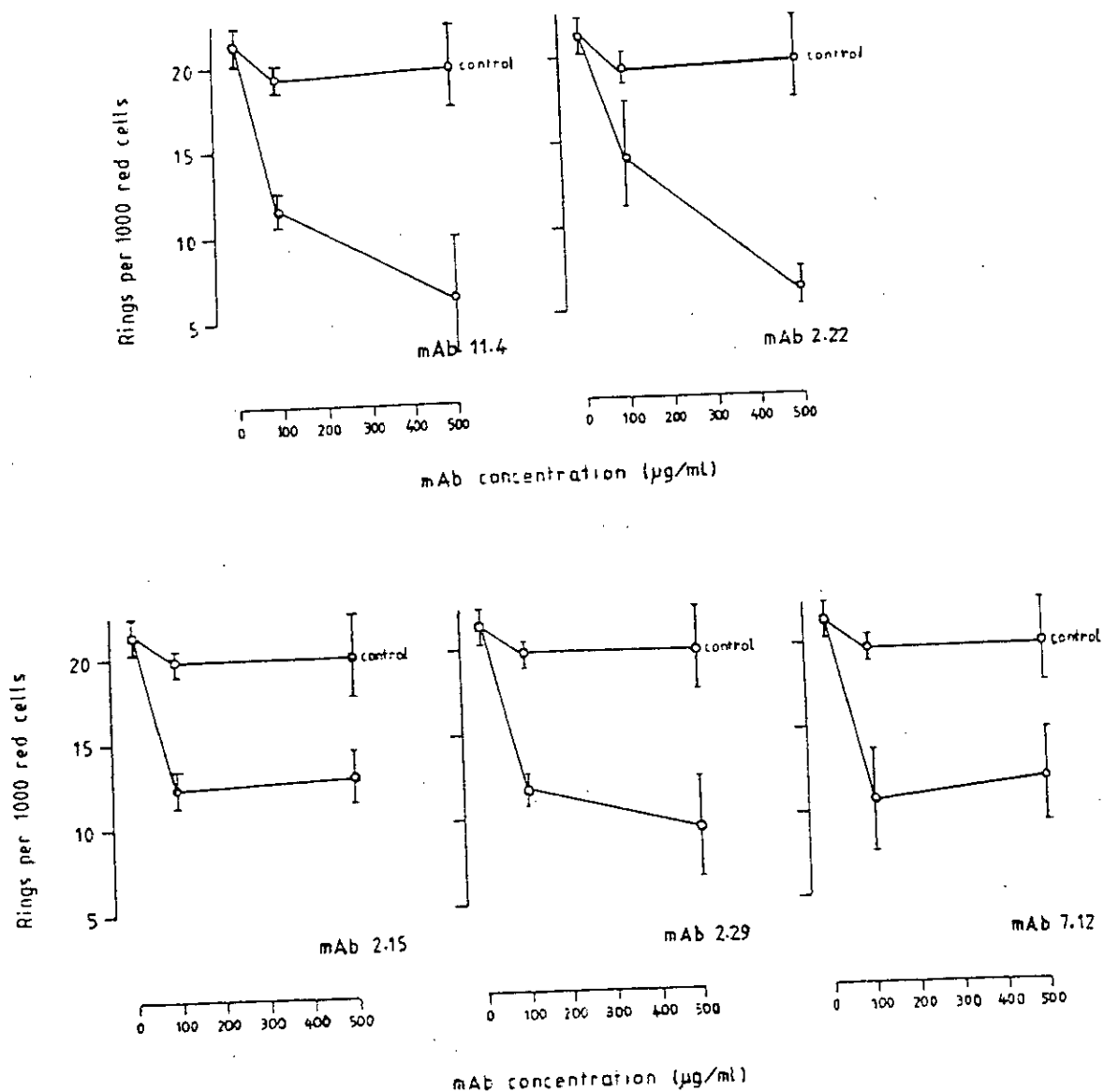


Figure. 3.3. Graphs show the *in vitro* growth inhibitory effects of the monoclonal antibodies against the RAP-1 protein (Harnyuttanakorn *et al*, 1992). The graph shows similar degree of *P. falciparum* growth inhibition.

Antibodies	Isotype	IFA titre	Inhibition of parasite growth <i>in vitro</i>	Immunoblot-reactive parasite antigens (kDa)
mAb 2.13	IgG ₁	10 ³	+++	82/65*
mAb 2.15	IgG ₁	10 ⁴	+	82/65
mAb 2.29	IgG ₁	10 ⁵	+	82/65
mAb 7.12	IgG ₁	10 ⁴	+	82/65
mAb 8.1	IgM	10 ²	-**	82/65
mAb 11.4	IgG ₁	10 ⁵	+	-
RpAb anti-β-galactosidase	nd	nd	nd	nd
RpAb anti-purified RAP-1 protein*	nd	10 ⁵	nd	82/65

Table. 3.1. Table shows the antibodies and monoclonal antibodies used in this thesis. The data and the monoclonal antibodies were kindly provided by Dr. Jana McBride (McBride, personal comm.). nd = not determined, - = negative result, * data from Ridley *et al*, 1990, ** data from Hope, 1984.

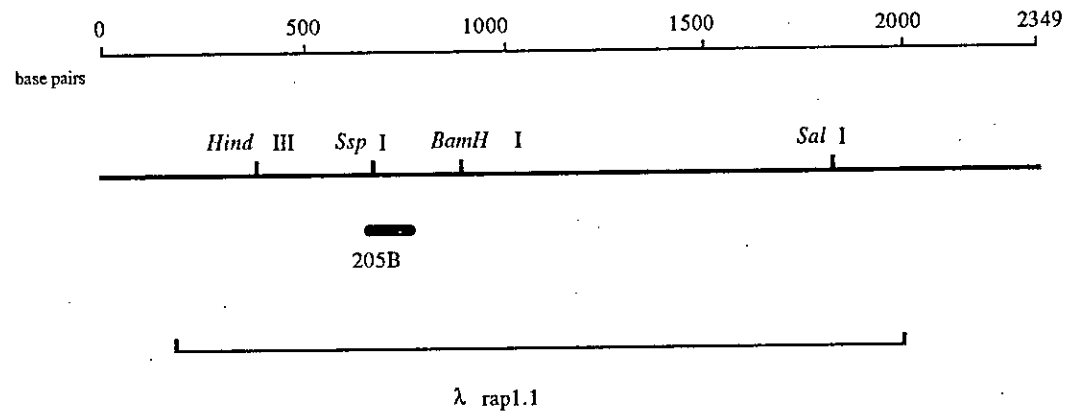


Figure 3.4. The diagram showed the fragment of *rap-1* gene which was subcloned into the λ gt11. Some unique restriction sites in the *rap-1* gene are also shown. The oligonucleotide B205 used in the screening procedure is shown as a thick line (not to scale).

known to be recognized by mAbs 2.13 and 7.12 (Ridley *et al*, 1990a). By expressing different fragments of the *rap-1* gene generated by restriction enzymes present in the *rap-1* gene sequence, the epitopes then can be mapped relatively to the restriction sites of the *rap-1* gene.

3.2.1. Subcloning and expression of the *rap-1* insert in *E. coli*.

DNA from the bacteriophage clone λ rap1.1 and pUR278 plasmid were purified as described in the method sections 2.2.2.1. and 2.2.2.2. respectively. The *EcoRI-SalI* fragment of the *rap-1* gene from the λ rap1.1 was subcloned into the *EcoRI-SalI* restriction site of the pUR278 plasmid at the C-terminus of the β -galactosidase gene in the correct frame. The *EcoRI-SalI* fragment encodes for 546 amino acid residues from amino acid 62 to 608 of the RAP-1 protein. The subcloning strategy is shown in the Fig. 3.5. The purified recombinant lambda DNA and the pUR278 plasmid were digested with *SalI* and partial digested with *EcoRI* (see method 2.2.2.4.). The digested lambda DNA and plasmid were ligated using the enzyme T4 DNA ligase as described in method section 2.2.2.7. The ligated DNA was then transformed into competent cells of the *E. coli* NM522 (see method 2.2.2.8.). Bacterial colonies were screened with radiolabelled internal oligonucleotide B205, as shown in Fig. 3.4., (see method 2.2.2.12). A transformant which contained an *EcoRI-SalI* fragment of the *rap-1* gene was detected. To ensure the purity of the clone obtained, the positive clone was purified by re-streaking to give a single colony (see method 2.2.1.1.) and rescreened by using the same method. The double screening method was also used for subsequent subcloning procedures.

Eight positive clones from the second screening were obtained. The recombinant clones were tested for the ability to produce the β -galactosidase-RAP-1 fusion protein. The total cell extracts of uninduced and induced sample from the recombinant clones were subjected to SDS-PAGE gel electrophoresis (see method

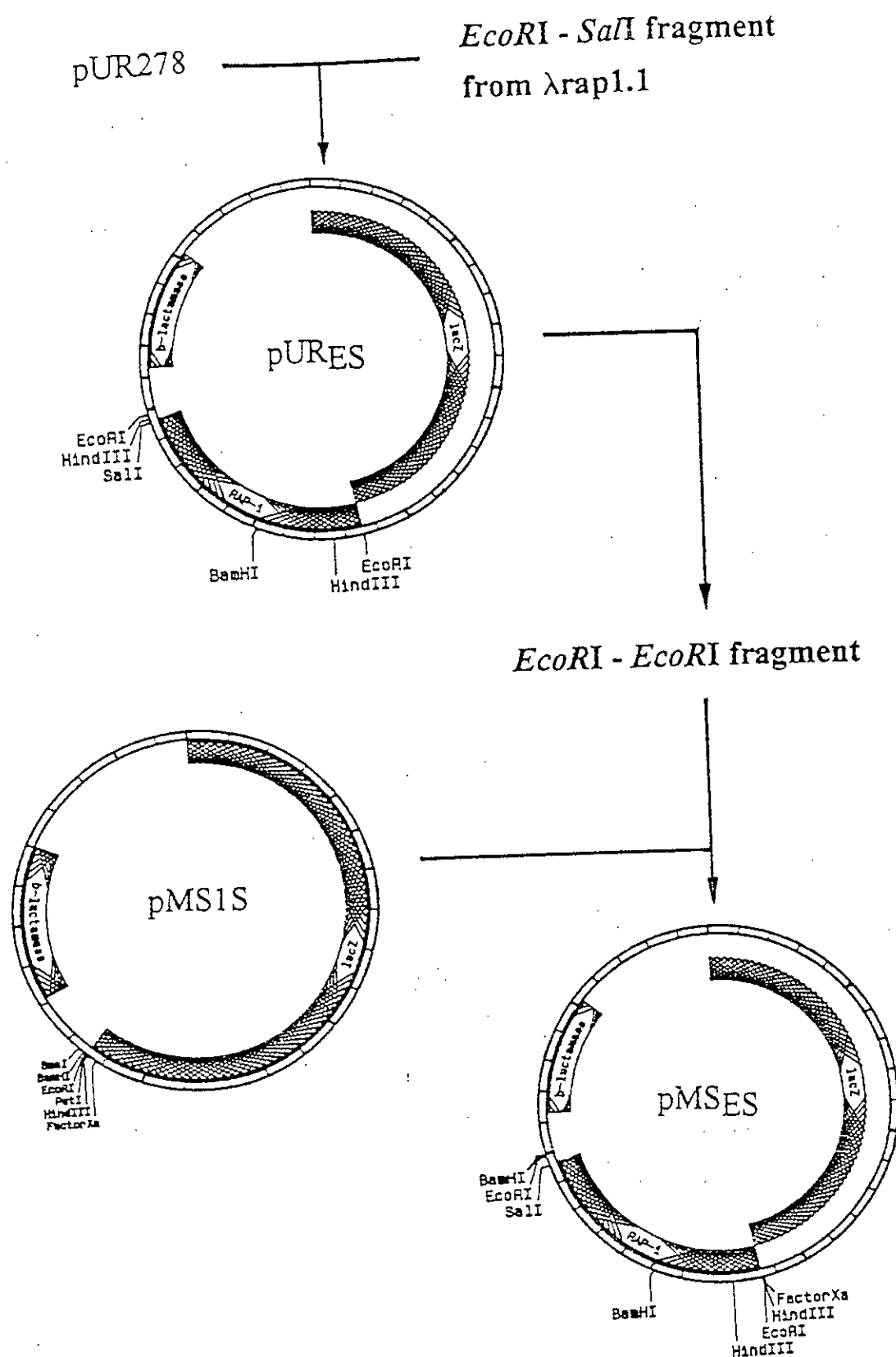


Figure. 3.5. Diagram shows the subcloning strategies used for generating the pMSES and pURES from expression plasmid pMS1S and pUR278 respectively. The pUR278 plasmid was digested with *SalI* and partial digested with *EcoRI* before the subcloning.

2.2.1.4. and 2.3.1.). The result is shown in Fig. 3.6. Only one clone, UR_{ES}, expresses a few high molecular weight protein bands which were strongly recognized by the rabbit polyclonal antibodies (RpAb) against the RAP-1 protein (Fig. 3.7.). Some low molecular weight proteins were also detected as minor components. The molecular weight of the highest expression protein band is about 180 kDa which corresponds to the size of the fusion protein expected. The transformant extract in lane 4 also gave positive bands in similar pattern to the UR_{ES} extract but with noticeably weaker signal. Other total parasite extracts in lane 2, 6 and 7 were weakly recognized by the anti-RAP-1 RpAb. However, detected protein bands in these samples have lower molecular weight than the size of the fusion protein expected. It is still unclear how these proteins were generated.

The fusion protein from the UR_{ES} clone was also recognized by the inhibitory monoclonal antibody 2.13 and RpAb against β -galactosidase protein (Fig. 3.8.) as three major protein bands all of which are bigger than the size of the β -galactosidase protein (116 kDa marker). For subsequent studies, only the UR_{ES} clone was used.

To investigate whether the three major bands were the result of degradation or premature termination, UR_{ES} cells were collected at different time intervals after induction. The total cell extracts were run on the SDS-PAGE gel and were analyzed by western blot. The results are shown in Fig. 3.9. Three major bands were detected in all samples after 30 minutes of induction with some minor bands after 120 mins of induction. There was no evidence of time dependent protein degradation. This suggested that those three major bands may be generated by early termination of the transcription or translation process (see discussion below).

The success of the subcloning process was also confirmed by double-strand sequencing (see method 2.2.2.15). The flanking regions of the *rap-1* gene insert of the pUR_{ES} were sequenced. The sequences showed that the *rap-1* insert had been cloned in frame with the *lacZ* gene and the restriction site *SaII* at the 3' end is also maintained.

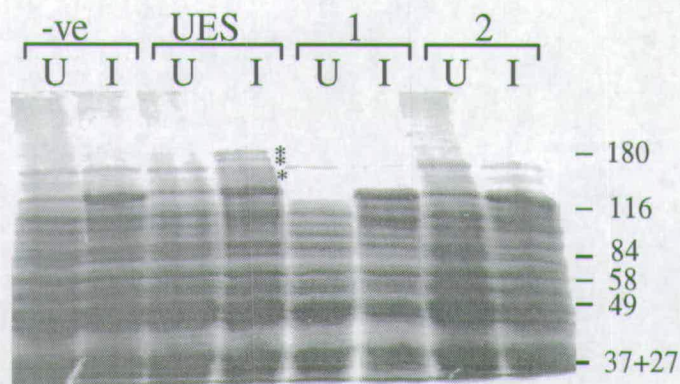


Figure 3.6. The eight total cell extracts of the transformants which positive with the radiolabelled nucleotide probe were run on the SDS-PAGE gel and were stained with coomassie blue (only three clones were shown here, UES, 1 and 2). Only the UES clone showed extra bands (star dots) in the induced sample (I) which were not present in uninduced sample (U). The negative control (-ve) are the total extracts of *E. coli* NM522 which carries pUR278 plasmid. The molecular weight markers are shown in KDa.

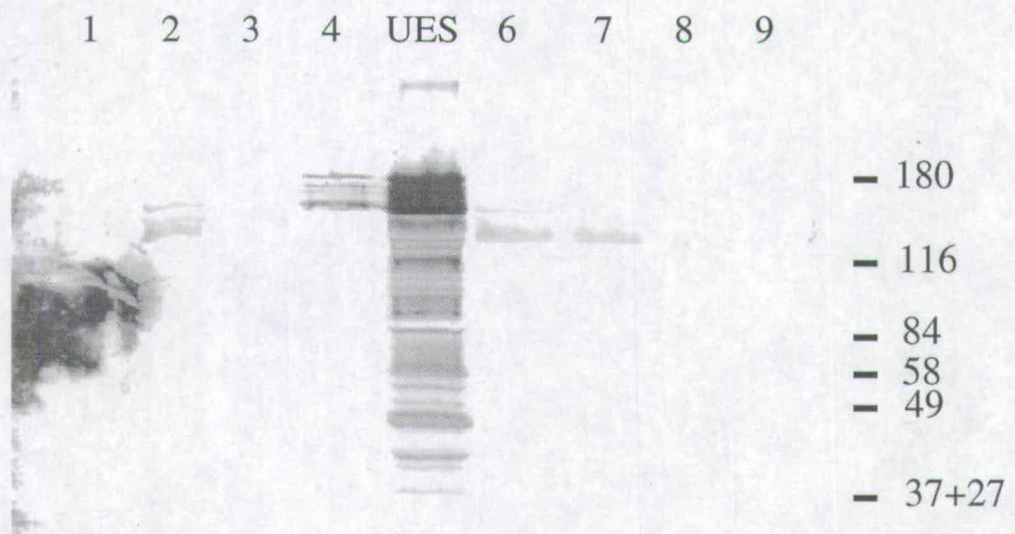


Figure 3.7. The total cell extracts of the eight transformants from induced culture were examined for fusion proteins by western blot analysis (lane 1-8). Lane 9 was a negative control. The anti-RAP-1 RpAb was used as a probe. The UES clone (lane 5) produces the fusion protein which were strongly recognized by the anti-RAP-1 RpAb used.

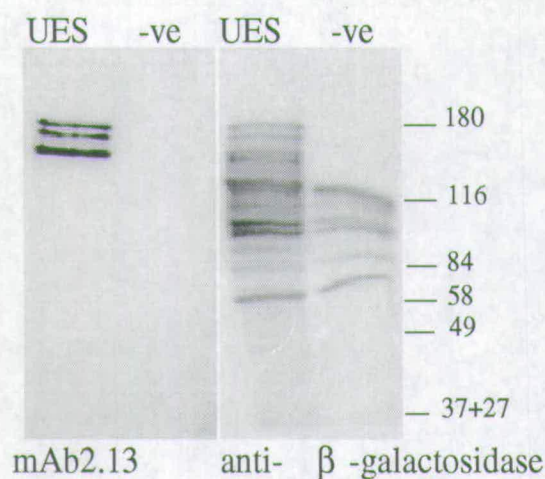


Figure 3.8. The Western blot of the UES clone total cell extract was probed with mAb2.13 and anti- β -galactosidase RpAb. For comparison, the total cell extract of *E. coli* NM522 which contains pUR278 also blot onto the same membrane (-ve).

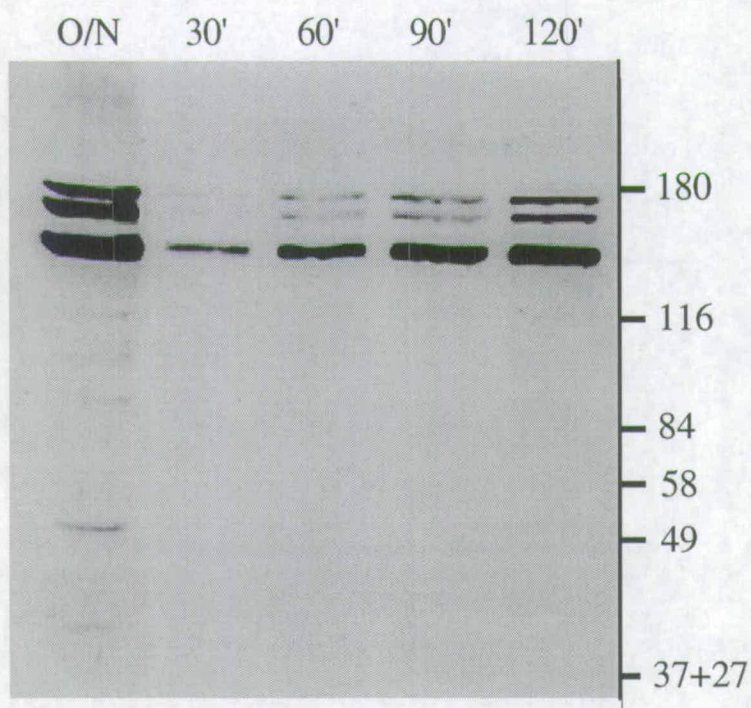


Figure 3.9. The western blot of the total cell extract from MES clone after 30, 60, 90 and 120 mins induction compared with overnight induction. The degradative products were detected after 120 mins of induction. The total cell extract of UES clone also give the same result.

3.2.2. Subcloning and expression of the other restriction fragments from the *rap-1* gene.

To facilitate the subsequent subcloning, pMS1S as well as the pUR278 were used. The *rap-1* insert from the pURES was cut with *EcoRI* and was subcloned into pMS1S at the 3' end of the *lacZ* gene. After the subcloning, the *rap-1* insert will stay in frame with the *lacZ* gene without further manipulation. The *BamHI* and *HindIII* restriction site on the pMS1S plasmid were used for further subcloning. The subcloning strategy is described below.

The fragment of the *rap-1* gene in the recombinant plasmid from URES, pURES, was cut with *EcoRI* and purified. The purified fragment was subcloned into the *EcoRI* restriction site of a pMS plasmid at the C-terminus of the β -galactosidase gene. The recombinant plasmid was transformed into *E. coli* NM522 and was screened with the same radiolabelled internal oligonucleotide as the one used in the screening of URES clone. Seventeen positive clones were obtained and analyzed by western blot (data not shown).

Detection of the fusion protein by western blot analysis using mAb 2.13 revealed that only seven clones produced fusion proteins. Again, the fusion protein was detected as three major bands at a higher molecular weight than wild type β -galactosidase, similar to that shown in Fig. 3.8. The highest band of the fusion protein migrated as 180 kDa protein. The presence of the 3 major bands at different time point after induction was also similar to that described in Fig. 3.9. (data not shown).

An expressing clone, MS_{ES}, was selected for further study and subcloning. The sequences at the flanking regions of the insert were also determined. The result confirmed that the insert was in frame with the *lacZ* gene.

Other constructs also obtained by using the available restriction sites on the plasmid vectors and the *rap-1* insert as shown in Fig. 3.10, 3.11 and 3.12. A pMS_{EB}

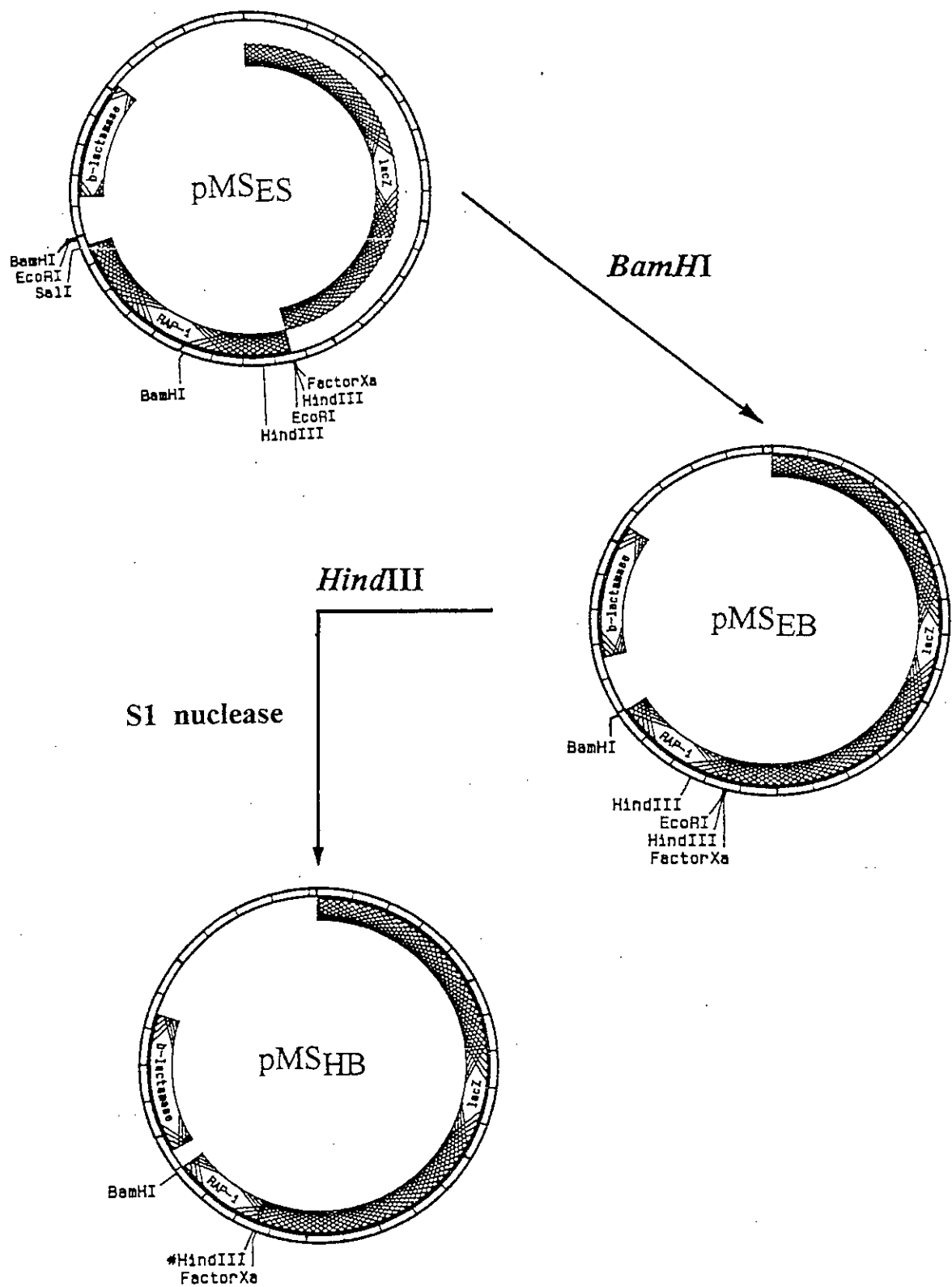


Figure. 3.10. Diagram shows the subcloning strategies used for generating the pMSEB and pMSHB from the recombinant plasmid pMSES.

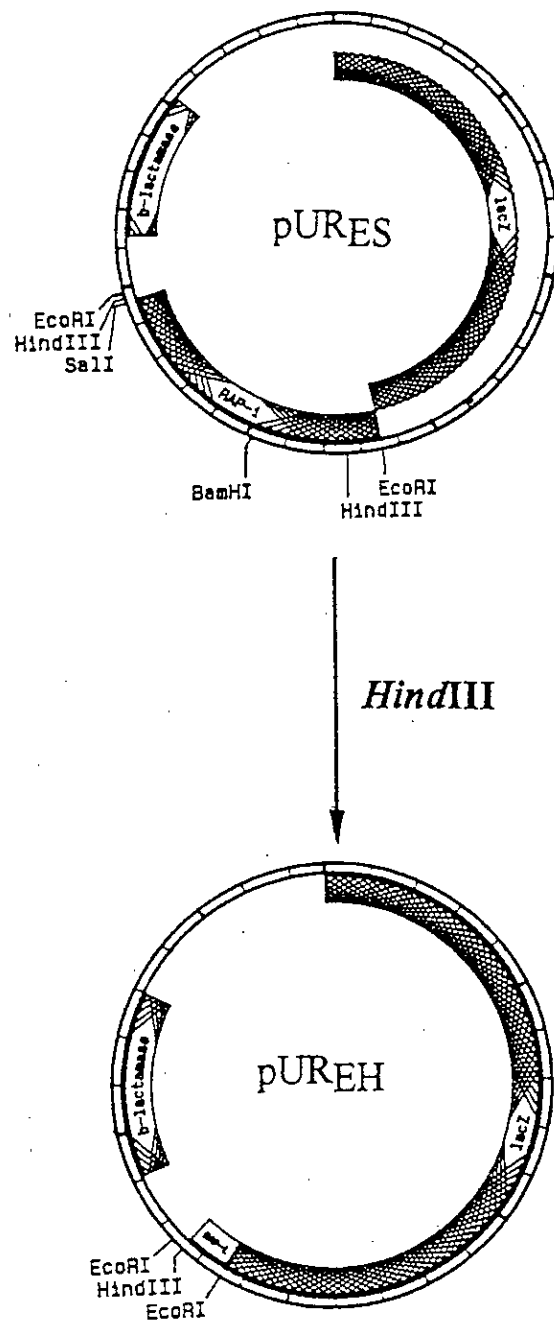


Figure. 3.11. Diagram shows the subcloning strategies used for generating the pURE_H from the recombinant plasmid pURE_S.

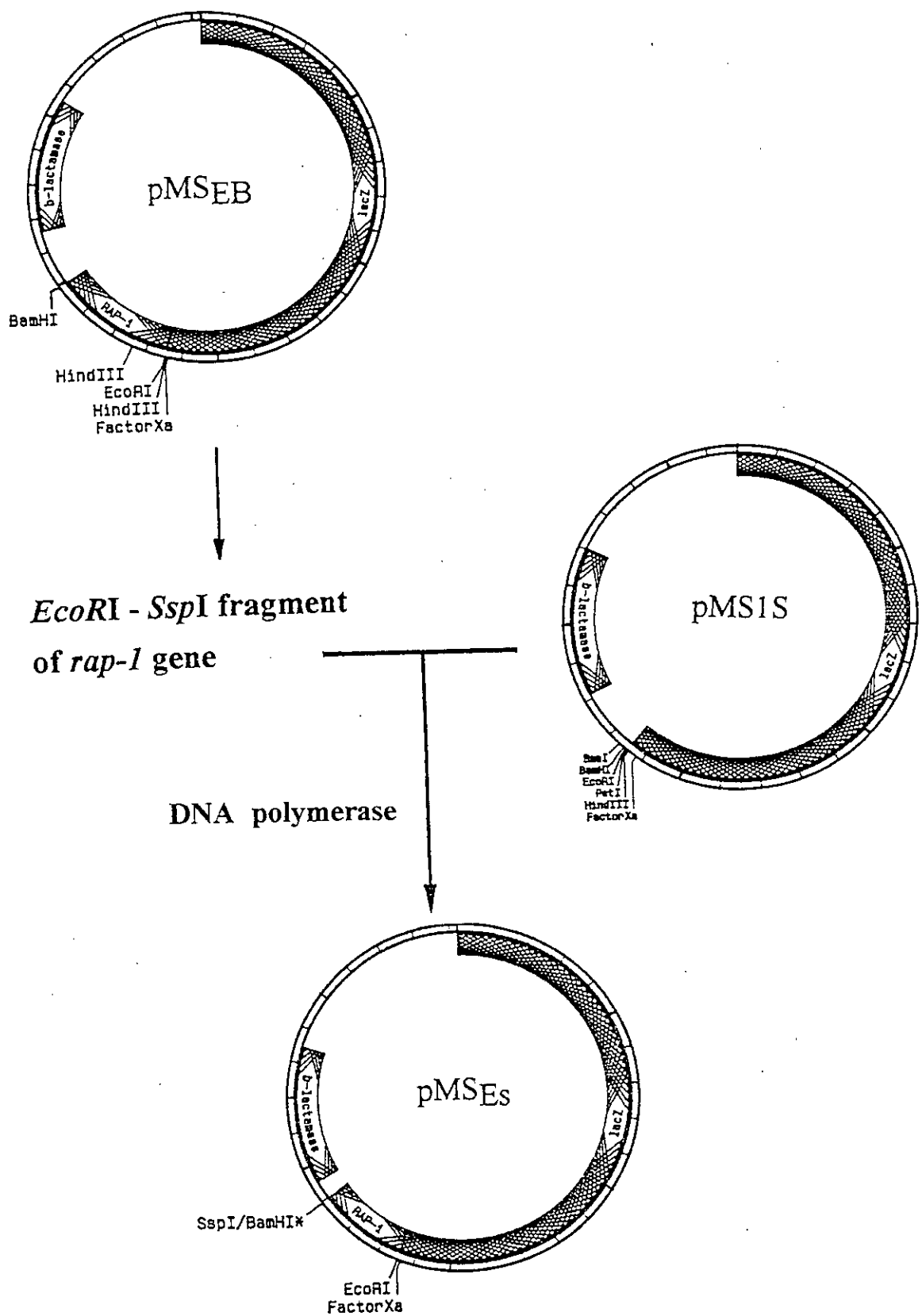


Figure. 3.12. Diagram shows the subcloning strategies used for generating the pMSEs using the purified *Eco*RI-*Ssp*I fragment from recombinant plasmid pMSEB.

construct was generated by digesting the pMS_{ES} with the *Bam*HI restriction enzyme (Fig. 3.10). The bigger DNA fragment product, which is composed of the pMS1S vector and *Eco*RI-*Bam*HI fragment of the *rap-1* gene, was purified and religated. The recombinant plasmid was transformed into the *E. coli* NM522. Using similar strategy pMS_{HB} (contains the *Hind*III-*Bam*HI fragment of the *rap-1* insert) and pUR_{EH} (contains the *Eco*RI-*Hind*III fragment of the *rap-1* insert) were generated from pMS_{EB} and pUR_{ES} respectively (Fig. 3.10 and 3.11 respectively). The 5' overhang generated by *Hind*III digestion of the pMS_{EB} was digested with S1 nuclease to correct the translational frame before religation to produce pMS_{HB}.

Another construct, pMS_{ES} (contains the *Eco*RI-*Ssp*I fragment of the *rap-1* insert), was generated by different strategy (Fig. 3.12). The pMS_{EB} was digested with *Eco*RI and *Bam*HI restriction enzyme. The *Eco*RI-*Bam*HI fragment of the *rap-1* insert was purified (see method 2.2.2.10.) and digested with *Ssp*I. The bigger fragment, which contains the *Eco*RI-*Ssp*I fragment, was purified and ligated to the pMS1S digested with *Eco*RI and *Bam*HI. The uncomplementary end between *Ssp*I and *Bam*HI was blunt ended by the use of DNA polymerase (see method 2.2.2.5). The linear DNA was then circularized by a second ligation reaction.

All the recombinant plasmids were screened using an internal oligonucleotide as described above. Western blot analysis of the fusion proteins showed a major fusion protein band at expected sizes for all of the constructs. These proteins were recognized by both RpAb against the RAP-1 protein and RpAb against β -galactosidase, except the fusion protein from UR_{EH} which is not recognized by RpAb against the RAP-1 protein (data not shown). The plasmids were also subjected to sequencing (see method 2.2.2.15) at the flanking regions between insert fragments and the vectors to confirm the accuracy of the subcloning (data not shown).

Fusion proteins generated by the recombinant clones, which contain different part of the *rap-1* gene, were then used for epitope mapping of inhibitory monoclonal antibodies.

3.2.3. The epitope mapping of the inhibitory monoclonal antibodies using the fusion proteins.

The successful expression of the several restriction fragments of the *rap-1* inserts allowed the epitopes of the inhibitory monoclonal antibodies to be mapped. Only fusion proteins which contain the epitope of a monoclonal antibody will be recognized by the monoclonal antibody tested. By comparing the sequences of the *rap-1* inserts in the positive clones, the epitope can be mapped correspondingly to the restriction site.

The total cell extracts of induced culture of all the constructs was run on SDS-PAGE gels and the proteins were transferred on to nitrocellulose membranes. Western blots of the fusion proteins were probed with mAbs 2.13, 2.15, 2.29, 7.12, 8.1 and 11.4. All the monoclonal antibodies gave identical results. A western blot is shown in Fig. 3.13. The results showed that the epitopes of all monoclonal antibodies were encoded by the *Hind*III-*Ssp*I fragment of the *rap-1* gene.

3.3. Discussion.

Restriction fragments of the *rap-1* gene have been successfully expressed in fusion protein forms. The fusion proteins are under the control of the *lacZ* promoter and operator. This expression system was proved to be very effective by western blot analysis as the fusion proteins were detected only after the induction with IPTG.

The reason for the appearance of the fusion protein as the three major bands as detected by mAb 2.13 is still unclear. In *E. coli* cell, a fusion protein may be digested by a scavenger protease (random degradation process) or a specific protease (cleavage at a specified amino acid sequence). The early co-appearance of the three major bands and the exhibition of degradation process giving rise to smaller products

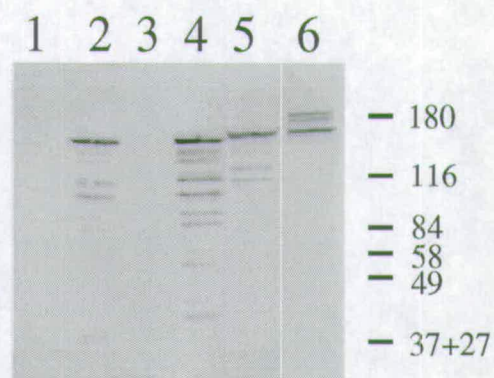


Figure. 3.13. Epitope mapping of the inhibitory monoclonal antibodies using fusion protein generated from different fragments of restriction enzymes digested *rap-1* gene. All the monoclonal antibodies gave the same result. Here, only a western blot of the total cell extract of the recombinant clones is shown. The blot was probed with mAb 2.29. 1) MS, 2) MS_{HB}, 3) UR_{EH}, 4) MS_{ES}, 5) MS_{EB}, 6) MS_{ES}.

after 120 mins of induction suggested that a random degradation process is unlikely to be the cause of triplet pattern.

Though the induction effect on fusion protein expression is different from the fusion protein which was shown to be specifically cleaved by membrane protease OmpT (Hellebust *et al*, 1989), the specific cleavage of the RAP-1 fusion protein cannot be ruled out. All the fusion proteins may be quickly cleaved by specific protease into lower molecular weight peptides at the beginning of the induction. As more of the fusion proteins were produced, some of the full length fusion proteins can be detected because the specific protease which only generated in a limit amount in a bacterial cell cannot digest all of the fusion protein. Other possible explanation is that they may be caused by the early transcription or translation process which causes shortening of mRNA and the fusion protein.

The previous work suggested that mAb 2.13, 2.15, 2.29, 7.12 and 8.1 recognized linear epitope protein on the RAP-1 (Clark *et al*, 1987 and Ridley *et al*, 1990a). The results from this experiment lead to the same conclusion. However, the mAb 11.4 which was previously reported to recognize a non-linear epitope (Clark *et al*, 1987) was able to detect β -galactosidase-RAP-1 fusion protein on the western blot. It should be noted that the SDS extracted RAP-1 antigen (2% SDS) was immunoprecipitated by the mAb 11.4 in the same report but not by another mAb, mAb 2.22, which recognized a non-linear epitope of the RAP-1 protein. This suggested that the epitope of mAb 11.4 may be partly formed by the primary structure of the RAP-1 protein.

The western blot analysis of the fusion protein from different constructs suggested that the epitope of all monoclonal antibodies tested locate between *Hind*III and *Ssp*I restriction sites in the *rap-1* gene as shown in Fig. 3.14. These mAbs were reported to recognize the 80 kDa protein of the RAP-1 protein and its processing product, 65 kDa polypeptide (Clark *et al*, 1987 and Ridley *et al*, 1990a). The N-terminus of the a 65 kDa polypeptide was recently sequenced and the cleavage site

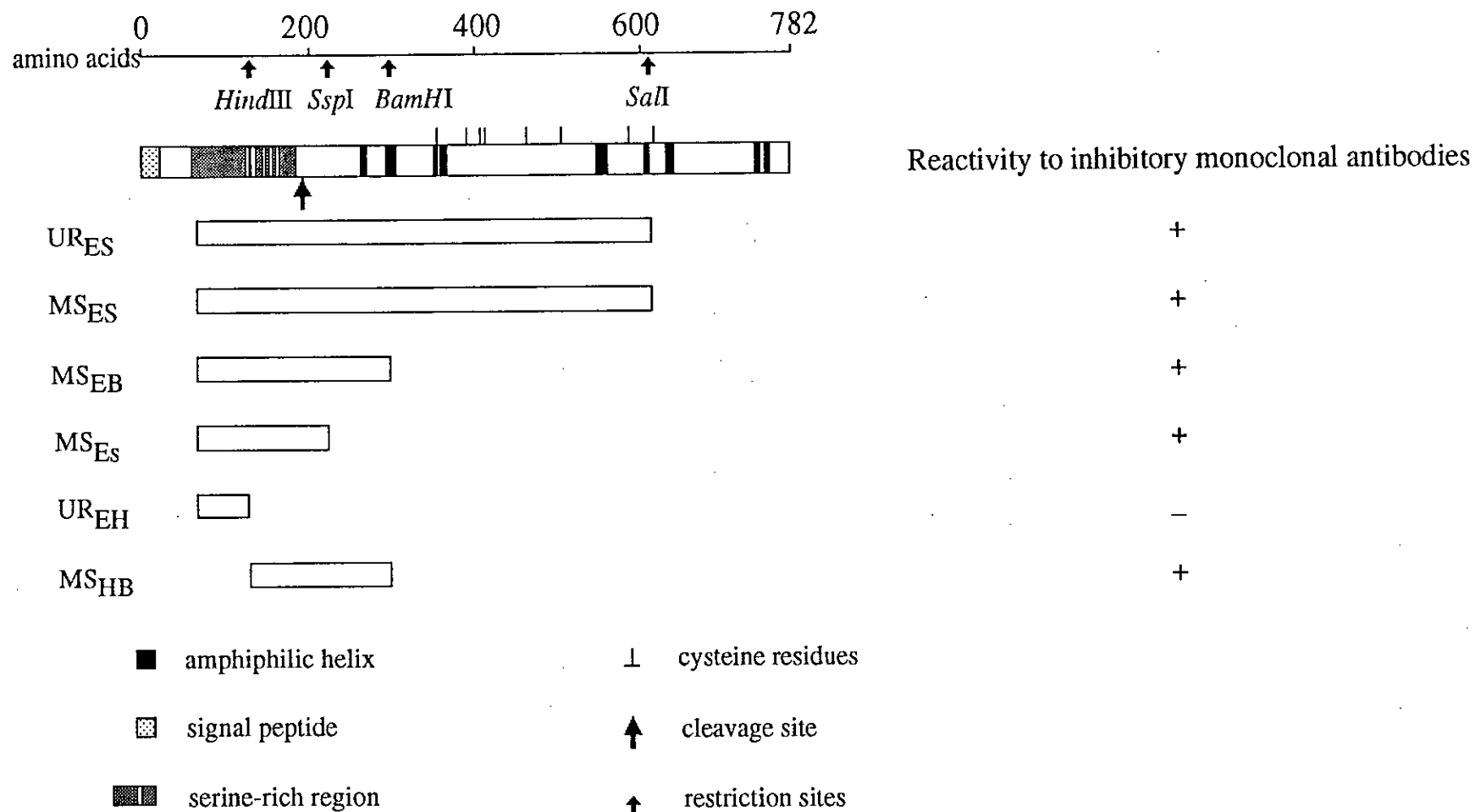


Figure. 3.14. The summary of the epitope mapping using fusion protein from different constructs which had been generated by restriction enzymes. The diagram shows the *rap-1* gene fragment in each clone compared with the restriction site and schematic representation of the RAP-1 protein.

was located between A₁₉₀ and D₁₉₁ amino acid residues (Ridley *et al*, 1991). These data indicated that the epitopes of all mAb tested are probably located between amino acid residue D₁₉₁ and K₂₁₉.

To confirm and further localize the epitopes of these inhibitory monoclonal antibodies, exonuclease III was used to produced nested deletion of the recombinant protein. This work is described in Chapter 4.

Chapter 4

Epitope mapping of the RAP-1 protein using exonucleaseIII

4.1. Introduction.

4.1.1. The importance of an inhibitory epitope.

An antibody-antigen binding reaction involves the recognition of a small region on a given antigen by amino acid residues in the antigenic binding site of the antibody molecules. The antibody-binding region of an antigen is called an epitope. The epitope may be composed of continuous amino acid residues on the primary structure of a protein or amino acid residues which are brought together as the result of protein folding. The former epitope is called a continuous epitope, the latter, is called a discontinuous epitope.

Binding of an antibody, such as an inhibitory monoclonal antibody, to an epitope on a protein may have a significant effect on the protein structure and/or the protein function. Hence, it is believed that the determination of such an epitopes on a protein (epitope mapping) may contribute to an understanding of both the immunological and functional nature of the protein studied.

4.1.2. Epitope mapping techniques.

In the past few years, several approaches have been developed to identify the epitopes which are recognized by monoclonal antibodies. X-ray crystallographic techniques have provided the most detailed information. Theoretically, this technique can identify both continuous and discontinuous epitopes. However, the process is very expensive and time-consuming. Up until now, only a few antigen-antibody complex had been studied by this method (Padlan *et al*, 1989 and Amit *et al*, 1986).

Continuous epitopes are the major interest for epitope mapping studies because they are more easy to identify than discontinuous epitopes. Several groups of researchers have applied a variety of approaches for continuous epitope mapping. For example, non-defined epitope mapping of some monoclonal antibodies against the MSP-1 protein of the *P. falciparum* was carried out by the determination of naturally processing and chemical cleavage sites of this protein (Cooper *et al*, 1992). The more accurate mapping of eptiopes may be performed by identification of fragments following limited proteolysis of the antigen-antibody complex using NMR (Suckau *et al*, 1990). In the past few years, several groups of scientists have mapped epitopes by the method described by Geysen and his colleagues in which overlapped peptides are synthesized on solid supports (Geysen *et al*, 1987, Neurath *et al*, 1990 and Ohlin *et al*, 1989). Alternatively, site-direct mutagenesis has been used (Smith *et al*, 1991 and Smith and Benjamin 1991). Recently, a new technique has been developed for epitope mapping by the use of filamentous phage expression library of random oligonucleotides (Stephen and Lane, 1992).

Recombinant proteins can also be used for the preliminary mapping of epitopes because they provide abundant material for study and are easy to manipulate. Small fragments of gene can be subcloned and expressed in a bacterial cell. Expressed proteins which are recognized by monoclonal antibody should carry the epitope of the monoclonal antibody probe. By comparing the sequences of expressed proteins, it is possible to map the epitope into a given region. There are several methods used to generate small fragment for this purpose such as the use of restriction enzymes (Gangloff *et al*, 1992), DNaseI treatment (Ware *et al*, 1988) or sonication (Piétu *et al*, 1992).

4.1.3. ExonucleaseIII.

Among the enzymes used in molecular biology, exonucleases also have a prospect for generating different lengths of DNA for epitope mapping, especially exonuclease III. Exonuclease III is an exonuclease which can specifically digest DNA in a 3' to 5' direction from 5' protruding or blunt ends. A 3' protruding end is resistant to digestion by this enzyme. The rate of digestion can be controlled by adjusting the reaction temperature between 4°C (25 bp/min) to 45°C (600 bp/min). This allows the progressive deletion of a DNA fragment by removing small aliquots at time intervals from a reaction. The single strand overhang of the digested DNA is blunt-ended after treatment with S1 nuclease and Klenow fragment of DNA polymeraseI.

In this chapter, the inhibitory monoclonal antibody epitopes were mapped using progressively deleted recombinant proteins generated by exonuclease III digestion of a *rap-1* gene insert in a recombinant plamid.

4.2. Results.

4.2.1. ExonucleaseIII digestion.

The plasmid, pMS_{EB}, from an expression clone which expresses the epitopes of all inhibitory monoclonal antibodies (see Chapter 3) is subjected to the exonuclease III digestion. The strategy is outlined in Fig. 4.1. To generate progressively deleted recombinant plasmid, the pMS_{EB} was purified by the use of PEG-preparation method (see method 2.2.2.2.). The plasmid was digested with the *Bam*HI restriction enzyme and precipitated with ethanol (see method 2.2.2.4. and 2.2.2.2.). The plasmid was then digested with exonuclease III (see method 2.2.2.13) at 25°C. The nested deletion plasmids were blunt-ended by S1 nuclease and Klenow treatment. They were circularized with T4 DNA ligase and transformed into the *E. coli*

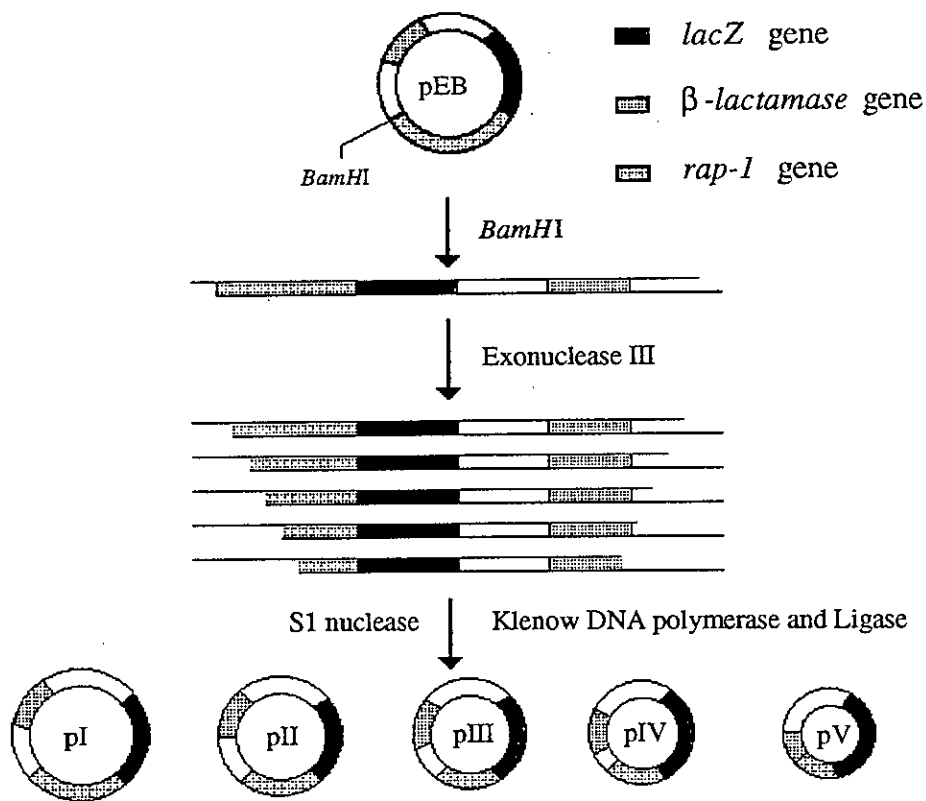


Figure. 4.1 Schematic diagram shows the strategy of exonuclease III digestion in this study. The plasmid pEB was cut at the C-terminus of the inserted *rap-1* gene fragment with *Bam*HI. The linearized plasmid was then digested with exonuclease III. The stepwise deletions of the inserted fragment were collected at time intervals during the digestion. The plasmids were circularized with T4 DNA ligase after the S1 nuclease and Klenow DNA polymerase treatment. The deleted plasmids were transformed into *E. coli* NM522 and cultured on agar supplemented with ampicillin.

NM522. One hundred colonies from each time point of the first five time point for appropriate constructs were handed-picked and screened.

4.2.2. Screening of expressed clones.

The screening strategies were based on the colony hybridization (method 2.2.2.12.) and antibody colony screening by mAb 2.13 (see method 2.3.2.2). As the results of the previous chapter showed that the epitopes of the inhibitory monoclonal antibodies studied were located between the *Hind*III and *Ssp*I site, three oligonucleotides were used as probes, namely 999A, 284B and 205B (Fig. 4.2.). The 999A oligonucleotide contains a part of the *rap-1* gene sequence between the cleavage site and the *Hind*III restriction site. The 284B sequence is located between the cleavage site and the *Ssp*I restriction site and the oligonucleotide 205B has a nucleotide sequence identical to the *rap-1* gene just downstream from the *Ssp*I restriction site. Clones which contain the 999A and 284B sequences but gave negative signals to the 205B oligonucleotide were selected. The positive clone distribution at different time points were shown in Table 4.1. The gradually decrease of positive colonies, as the digestion times were extended, suggested that the exonucleaseIII progressively digested the *rap-1* gene fragment.

To screen for fusion protein expressed by bacteria clones, the selected colonies were screened with mAb 2.13. Selected colonies were purified and handed-picked onto grid-nitrocellulose membrane which were placed on the L-broth agar. After induction by placing the nitrocellulose on LB agar containing IPTG, the cells were lysed. Expression proteins on the nitrocellulose were detected using the mAb 2.13 and alkaline phosphatase conjugated second antibody. After incubation with alkaline phosphatase substrate, the expressed protein recognized by the mAb was seen as a colony-shaped dark-purple mark on the grid-nitrocellulose (Fig. 4.3.). Antibody

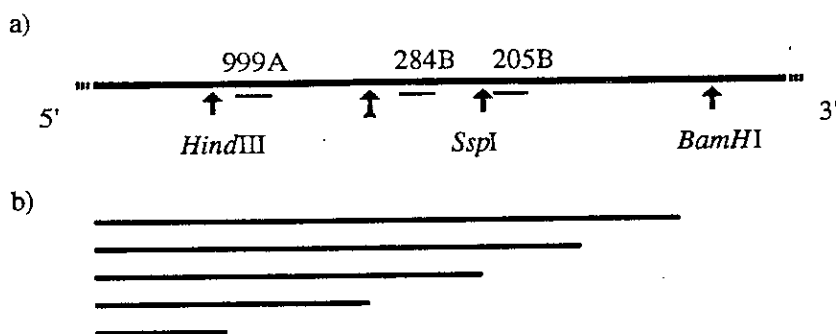


Figure. 4.2. Schematic diagrams shows a) the location of all nucleotide probes used in this study compared with the *HindIII*, *SspI* and *BamHI* restriction sites on the *rap-1* gene. The cleavage site of the RAP-1 protein is also shown as a tailed arrow. The digestion of the *rap-1* gene by exonuclease III was result to progressively deletion as shown in b).

Time points	999A	284B	205B
1	54	23	3
2	54	14	2
3	42	2	1
4	36	0	0
5	29	0	0

Table. 4.1. Table shows the positive clones selected from 100 colonies by oligonucleotide probes at each time point of exonuclease III digestion. All the 205B-positive clones gave positive result when probed with 284B and 999A probes. The 284B-positive clones also gave positive result with 999A.

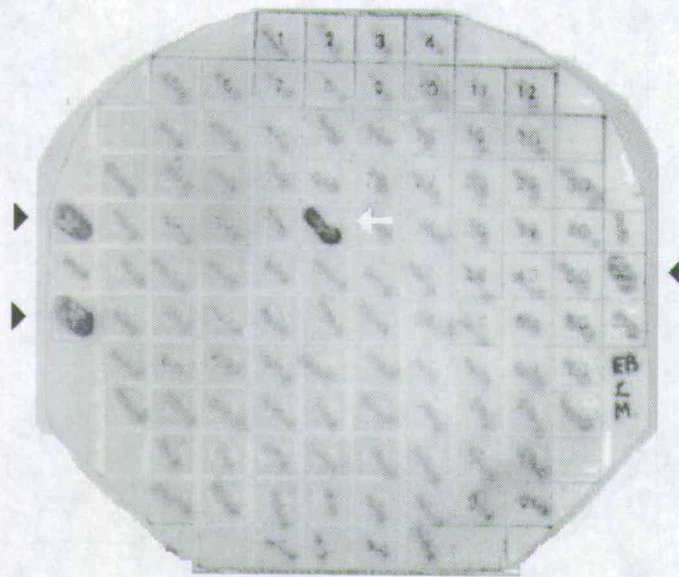


Figure 4.3. Exonuclease III deleted clones were screened with mAb 2.13 for recombinant proteins which contain mAb 2.13 epitope. This picture shows a transformant (white arrow) which give a positive result in antibody colony screening. The MES clone, described in Chapter 3, also included on the membrane as a positive control (arrow heads).

colony screening results showed that only a small number 284B-positive clones, 5 clones from the first time point and 1 clone from the second time point (Table 4.2A), expressed recombinant proteins which are recognized by the mAb 2.13. The rest of the 284B-positive clones gave negative results. The results suggested that the epitope of mAb 2.13 is located downstream of the 284B oligonucleotide sequence and the epitopes were identified by sequences comparison of the *rap-1* gene fragment in these clones.

4.2.3. Epitope mapping of the inhibitory monoclonal antibodies.

To define the epitope of the mAb 2.13, three mAb 2.13-positive clones and seven mAb 2.13-negative clones were sequenced by double strand sequencing method using dideoxy-termination technique. The C-terminal amino acid sequence of the *rap-1* inserted fragments were compared with their reactivity to mAb 2.13 and the results are shown in Table 4.2B. The comparison of the amino acid sequence revealed that the epitope of mAb 2.13 is diminished in clone 17 of which the ELYPT₁₂₁ peptide is deleted from the recombinant protein.

In order to confirm the epitope mapping results of mAb 2.13 and to characterize the epitope of other inhibitory monoclonal antibodies, fusion proteins from exonucleaseIII generated constructs were tested with rabbit anti-RAP-1 polyclonal sera and other monoclonal antibodies. The total extracts from seven clones, namely 3, 5, 6, 17, 31, 10 and 8 all of which carried different length of *rap-1* gene were subjected to western blot analysis (Fig. 4.4.). All recombinant proteins from the exonuclease III deletion clones are recognized by the rabbit anti-RAP-1 polyclonal serum in the western blot analysis (Fig. 4.4A.). The analysis also showed that all of the inhibitory monoclonal antibodies fail to recognize the fusion protein from clone 17 but not in clone 6 (Fig. 4.4B. and 4.5.). The summary of the western blot analysis is shown in Fig. 4.6.

A)

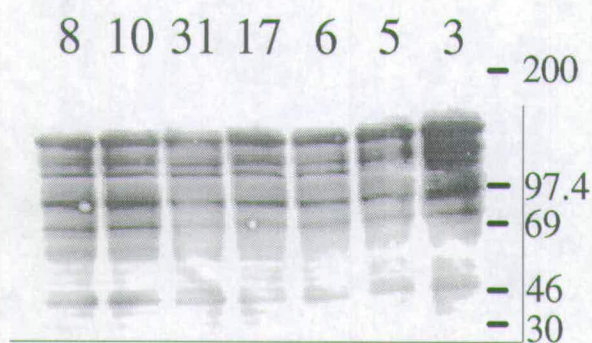
Time point	Clones positive with mAb 2.13	Clones negative with mAb 2.13
1	1, 2, 3, 4, 5	7-21
2	6	22-32
3	-	33

B)

RAP-1	↓ --GADEEAPPAPKNTLTPLEELYPTNVNLFNYKYSLNNMEE--	
Clones	C-terminus sequences	mAb2.13
3	--GADEEAPPAPKNTLTPLEELYPTNVNLFNYKYS	+
5	--GADEEAPPAPKNTLTPLEELYPTNVNLFNYKY	+
6	--GADEEAPPAPKNTLTPLEELYPT	+
17	--GADEEAPPAPKNTLTPLE	-
9, 31	--GADEEAPPAPKNTLT	-
10	--GADEEAPPAPKNTL	-
8, 19, 25	--GADEEAPPAPKN	-

Table. 4.2. Thirty-three clones were obtained as a result of deletion in the *HindIII*-*SspI* region of the *rap-1* gene insert which gave a positive signal to 999A and 284B but not to 205B. About 18% of these clones gave a positive signal while the rest gave a negative signal when probed with the mAb 2.13 (A). Ten clones were sequenced at the 3' terminus of the *rap-1* insert. The C-terminus amino acids encoded by each clones and their reactivity against mAb 2.13 are compared in table B. The relevant sequence of the *rap-1* gene is shown on the top of the table. The cleavage site of the RAP-1 protein was indicated by arrow (between A₁₉₀ and D₁₉₁).

A)



B)

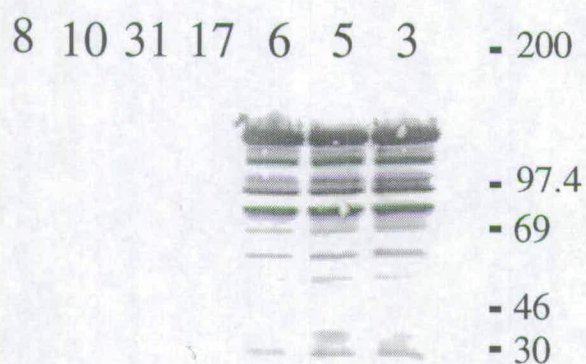


Figure. 4.4. Western blot analysis of the total cell extracts from the exonuclease III nested deletion clones were probed with anti- β -galactosidase rabbit polyclonal antibody (A) and mAb 2.13 (B).

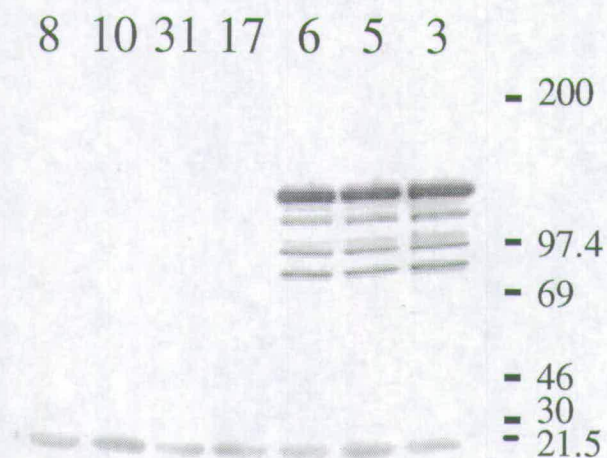


Figure. 4.5. This figure shows western blot analysis of the nested deletion clones, 3, 5, 6, 17, 31, 10 and 8. All of the inhibitory monoclonal antibodies gave the same result. These monoclonal antibodies only recognize the fusion protein from clones, 3, 5 and 6. This membrane was probed with mAb 11.4.

Clones	C-terminus sequences of the RAP-1 inserts	Blot-reactivity with RpAb	Blot-reactivity with inhibitory mAb
RAP-1	--DEEAPPAPKNTLTPLEELYPTNVNLFNYKYSLNNM--		
EBX3	--DEEAPPAPKNTLTPLEELYPTNVNLFNYKYS	+	+
ESs	--DEEAPPAPKNTLTPLEELYPTNVNLFNYK	+	+
EBX6	--DEEAPPAPKNTLTPLEELYPT	+	+
EBX17	--DEEAPPAPKNTLTPLE	+	-
EBX31	--DEEAPPAPKNTLT	+	-
EBX10	--DEEAPPAPKNTL	+	-
EBX8	--DEEAPPAPKN	+	-
minimum size of epitope	LTPLEE		

Figure. 4.6. Epitope mapping of RAP-1 protein by using ExonucleaseIII to produce different length of beta-galactosidase fusion protein.

[**RpAb** - rabbit polyclonal antibody against purified RAP-1; **mAb** - monoclonal antibody 2.13, 2.15, 2.29, 7.12, 8.1, 11.4]

4.3. Discussion.

The exonuclease digestion of the *rap-1* gene inserted fragment in pMS_{EB} gave definitive results. The rate of the exonuclease III digestion was similar to the predicted rate at 25°C (90 base pairs/min.). This was confirmed by the absence of the 284B-positive clone after third time point (3 mins after the beginning of the digestion reaction). Because the 284B probe sequence is 281 base pairs apart from the *Bam*HI restriction site, the calculation suggested that the overall rate of digestion reaction was approximate 94 base pairs/min.

Detection of the expressed proteins from the 999A- and 284B-positive clones suggested that ELYPT₁₂₁ are a part of the epitopes of all inhibitory monoclonal antibodies used in this study. This confirms the results in the previous chapter which located the epitopes in this part of the RAP-1 protein. It also suggested that the inhibitory epitopes of RAP-1 protein recognized by these monoclonal antibodies are close to each other. On the other hand, the possibility that these inhibitory monoclonal antibodies recognized the same epitope cannot be rule out. The presence of the inhibitory monoclonal antibodies against this RAP-1 region in different mice suggested that this region of the RAP-1 protein is highly immunogenic in mice.

It is of interest that this epitope-containing region is adjacent to the cleavage site of the RAP-1 protein between A₁₉₀ and D₁₉₁ (Ridley *et al*, 1991). It is possible that the inhibition effect of the monoclonal antibodies could be result from the interference of the proteolytic cleavage which may be an important aspect of RAP-1 protein function.

The results from this chapter showed that exonuclease III can be used for epitope mapping purpose by using recombinant DNA technology. The striking results of the epitope mapping point out that the epitopes of all monoclonal antibodies are located in a small region on the RAP-1 protein. This raised the hope that this highly immunogenic region may be used as a malaria vaccine component. However, to further

characterize the epitopes of all inhibitory monoclonal antibodies, the Geysen method was applied (Geysen *et al*, 1987). In the next chapter, two series of overlapped synthesized peptides were generated and used for the epitope mapping.

Chapter 5

Further characterization of inhibitory epitopes of RAP-1 protein using overlapping synthesized peptides.

5.1. Introduction.

5.1.1. Principle of peptide synthesis.

In order to chemically synthesize a peptide *in vitro*, there are several factors to be considered. Firstly, the peptide bond forming reaction is not autonomously driven. Secondly, in some amino acids, chemically active side chains may take part in reactions and produce undesired by-products. Thirdly, the synthesis of a synthetic peptide must be specifically controlled to assure the amino acid sequence required.

The peptide bond is a bond which is formed between two amino acid residues. The carboxylic acid group of one amino acid and amino group of the other amino acid combine to form an amide bond (peptide bond) with the elimination of a water molecule. The peptide bond forming reaction is described in Fig. 5.1. It can be autonomously driven by replacement of the hydroxyl group of the carboxylic acid group of amino acid with an electron-withdrawing group such as pentafluorophenyl group and 3,4-dihydro-4-oxo-benzotriazine-3-oxy group. A negative inductive effect, created from the electron-withdrawing group, results in a higher electrophilicity of the carbon atom at the carbonyl group. This enhances the possibility of reaction between the carbon atom of the carbonyl group and the nitrogen atom of the amino group of another amino acid residue which has a free-pair of electrons and is hence very nucleophilic.

Because many amino acid residues contain reactive side chains which may participate in the peptide synthesis reaction and generate unwanted by-products, these side chain groups have to be protected by specially designed protecting groups. The

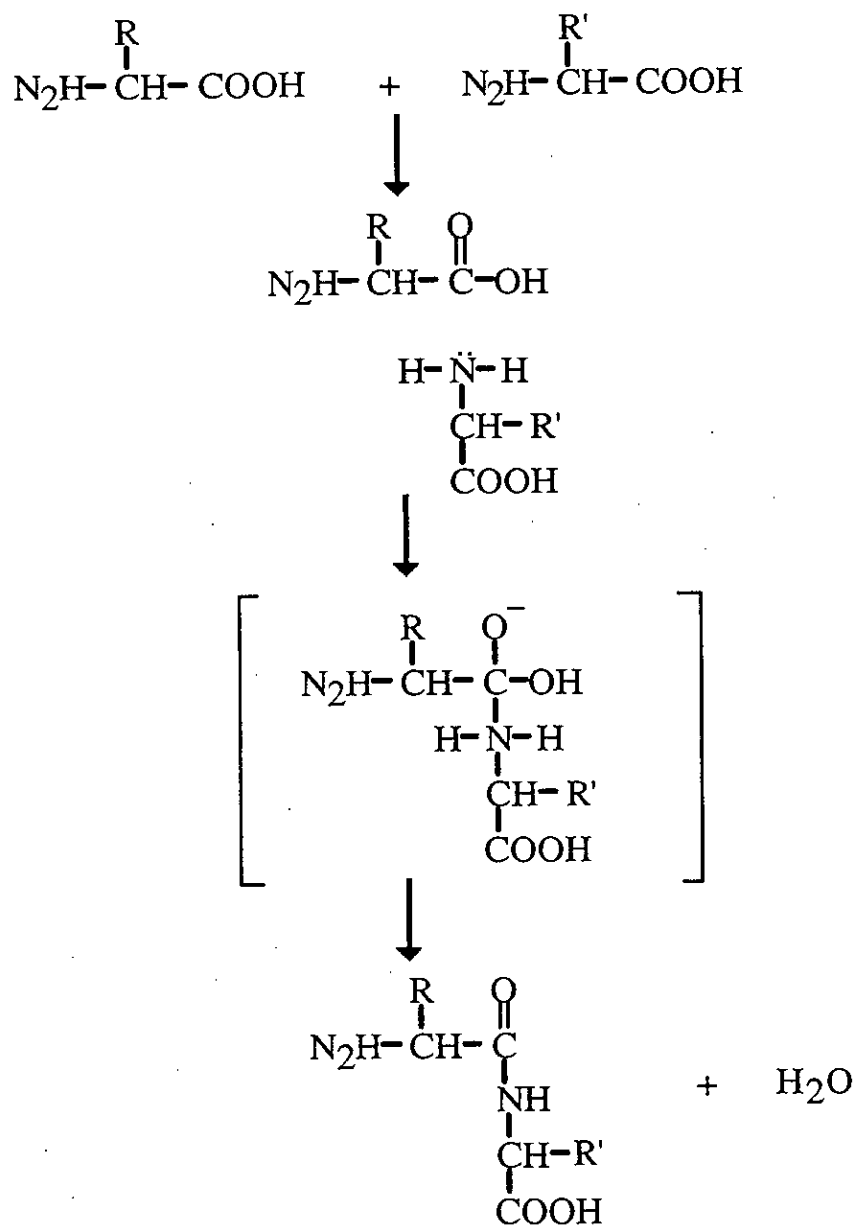


Figure 5.1. Diagram shows the peptide bond formation between two amino acids. A α -amino group of one amino acid reacts with the carboxylic group of another amino acid. The formula show in parenthesis is intermediate structure during peptide bond formation. After the reaction is complete, one molecule of water is obtained.

necessity and the strategy employed for side chain protection largely depends on the synthesis conditions. However, there are general criteria for the properties of protecting groups used; (i) they should suppress the intrinsic reactivity of the amino acid side chains (ii) the protecting groups must not interfere and take part in the synthesis reaction (iii) they are completely stable during peptide synthesis and the deblocking reaction in which the α -amino group of the amino acid is deprotected (see below) because side chain protecting groups are only removed after the end of synthesis process. (iv) they can be removed under a mild condition which has no effect on synthesized peptide.

The sequence of an *in vitro* synthesized peptide is controlled by allowing only one peptide bond formation between solubilized amino acids and extended synthesized peptide (Fig. 5.2.). The amino terminus of a solubilized amino acid is modified with a blocking group. After a single amino acid is added to the N-terminus of the synthesized peptide, the peptide bond formation to other amino acid is inhibited by the blocking group at the N-terminus of the newly incorporated amino acid. Another amino acid may be added to the peptide only when the blocking group is removed. Similar criteria to the protecting groups for choosing the appropriate blocking groups may also be applied.

5.1.2. Pepscan system.

In this experiment, the epitopes of all monoclonal antibodies are finely mapped using series of overlapping peptides. The peptide synthesis technique used in this study is based on solid-phase peptide synthesis (Atherton and Sheppard, 1989). In the conventional solid-phase peptide synthesis, the peptides are synthesized one amino acid at a time by attachment of the C-terminus end residue to an insoluble polymer (a resin) as shown in Fig. 5.3. After a complete process of synthesis, the excess amino acid and by-products are washed off and the next residue is added to the

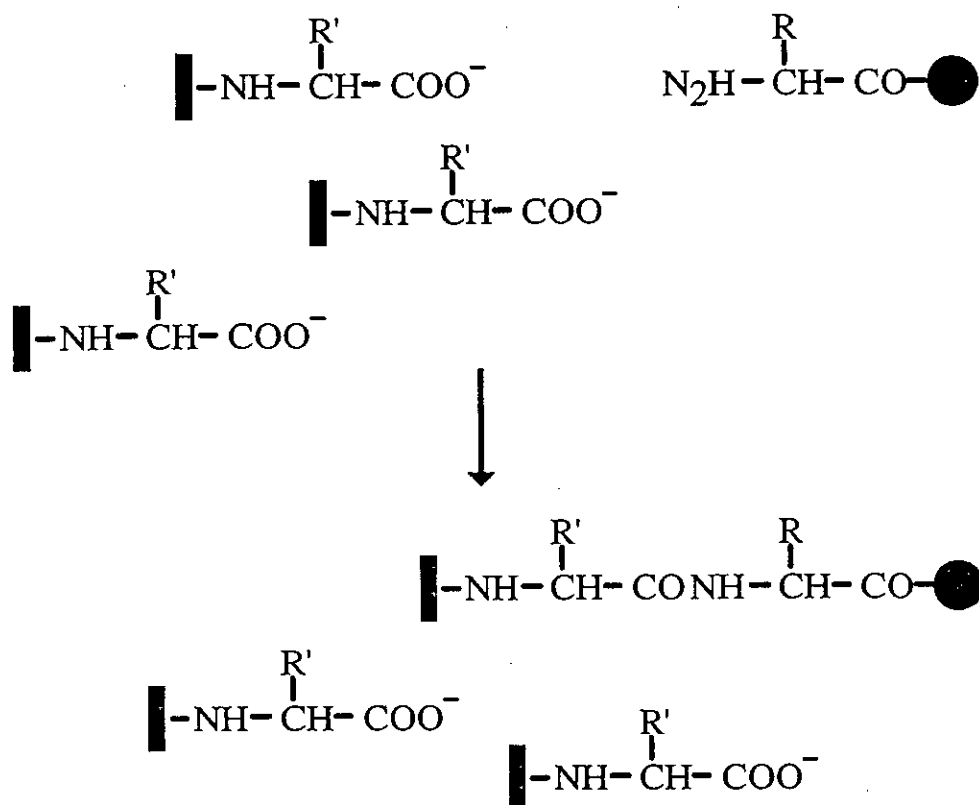


Figure 5.2. Diagram shows the use of a blocking group to limit peptide bond formation. The α -amino group of amino acid bound solid phase (black circle) can form a peptide bond with the activated amino acid in the reaction solution but further reaction is prevented by the blocking group (black thick line) at the N-terminal of synthesized peptide. The blocking group must be removed before next amino acid is added.

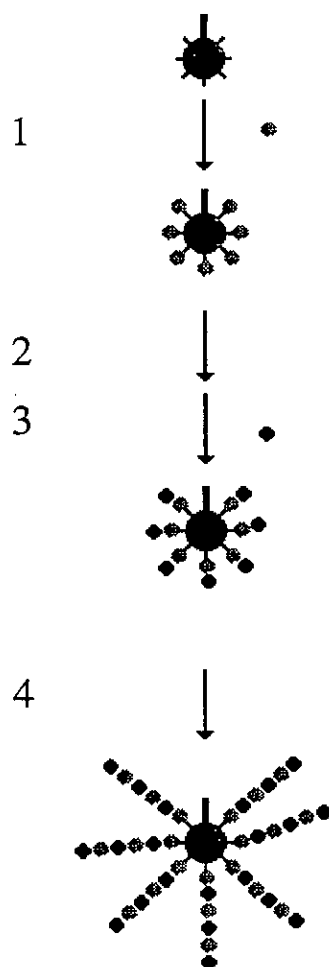


Figure. 5.3. Diagram shows the peptide synthesis used by Pepscan system based on solid-phase peptide synthesis. 1) The modified amino acid at the C-terminus of the peptide is added to a specially-prepared polyethylene pin. The N-terminus of the added amino acid is blocked by the Fmoc group (see text), so only one amino acid is added to the pin. Side chain groups of some amino acids are also modified by the protecting groups to ensure that only the peptide bond is formed in the reaction between amino acids. 2) The excess amino acids are washed off before the Fmoc group is removed at the alkaline pH. 3) The next modified amino acid is added to the pin. 4) After repeating step 1-3 several times the desired peptides were obtained bound to the polyethylene pin. The positive charge at the N-terminus of the peptide is removed by acetylation. All protecting groups are also removed by treatment with acid.

resin. The synthesis cycle will be repeated until the peptides contain all the amino acids required. The peptide is separated from the resin only after the completion of peptide synthesis.

Recently, Geysen and his colleagues modified the solid-phase peptide synthesis for the epitope analysis purposes (Geysen *et al.*, 1987). A peptide synthesis kit is commercially available (Pepscan, see materials 2.1.5). Although a peptide is synthesized from the C-terminus residue, like normal solid-phase peptide synthesis, a polyethylene rod, instead of a resin, acted as a solid supporter as shown in Fig. 5.3.

In the Pepscan system, active ester forms (pentafluorophenyl ester and 3, 4-dihydro-4-oxo-benzotriazine-3-oxy ester) of amino acids are used because they are stable, easy to prepare and their structures cannot generate significant side reactions. The rate of peptide bond formation of these active esters is easily increased in the presence of hydroxybenzotriazole, a catalyst. The peptide synthesis reaction is set up in dimethylformamide solvent.

Several protecting groups are used for the inhibition of side-chain reaction because the amino acid side-chains of different amino acids have different chemical reactivities. All the protecting groups are acid-labile, i.e. they can be removed under the acid condition. Some amino acid residues contain side-chains which are considered as non-reactive in the synthesis conditions used in this system, so side-chain protection is not needed. These amino acids are A, F, G, I, L, M, N, P, Q, W and V. Some amino acids use t-butyl group as a protecting group, for example S, T, Y. Because the S-t-butyl derivative of the cysteine is very stable at the acid condition, a triphenylmethyl group is introduced as a protecting group for this amino acid. The carboxy groups of the D and E residues are also protected by a similar group, the t-butoxy group. The amino group at the lysine side-chain and the imidazole ring of histidine are protected by another protecting group, t-butoxycarbonyl. The guanidino group of arginine is highly nucleophilic and can not be adequately suppressed by the

t-butoxycarbonyl group, thus, a 4-methoxy-2, 3, 6-trimethylbenzene sulphonyl group is used.

The 9-Fluorenylmethoxycarbonyl (Fmoc) group is used to block the α -amino group of all amino acids used in the Pepscan system. The advantage of the Fmoc group against other blocking groups is that it can be readily removed by secondary bases (piperidine) in dimethylformamide without any effects on the acid labile protecting groups (as described above) and the fully synthesized peptide.

The peptide synthesis and epitope mapping procedure are simplified by assembly of the ninety-six polyethylene rods into a polyethylene holder in the microtiter plate format. Each polyethylene rod represents as a solid support in one peptide synthesis reaction. This means that ninety-six different peptides can be synthesized at once. For epitope analysis, peptides are synthesized over a region of interested protein with one amino acid shift from N- toward C-terminus as shown in Fig. 5.4. The series of overlapped peptides are then subjected to an ELISA technique using microtiter plate and the reactive peptides were determined. The advantage of this technique is that the polyethylene rods can be reused after the bound monoclonal antibody is washed off by sonication in the presence of SDS and mercaptoethanol.

Many researchers have been reported the success of epitope mapping by Pepscan system (Geysen *et al*, 1984, Papsidero *et al*, 1989, Epping *et al*, 1988, Hill *et al*, 1989, Virji and Heckels, 1989, Ramasamy and Geysen, 1990, Ewing *et al*, 1990, Radford *et al*, 1990 and Napier and Venis, 1992). Most of these experiments based on synthesized peptides about 6-8 amino acids long. This chapter described the attempt to map the epitopes of inhibitory monoclonal antibodies against RAP-1 protein by Pepscan system using overlapping hexapeptides and decapeptides.

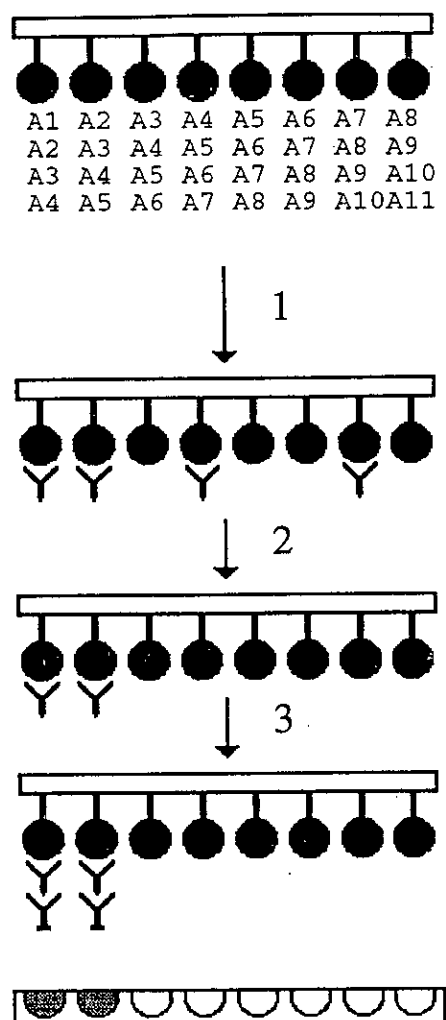


Figure. 5.4. Digram shows the epitope detection used in a Pepsan system for peptides covered from residues A1 to A11. The epitope mapping is simplified by the synthesis of overlapping peptides on the pins assembled in the microtiter plate format. The pins are incubated with an antiserum in the presence of BSA (1) and the non-specific binding antibodies are washed off by detergent mixture (2). The peptides containing the epitope sequence are detected by exposing the pin to the horseradish-peroxidase conjugated second antibody. Positive signals can be read by the microtiter plate reader at OD₄₁₀ after azino-di-3-ethyl-benzthiazodinsulphonate is added (3)

5.2. Results.

5.2.1. Peptides synthesis.

Two sets of overlapping peptides were synthesized, a hexapeptide set and a decapeptide set. Their sequences cover the epitope of inhibitory monoclonal antibodies identified by exonuclease III experiment (see Chapter 4). The hexapeptide set represents amino acid V₁₀₂ to amino acid S₂₉₄ of the RAP-1 protein as shown in Fig. 5.5. Each peptide in the set has five amino acid residues identical to the adjacent peptide so that each of the peptide sequences shifts one amino acid residue along the RAP-1 protein sequence. Another set contains 10 amino acids long synthesized peptides each of which overlap 9 amino acids to adjacent peptide. The decapeptide set covers the RAP-1 protein from A₁₉₀ to S₂₂₁ (Fig.5.5). All the peptides in this set were synthesized as duplication to ensure the fidelity of the results.

The peptide synthesis procedure was carried out as suggested by the manufacture manual (see method 2.3.3.). The dimethylformamide (DMF) used in this study is specially purified for peptide synthesis purposes so no pretreatment of the DMF was required. The peptide bond forming reaction was performed in a lid-black plastic box to protect from light (DMF can easily decompose in the presence of light) and evaporation. The box was incubated at 30°C for at least 18 hours to increase the coupling reaction rate.

5.2.2. Epitope mapping of the inhibitory monoclonal antibodies.

The hexapeptide and decapeptide set were incubated with the inhibitory monoclonal antibodies and the epitopes were determined by horseradish peroxidase conjugated second antibody. No binding of the inhibitory monoclonal antibodies could be detected among synthesized hexapeptides (data not shown).

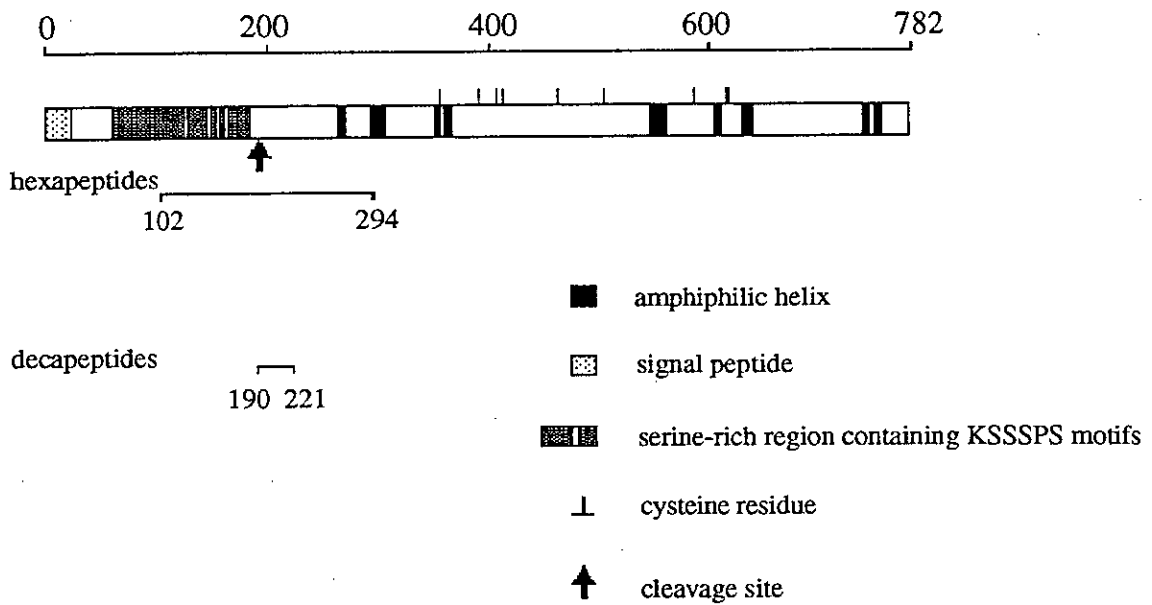


Figure. 5.5. Diagram shows amino acid sequences of the RAP-1 protein which were synthesized as the hexapeptides and decapeptides. The diagram of the whole RAP-1 protein is given for reference.

All the monoclonal antibodies, except mAb 11.4, bind to a decapeptide, TLTPLEELYP₂₁₀ and LTPLEELYPT₂₁₁ as shown in Fig. 5.6., 5.7. and 5.8. The mAb 2.13, 7.12, 2.29 gave higher signal with the peptide TLTPLEELYP₂₁₀ than the peptide LTPLEELYPT₂₁₁ while the signal given by the mAb 2.15 showed an opposite nature. Although the mAb 8.1 gave similar level of the signal at the TLTPLEELYP₂₁₀ and LTPLEELYPT₂₁₁ peptide, it also binds strongly to the peptide NTLTPLEELY₂₀₉ (Fig. 5.8.). The mAb 11.4 does not react with either synthesized hexa- and decapeptides (Fig. 5.8.).

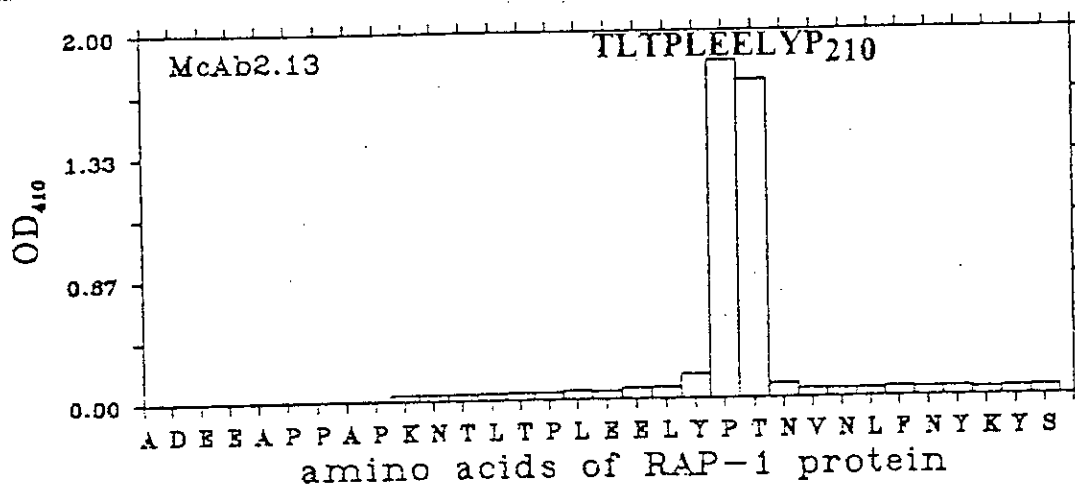
5.3. Discussion.

5.3.1. Epitope mapping results.

The failure of the inhibitory monoclonal antibodies to bind to synthesized hexapeptides in the first experiment suggested that the epitopes of these monoclonal antibodies are bigger than 6 amino acid residues. Indeed, the results from the binding of synthesized decapeptides confirmed this conclusion. The inhibitory monoclonal antibodies, 2.13, 2.15, 2.29, 7.12 and 8.1 bound to the TLTPLEELYP₂₁₀ and LTPLEELYPT₂₁₁ decapeptides near to the cleavage site of the RAP-1 protein. These confirmed the results of the epitope mapping by exonuclease III digestion (see Chapter 4).

The mAb 11.4 has no detectable reactivity against both synthesized hexa- and decapeptides. There are two possibilities which may explain this finding. First, the epitope of this monoclonal antibody may be bigger than 10 amino acid residues so that the mAb 11.4 can not bind to the synthesized peptides smaller than its epitope size. Second, the epitope of may be composed of a certain secondary structure as the result of the protein folding which depends only on amino acid sequence in this area of the

A)



B)

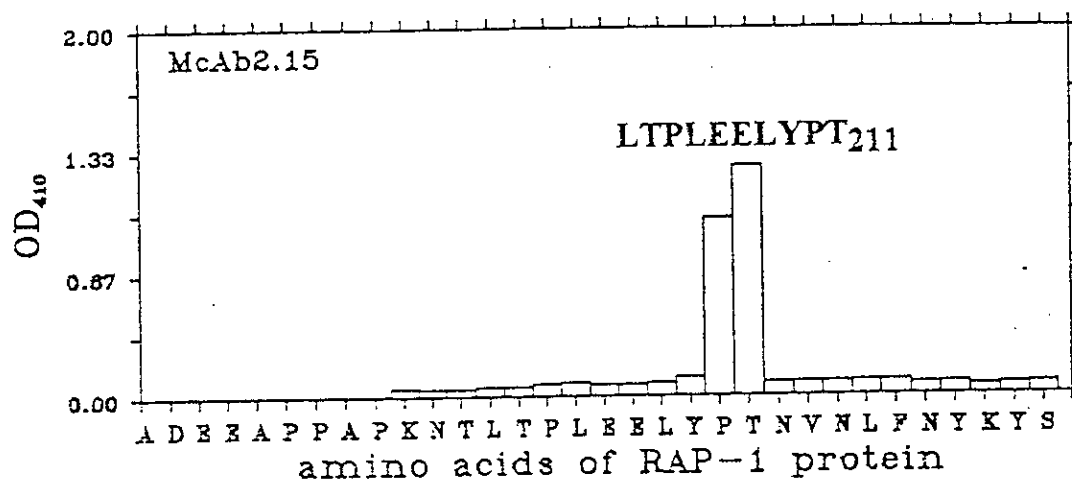
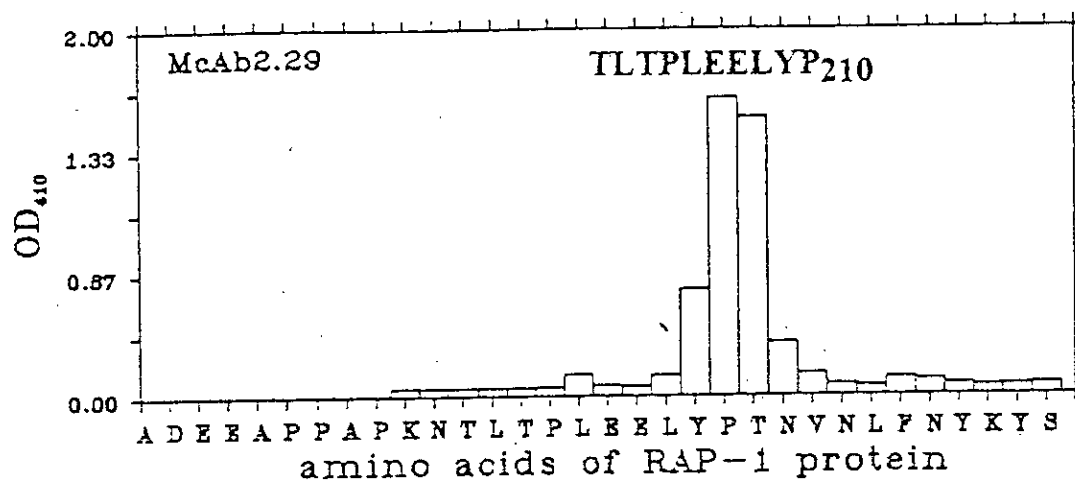


Figure. 5.6. Graph shows the binding of the mAb 2.13 (A) and 2.15 (B) to a series of the decapeptides. The sequence of the peptides which gave the highest signals also shown in each graph.

A)



B)

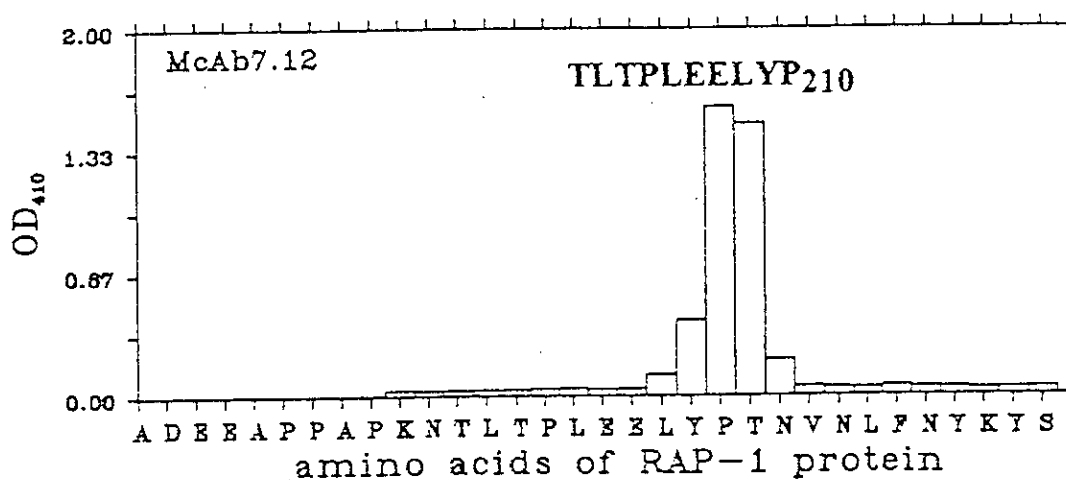
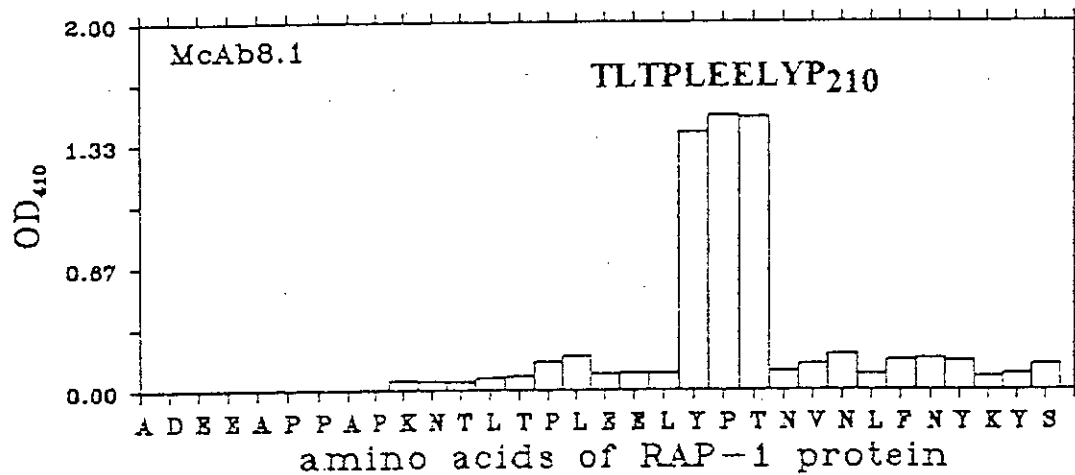


Figure. 5.7. Graph shows the binding of the mAb 2.29 (A) and 7.12 (B) to a series of the decapeptides. The sequence of the peptides which gave the highest signals also shown in each graph.

A)



B)

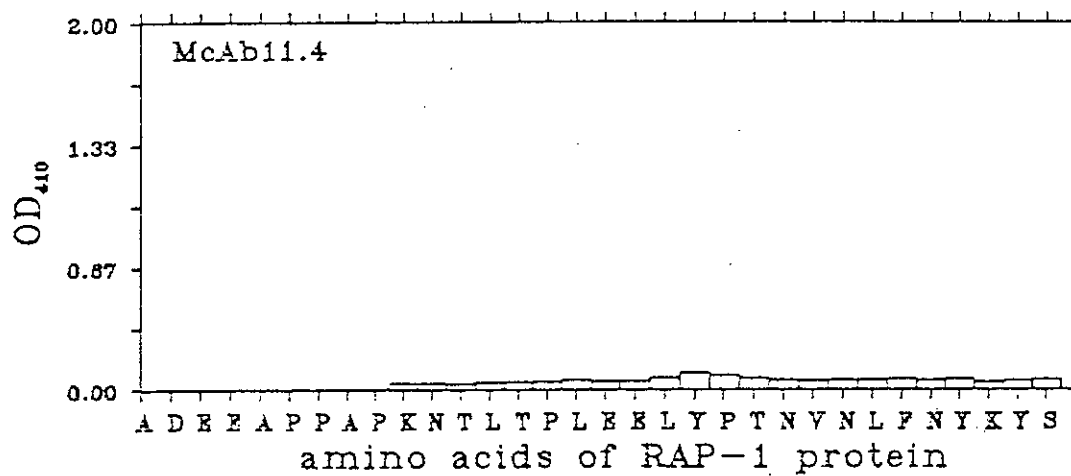


Figure. 5.8. Graph shows the binding of the mAb 8.1 (A) and 11.4 (B) to a series of the decapeptides. The sequence of the peptides which gave the highest signals also shown in each graph.

RAP-1 protein. This type of epitope may be also contributed, in part, by amino acid sequence in the related region.

Although most of the monoclonal antibodies bind to the peptides in the same region, their binding to the pin-bound peptides gave slightly different patterns of the binding signals. The mAb 2.15 preferably binds to LTPLEELYPT₂₁₁ rather than TLTPLEELYP₂₁₀ while other monoclonal antibodies gave higher signal binding at pin-bound TLTPLEELYP₂₁₀.

The epitope identified by mAb 2.13, 2.15, 2.29, and 7.12 is considered to be the biggest reported epitope identified by Pepscan system so far. It is possible that not all of the amino acid residues in the identified epitope are bound by the monoclonal antibodies and the binding affinity with the RAP-1 native form is enhanced by other amino acid residues which come close together by the protein folding.

The systematic replacement of an amino acid at each position in epitope studies, replacement net analysis, suggested that only some amino acids in the epitopes identified by the Pepscan system are crucial for antibody binding and a change in these amino acid residues results in the destruction of the antibody binding site (Geysen *et al*, 1984, Geysen *et al*, 1985 and Radford *et al*, 1990). One of these experiments also showed that the crucial amino acids for antibody binding are the overlapped amino acid residues of the peptides bound to the antibody studied (Radford *et al*, 1990). These finding implies that the amino acids which play an important part in the mAb 2.13, 2.15, 2.29, and 7.12 binding are located in nonapeptide LTPLEELYP₂₁₀ and octapeptide LTPLEELY₂₀₉ for the mAb 8.1. One characteristic of the LTPLEELYP₂₁₀ epitope is the near-symmetrical amino acid sequence at the N and C terminus of the peptide and in the middle of the identified epitope.

Results from this experiment proved that the recombinant proteins generated by exonuclease III digestion of the RAP-1 can correctly map the epitopes of monoclonal antibodies. A major advantage is that the exonuclease III digestion method

also revealed an epitope of mAb 11.4 which can not be detected in the Pepscan system. However, the Pepscan system was significantly able to ensure the amino acid sequence of the epitopes.

5.3.2. Further works in Chapter 6 and 7.

The reactivity of these inhibitory monoclonal antibodies from 3 different mice against the same epitope shows that this inhibitory epitope may be a highly immunogenic region on RAP-1 protein in mice when immunized with whole parasite (native form of the RAP-1 protein). However, this phenomenon may not occur in human which has a similar but not identical immune system to mouse. Another point of interest is whether the immunogenicity of a RAP-1 recombinant protein is identical to that of the native form. Both of these points will be studied and discussed in Chapter 6.

The *rap-1* gene was considered as a conserved gene among variety of isolates based on the monoclonal antibodies used in this study (Clark *et al*, 1987). It is now clear that these monoclonal antibodies are directed against the same region of the RAP-1 protein. This suggests that the conservation of the *rap-1* gene needs to be re-assessed. In Chapter 7, the conservation of the *rap-1* gene is again studied and discussed.

Chapter 6

Immunogenicity studies of the RAP-1 protein in different animals and epitope mapping of monoclonal antibodies against a bacterial expression protein.

6.1. Introduction.

6.1.1. A problem of a malaria vaccine.

An important problem related to the malaria vaccine design is the necessity to raise an immune response to a protein target which can protect human from malaria infection. Unfortunately, several attempts of human immunization experiments using short peptides, or irradiated parasites led to unsuccessful results as discussed in Chapter 1. This may either be because the protein target cannot elicit the protective immune response or the 'inhibitory epitopes' fail to be recognized by the human immune system.

The native-form of the RAP-1 protein can stimulate mice to produce antibodies which can inhibit parasite growth *in vitro* (see Chapter 3). The results from previous chapters located the epitopes of all the inhibitory monoclonal antibodies in the same region. This suggested that the binding of monoclonal antibodies to this 'inhibitory epitope' leads to the interference of RAP-1 protein function and/or its structure and so, inhibits parasite growth *in vitro*. Because several monoclonal antibodies from mice raised by the native form of the RAP-1 protein are directly against these 'inhibitory epitopes', it seems that this region of the RAP-1 protein is highly immunogenic in mice.

However, it can not be implied that this 'inhibitory epitopes' is also able to elicit protective immune responses in other animals, especially humans. Though the antigenicity is a non-intrinsic property of a protein, it still depends largely on the host immune system to recognize a particular epitopes. Studies of the antigenicity of

several proteins in different hosts suggest that the antigenicity of a given protein in a host is influenced by structural differences between the protein and the host protein as well as the host's immunological regulatory mechanisms (Benjamin *et al*, 1984). Recently, it has been shown that the denatured form of the RAP-1 protein can elicit a protective immune response in *Saimiri* monkey and that the RAP-1 protein was recognized by the Nigerian human immune sera pool (Ridley *et al*, 1991). None of the epitope binding sites of these sera have been identified. It would be of interest to know whether the 'inhibitory epitope' defined in this study are also recognized by human immune response.

6.1.2. Monoclonal antibodies against a RAP-1 expression protein.

During the course of this study, another series of monoclonal antibodies were raised against the expressed RAP-1 protein and were available for epitope mapping. The expressed RAP-1 protein came from a bacterial expression system covering amino acid 23 to 711 of the RAP-1 protein. Thirteen monoclonal antibodies were obtained (see Table 6.1.). However, only eleven of these monoclonal antibodies specifically recognize the RAP-1 protein on western blot of the total parasite extracts. No data on the inhibitory effect of these monoclonal antibodies has yet been obtained.

Because recombinant RAP-1 protein has a prospect for use as a malaria vaccine, it is necessary to investigate the ability to generate a protective immunity with the recombinant RAP-1 protein. The epitope of the monoclonal antibodies generated against the recombinant RAP-1 protein must therefore be determined and compared to the 'inhibitory epitope' recognized by monoclonal antibodies raised against the native protein. If the recombinant RAP-1 protein can generate antibodies against the 'inhibitory epitope', it is then possible to use this recombinant protein as a malaria vaccine.

Antibodies (cell culture supernatants)	Reactivity against the total parasite extracts
mAb 1.7	80/65
mAb 1.8	80/65
mAb 1.14	80/65
mAb 1.15	80/65
mAb 1.22	recognizes only the RAP-1 precursor
mAb 1.25	80/65
mAb 2.5	80/65
mAb 2.16	not specific to the RAP-1 protein
mAb 2.21	80/65
mAb 2.25	80/65
mAb 4.9	80/65
mAb 5.2	80/65
mAb 8.2	negative result

Table 6.1. The list shows monoclonal antibodies generated from Hoffman-La Roche. The mAb 1.22 was excluded from this study because its epitope is located at the N-terminus of the RAP-1 protein. The mAb 2.16 and 8.2 was also excluded.

6.1.3. Characterization of the RAP-1 protein immunogenicity.

This chapter is divided into two parts. The first part describes the attempt to establish the immunogenicity of the RAP-1 protein in different animals, especially human. Some epitopes recognized by Nigerian human immune sera pool and rabbit antisera raised against the native form of the RAP-1 protein were identified and compared. The second part is devoted to the epitope mapping of the monoclonal antibodies raised against the bacterial expressed RAP-1 protein in order to compare the antigenicity of native and non-native RAP-1 protein.

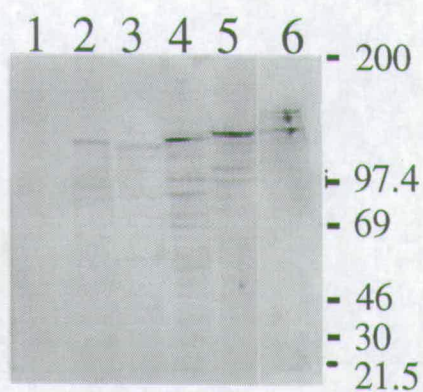
6.2. Results.

6.2.1. Comparison of some epitopes recognized by immune human sera and rabbit polyclonal antibodies.

To locate some epitopes recognized by Nigerian immune human sera pool and rabbit polyclonal antibodies, both sera were probed against fusion proteins encoded by plasmid constructs generated by the subcloning of the restriction enzymes digested *rap-1* gene fragments (see Chapter 3). The results showed that the Nigerian immune sera pool recognizes all fusion proteins while the rabbit serum fails to recognize the fusion protein from bacteria clone UR_{EH} (Fig. 6.1.). The results suggested that the human and rabbit immune system react differently to the RAP-1 protein. In both cases, there may be more than one epitope located on different fragments of the fusion proteins. However, the possibility of cross reaction between those RAP-1 protein fragments can not be ruled out.

Both sera were also tested with the overlapped synthesized hexapeptides (amino acid 102-294) and decapeptide (amino acid 190-221). The human immune sera pool recognizes decapeptide APPAPKNTLT₂₀₃ with relatively high background. The

A)



B)

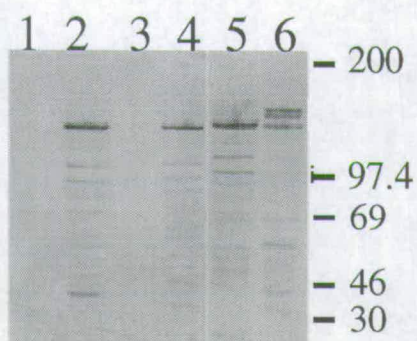


Figure. 6.1. Western blot analysis of the Nigerian immune sera pool (A) and rabbit serum (B) with the recombinant RAP-1 protein from bacteria clones generated by restriction enzymes. track 1.) MS, 2) MS_{HB}, 3) UR_{EH}, 4) MS_{ES}, 5) MS_{EB}, 6) MS_{ES}.

rabbit serum gave two peaks of the signal with the highest peaks at EAPPAPKNTL₂₀₂ and TPLEELYPTN₂₁₂ (Fig. 6.2.) The synthesized hexapeptides gave no strong positive signal by both sera (data not shown). This suggested that the region containing the 'inhibitory epitope' can stimulate an immune response in both human and rabbit. However, the exact location of epitopes recognized by both sera do differ.

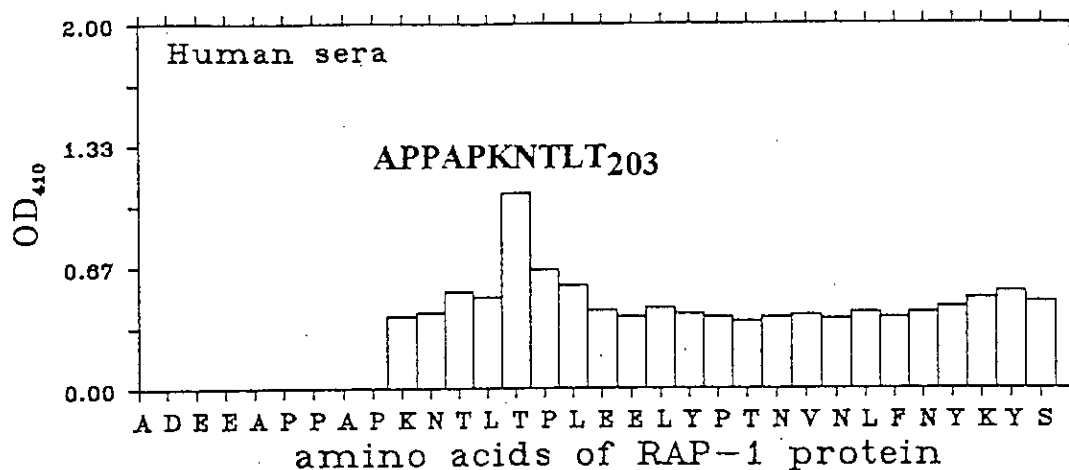
6.2.2. Epitope mapping using fusion proteins from different fragments of *rap-1* gene.

The monoclonal antibodies derived from 4 different mice (see Table 6.1.) were tested against fusion proteins from different fragments of the *rap-1* gene as described in Chapter 3. The fragments which contain the epitopes of these monoclonal antibodies were determined by western blot as previously described (see Chapter 3). The results showed that nine monoclonal antibodies from two mice, mAb 1.7, 1.8, 1.14, 1.15, 1.25, 2.5, 2.16, 2.21 and 2.25 recognized fusion proteins from all constructs except the fusion protein from the clone UR_{EH} (Fig. 6.3.). This suggests that these monoclonal antibodies recognize epitopes encoded by the *HindIII-SspI* fragment of the *rap-1* gene. The other two monoclonal antibodies from two other mice, mAb 4.9 and mAb 5.2 recognized fusion protein from all constructs except UR_{EH} and MS_{ES} which suggested that the epitopes of these monoclonal antibodies located in *SspI-BamHI* fragment (Fig. 6.4.). The results are summarized in Table 6.2.

6.2.3. Epitope mapping of monoclonal antibodies by Pepscan.

All epitopes of the monoclonal antibodies were specifically mapped using hexa- and decapeptides which had been previously synthesized (see Chapter 5). Only one block of synthesized hexapeptides which represent amino acid 196 to 294 were

A)



B)

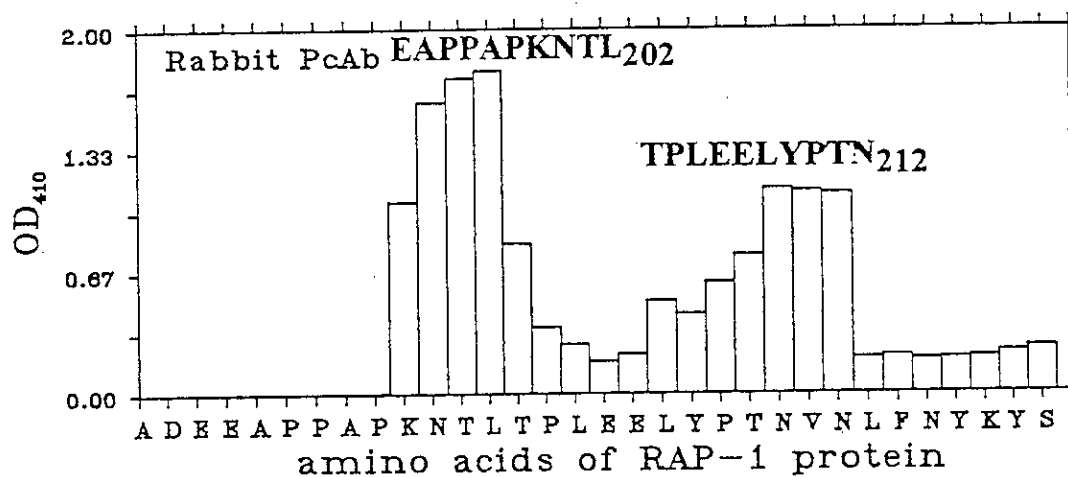
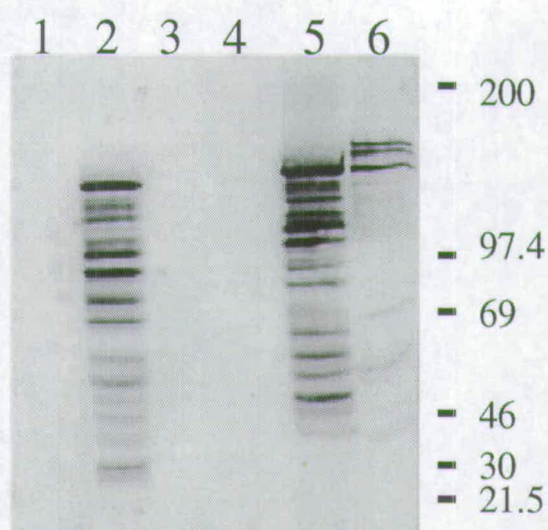


Figure. 6.2. Graphs show epitope scanning panels of Nigerian human immune sera pool (A) and rabbit serum (B) using decapeptides at dilution 1:100 and 1:1000 respectively. The amino acid sequences of high peaks were shown.



Figure 6.3. The western blot of total cell extracts from restriction enzymes generated clones was probed with monoclonal antibodies; track 1.) MS, 2) MS_{HB}, 3) UR_{EH}, 4) MS_{ES}, 5) MS_{EB}, 6) MS_{ES}. All of the monoclonal antibodies tested gave the same pattern except mAb 4.9 and 5.2. Only one western blot was shown here (probed with mAb 1.7).

A)



B)



Figure 6.4. Western blot analysis of mAb 4.9 (A) and 5.2 (B) with total cell extracts from restriction enzymes generated clones; track 1.) MS_{1S}, 2) MS_{HB}, 3) UR_{EH}, 4) MS_{ES}, 5) MS_{EB}, 6) MS_{ES}.

mAb	MS _{ES}	MS _{EB}	MS _{Es}	MS _{HB}	UR _{EH}
1.7	+	+	+	+	-
1.8	+	+	+	+	-
1.14	+	+	+	+	-
1.15	+	+	+	+	-
1.25	+	+	+	+	-
2.5	+	+	+	+	-
2.16	+	+	+	+	-
2.21	+	+	+	+	-
2.25	+	+	+	+	-
4.9	+	+	-	+	-
5.2	+	+	-	+	-

Table 6.2. The summary of the results of epitope mapping using western blot analysis of fusion protein from different plasmid constructs generated by restriction enzymes.

used in this experiment because all nine epitopes had been potentially mapped to this region of the RAP-1 protein. The synthesized decapeptides include amino acid 190 to 221. Two monoclonal antibodies, mAb 4.9 and mAb 5.2, which gave different signal pattern on the western blot from other monoclonal antibodies were probed only to the hexapeptides block since their epitopes lay outside the region covered by the decapeptides (see above).

The results indicated that these monoclonal antibodies can be divided into three different groups according to their epitopes (Fig. 6.5.). The first group of monoclonal antibodies recognize similar epitopes on hexapeptide EEFYD₂₄₈ and gave a negative result on synthesized decapeptides. These monoclonal antibodies are mAb 1.7, 1.8, 1.25, 2.21 and 2.25. The second group, mAb 1.14 and 1.15, recognized only the decapeptides NTLPLEELY₂₀₉. The third group, mAb 5.2, reacts with hexapeptide FLENQV₂₈₆. No reactivity against hexa- or decapeptides was observed with mAb 2.5, 2.16 and 4.9. This may either because their epitopes locate outside the synthesized peptide region or their epitopes are bigger than 6-amino acid in length. The summary of this study is shown in Fig 6.6.

6.3. Discussion.

6.3.1. Immunogenicity of the RAP-1 protein in different animals.

The studies of rabbit and human sera suggested that the immune responses in rabbit and human to the RAP-1 protein are distinguishable. However, the region near the 'inhibitory epitope' is recognized by both rabbit and human sera. The decapeptide APPAPKNTLT₂₀₃ recognized by the human sera pool was also overlapped with one of the rabbit sera epitopes EAPPAPKNTLT₂₀₂ and with the inhibitory epitopes TLTPLEELY₂₁₀. This results suggest that this region is a immunodominant region on the RAP-1 protein and the immune response to this

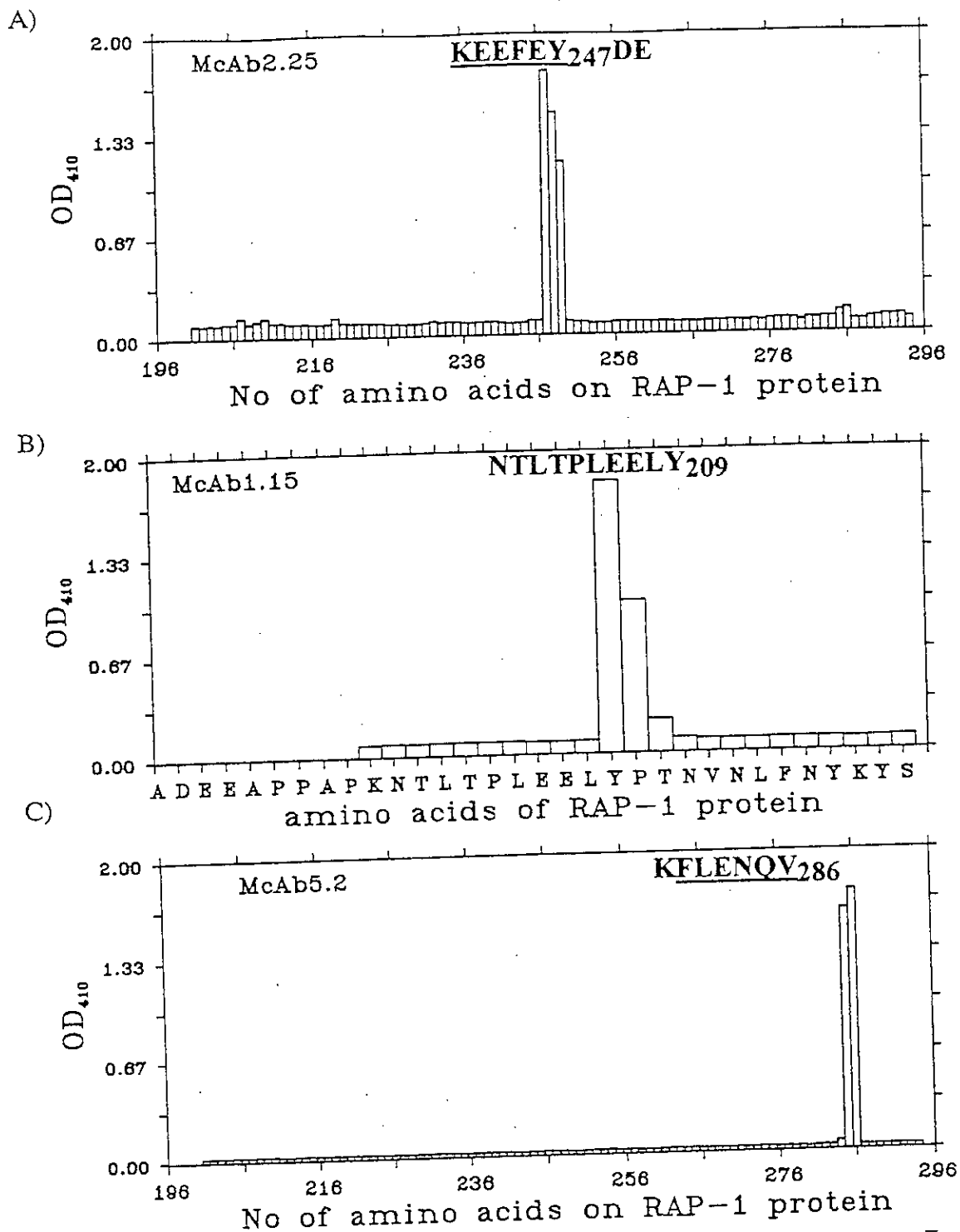


Figure. 6.5. Graphs show epitope scanning panels of three groups of monoclonal antibodies (only one graph from each group is presented here). The amino acid sequences of high peaks were shown. A) mAb 1.7, 1.8, 1.25, 2.21 and 2.25 group, B) mAb 1.14 and 1.15 group, C) mAb 5.2

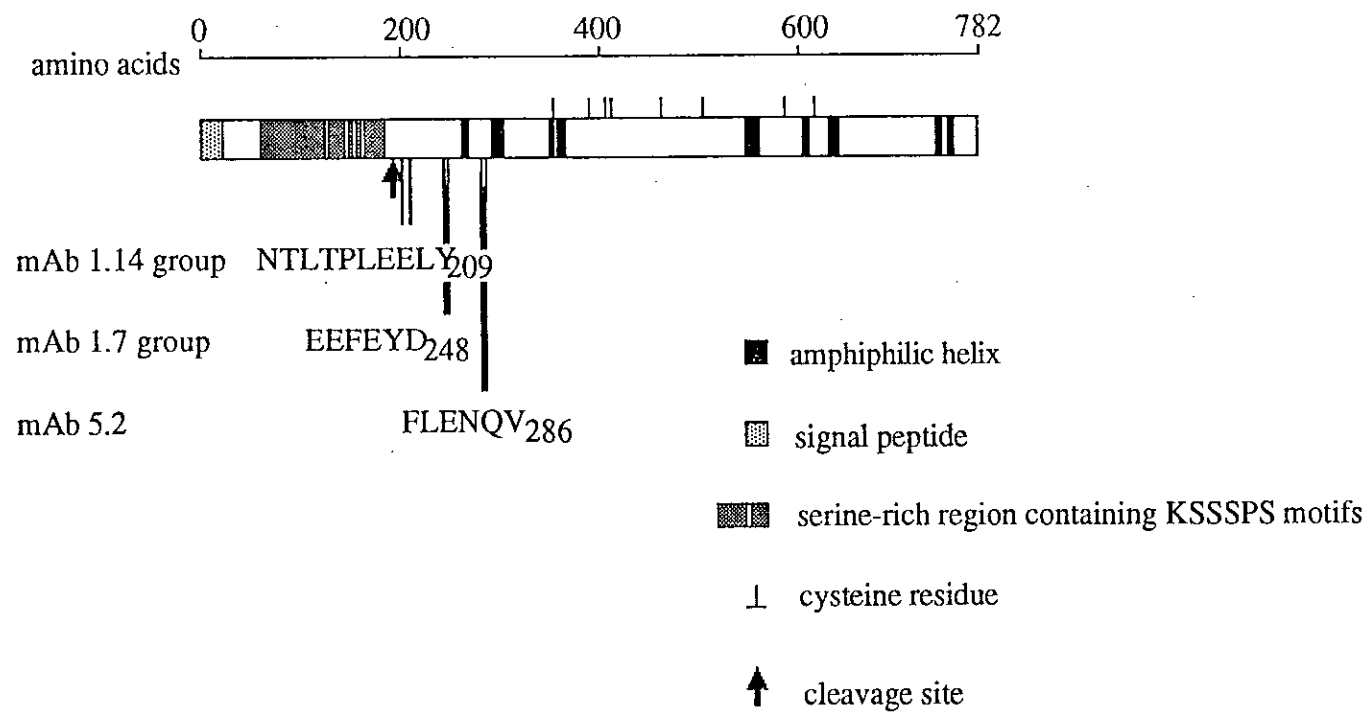


Figure 6.6. The summary diagram demonstrates the epitopes recognized by the monoclonal antibodies which were raised against the recombinant RAP-1 protein from bacterial expressing system.

particular region can occur in some animals including human.

6.3.2. Immunogenicity comparison between native and non-native form of the RAP-1 protein.

The epitope mapping of monoclonal antibodies indicated that the expressed protein (non-native form) of the RAP-1 protein demonstrates similar immunogenicity to the native form. However, a few epitopes from different region of the protein were identified compared to a single epitope recognized by inhibitory monoclonal antibodies. The significance of this study is that the recombinant protein was able to elicit an immune response against the 'inhibitory epitope' (TLTPLEELYP₂₁₀). The results also suggested that the EEFEYD₂₄₈ peptide may be highly immunogenic on the expressed RAP-1 protein molecule because most of the monoclonal antibodies recognized this epitope. The contradiction of the epitope mapping results between western blot of the fusion proteins and Pepscan may be caused by the cross-reaction among different fragments of the RAP-1 protein.

These studies contribute several important point toward the development of the RAP-1 protein as a malaria vaccine. They revealed that a specific region which contains 'inhibitory epitope' is recognized by rabbit, human as well as mice. Moreover, there is an evidence that the non-native form of the RAP-1 protein can generate immune response against the same region recognized by inhibitory monoclonal antibodies raised against the native form of the protein. This suggests that it may be possible to stimulate human immune responses against the inhibitory epitope region by using recombinant protein or a short synthetic peptide.

The next chapter of this thesis will described the study of *rap-1* gene diversity in different isolates and clones of *P. falciparum*. This information is required for establishment the importance of the RAP-1 protein as a malaria vaccine candidate.

Chapter 7

Sequence analysis of the *rap-1* gene in different isolates and clones of *P. falciparum* from different sources.

7.1. Introduction.

7.1.1. The need for sequence analysis of the *rap-1* gene.

Ideally, a good vaccine candidate must be able to stimulate a protective immune response against the majority, if not all, *P. falciparum* isolates. However, many of the *P. falciparum* genes display significant genetic polymorphisms, as was discussed in Chapter 1. Although the data from previous conservation studies of the RAP-1 protein using the group of monoclonal antibodies used in this study suggested that this protein is conserved among different isolates (Clark *et al*, 1987), the conservation of the *rap-1* gene is still in question, since these monoclonal antibodies appear to recognize a small amino acid sequence in the RAP-1 protein molecule (see Chapter 5). In this chapter, the degree of polymorphism exhibited by the *rap-1* gene was investigated in more detail. Several isolates of *P. falciparum* from a variety of geographical sources were selected. The *rap-1* alleles from these isolates were cloned, using the polymerase chain reaction (PCR) technique, and their sequences were compared.

7.1.2. Polymerase chain reaction (PCR) technique.

PCR is a technique in which a specific DNA fragment can be biochemically synthesized and amplified *in vitro* from purified genomic DNA or a cell lysate (Erlich, 1989). The PCR technique was originally made practicable by the discovery of a thermostable DNA polymerase isolated from a thermophilic microorganism,

Thermus aquaticus, namely *Taq* DNA polymerase (Gelfand, 1989). *Taq* DNA polymerase has an optimum activity at 75-80°C, depending on the nature of the DNA template and it is relatively stable at temperatures as high as 94-95°C. The principle of PCR reaction involves three main steps: (i) heat-denaturation of DNA, (ii) annealing of oligonucleotide primers and (iii) primers extension or polymerization step as shown in Fig. 7.1.. In the first step, the DNA template is heated up to about 90°C in order to separate double stranded DNA into single strand DNA. The temperature is then decreased. This allows the annealing of two oligonucleotides primers, each of which complements a flanking region on the opposite strands of a specified DNA fragment. The polymerization step is carried out after the temperature is raised to the optimum temperature, about 70°C. In this step, the *Taq* DNA polymerase synthesizes DNA from hybridized primers in the 5' to 3' direction. The two newly synthesized DNA fragment then act as a template in the another cycle of reactions. The target DNA fragment copies will be doubled in every cycle of the subsequent PCR reaction. Microgram quantities of a specified DNA fragment can be obtained after the PCR reactions are repeated many times.

Practically, these steps are easily manipulated in a single microcentrifuge tube and only a 'once and for all' preparation is needed for the multiple amplification cycles. The thermostable DNA polymerases diminish the need of adding fresh enzyme for each cycle and also increase the specificity and reaction yield as a result of the ability to use more stringent conditions (higher temperature) in the reaction. Thermo-cycling devices with their sophisticated software enable researchers to efficiently control the temperature and also the rate and time of temperature changes.

The PCR technique may also serve as a special tool for subcloning purposes. A subcloning procedure may be complicated by the need for a restriction site on a DNA fragment of interest. Using PCR, any restriction sites can be incorporated into both ends of the newly synthesized DNA fragments. This can be achieved because primers which contain a few mismatches or carry uncomplementary sequence at their

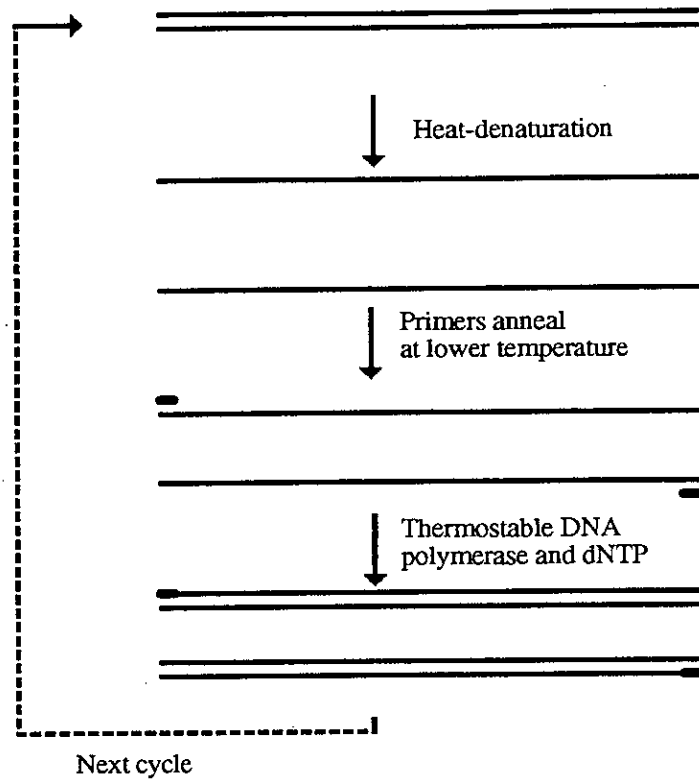


Figure 7.1. Simplified diagram showing the principle of the PCR technique. The double stranded DNA template is heat-denatured (typically at 90-95°C) into single strand DNAs. The temperature is then lowered to 40-60°C to allow the annealing of the primers. The primers are extended by the thermostable DNA polymerase at the optimum temperature about 70°C. The result is two identical double strand DNAs which can act as templates in the next cycle of PCR reaction.

5' end can still hybridize successfully to the DNA template and prime the reaction. By altering the primer sequence or adding restriction site sequences at the 5' end of the primer, the PCR products generated will also contain these restriction sites. Thus they can be digested and subcloned into a suitable restriction site in the plasmid vector.

7.1.3. PCR technical problems.

Like many methods, the PCR technique has its own problems. The reaction kinetics between a specific template and a set of primers differ depending on the primers used. The specificity of the PCR reaction may depend on many factors, for example concentration of the template DNA, Mg^{++} concentration, enzyme concentration, primer concentration, temperature, rate and time of temperature changes, contaminated substances in the reaction and pH (Saiki, 1989 and Gelfand, 1989). The reaction conditions need to be evaluated before the PCR reactions are performed and optimization is often an empirical process. The fidelity of the PCR product may also vary in different reaction conditions. This is because the DNA polymerases, e.g. *Taq* DNA polymerase, used in the PCR protocol lack 'proof-reading' activity, and some nucleotides may be misincorporated into the newly synthesized DNA. This problem is often minimized by optimizing the factors already mentioned above and lowering the number of cycles used. Recently, this problem has been minimized by the introduction of thermostable DNA polymerases which have 'proof-reading' activity.

Several *P. falciparum* genes have been analyzed by PCR techniques, for example, DHFR-TS (Thaithong *et al*, 1992), MSA-1 (Jongwutiwes *et al*, 1992), RAP-2 (Saul *et al*, 1992) and AMA-1 (Thomas *et al*, 1990). In fact, the *rap-1* gene from Honduras I/CDC isolate was also subcloned by this technique and its sequence determined (Howard, 1992). The conservation of the *rap-1* gene is also further investigated by the PCR technique in this chapter.

7.2. Results.

7.2.1. Polymerase chain reaction (PCR) and Southern blot analysis.

In this study, the *rap-1* genes of nine culture-adapted *Plasmodium falciparum* clones or isolates were amplified from genomic DNA by the PCR technique (see Method 2.2.2.14.). The clones or isolates are 3D7, HB3, GF881, K29, MAD20, NF54, T9/94, T9/96 and K1. Details about all clones and isolates are described in Table 7.1. Because the sequence of the primers used in this experiment were derived from the *rap-1* gene of the K1 allele (Ridley *et al*, 1990a), the K1 isolate was included as a positive control.

Two oligonucleotides, 142H and 143H, were used as primers in the PCR reactions. The sequence of both primers are shown in Fig. 7.2. and the flanking regions of the *rap-1* gene from K1 isolate is compared. *Pst*I and *Xba*I restriction sites were added to the 5' end of 142H and 143H primers respectively for future manipulation.

The PCR product from each isolate, including the K1 isolate, gave a single band by agarose gel electrophoresis as illustrated in Fig. 7.3. (lane 1-9). The size of all the PCR products was about 2.4 kb, the same size as that two reported *rap-1* alleles of which the sequence are known (Ridley *et al*, 1990a and Howard, 1992). This suggested that there is no notable insertion and deletion in the *rap-1* gene from any of the clones and isolates used in this study. No non-specific product was detected.

To verify the identity and homology of the *rap-1* gene, the PCR products of all isolates were transferred onto a Hybond-N membrane by Southern blotting and probed with the radiolabelled EcoRI-SalI fragment which had been purified from the recombinant clone, pMS_{ES} (as described in Chapter 3.). The probe is a part of *rap-1* gene from the K1 isolate which covers the nucleotides 182 to 1824. The transferred membrane was washed under high stringency conditions. The result is shown in Fig. 7.4.. The PCR products from all isolates and clones tested were hybridized to the

Isolates or Clones	Name	Origins	Remarks
Isolates	GF881	Gambia	
	K1	Thailand	from Kanchanaburi province.
	K29	Thailand	from Kanchanaburi province.
	MAD20	Papua	
	NF54	Netherlands	
Clones	3D7	Netherlands	derived from NF54 isolate.
	HB3	Honduras	derived from Honduras I isolate.
	T9/94	Thailand	derived from T9 isolate (Tak province).
	T9/96	Thailand	derived from T9 isolate (Tak province).

Table 7.1. Details of all isolates and clones used in this study.

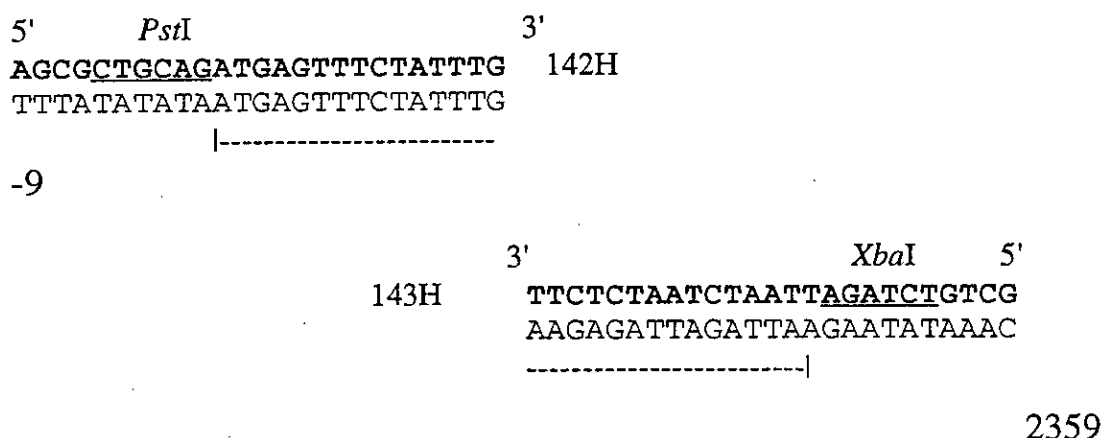


Figure 7.2. The sequence of primers used in the PCR experiment (in bold letters) shown in comparison with the sense strand of the *rap-1* gene sequence from K1 isolate (in normal letter). The restriction enzyme sites *Pst*I and *Xba*I, which were incorporated into the primer sequence are also shown (underlined). The RAP-1 coding region, starting with the initiating methionine (142H) and ending with the stop codon (143H) is shown as broken line. The numbers below, -9 and 2359, indicate positions of each end of the primers related to the *rap-1* gene.

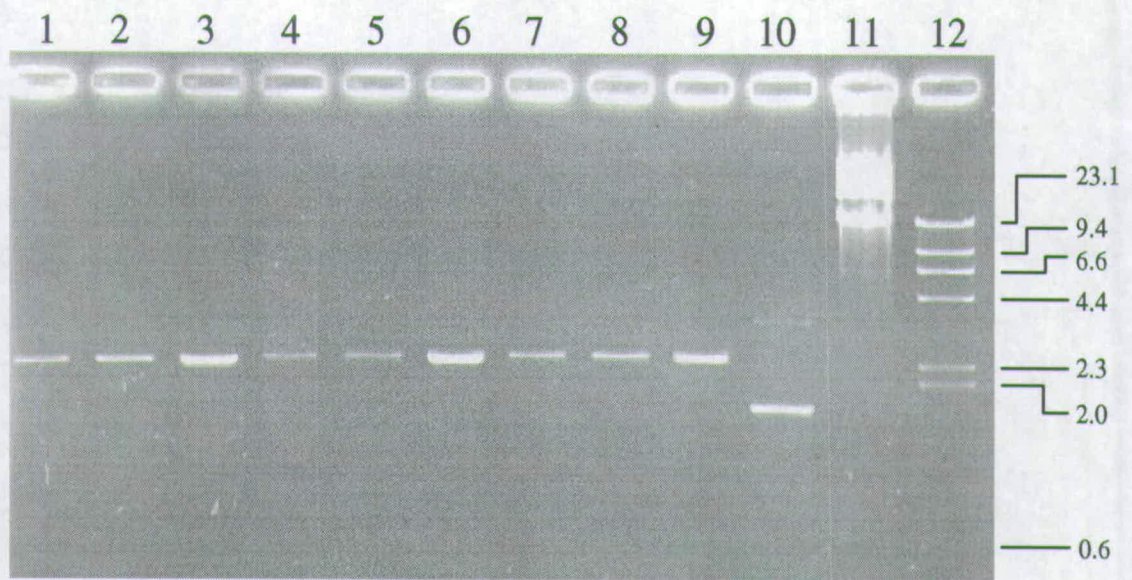


Figure 7.3. PCR products of different strains of *Plasmodium falciparum* are subjected to agarose gel electrophoresis (lane 1-9) and stained with ethidium bromide. 1.) T9/96, 2.) T9/94, 3.) NF54, 4.) MAD20, 5.) K29, 6.) K1, 7.) HB3, 8.) GF881, 9.) 3D7. Positive and negative control for hybridization analysis also included (lane 10 and 11 respectively). 10.) *EcoRI* -*SalI* fragment from pMS_{ES}, 11.) pMS1S vector without insert. Lambda markers cut with *HindIII* are shown in lane 12.



Figure 7.4. Southern blot analysis. The DNA fragments shown in Fig. 7.3. were transferred onto a nylon membrane. Hybridization was carried out at 40°C as described (see Method 2.2.2.12.) and the blot was wash at high stringency condition, 0.1 times SSC, 1%SDS at 65°C for 15 mins twice. 1.) T9/96, 2.) T9/94, 3.) NF54, 4.) MAD20, 5.) K29, 6.) K1, 7.) HB3, 8.) GF881, 9.) 3D7, 10.) *EcoRI*- *SalI* fragment from pME4, 11.) pMS1S, 12.) Lamda markers cut with *HindIII*.

probe, indicating a high level of homology between the K1 allele and the PCR products of all isolates and clones. However, this cannot rule out the presence of minor variation or blocks of variation sequence within conserved regions. To confirm the degree of conservation of the *rap-1* gene, the whole gene from different clones and isolates need to be sequenced and compared.

7.2.2. Subcloning of PCR products and sequence analysis.

As mentioned before, the thermostable DNA polymerase may misincorporate some nucleotides during PCR reaction. To ensure that the changes found are a result of differences in the *P. falciparum* genomic sequence, two independent PCR reactions were performed for each isolate and clone and their PCR products were separately subcloned into *Pst*I and *Sma*I sites of the pMS1S vector and transformed into *Escherichia coli* strain NM522 as illustrated in Fig. 7.5.. Therefore, the differences in DNA sequence between different isolates and clones could be easily confirmed by sequencing the products from the second PCR reaction.

Recombinant plasmids containing PCR generated DNA fragments from 3D7, HB3, GF881, K29 and T9/96 were obtained. Not all of the PCR products were able to subclone into the plasmid vector. The subcloning of PCR products from duplication PCR reactions of 3D7, K29 and GF881 were easily achieved. However, in the case of HB3 and T9/96, only one PCR product from each clone was successfully subcloned. Confirmation sequencing of the duplicate recombinant plasmids (kindly performed by R. Moon and A. Edgar) demonstrated that there is no misincorporation of nucleotide by thermostable DNA polymerase under the conditions used. This suggested that the HB3 and T9/96 allele sequences were generated by a high fidelity condition and can be represented as the genomic DNA sequences.

The PCR product fragments in the recombinant plasmids were sequenced by the dideoxy chain termination method (see Method 2.2.2.15.) using internal

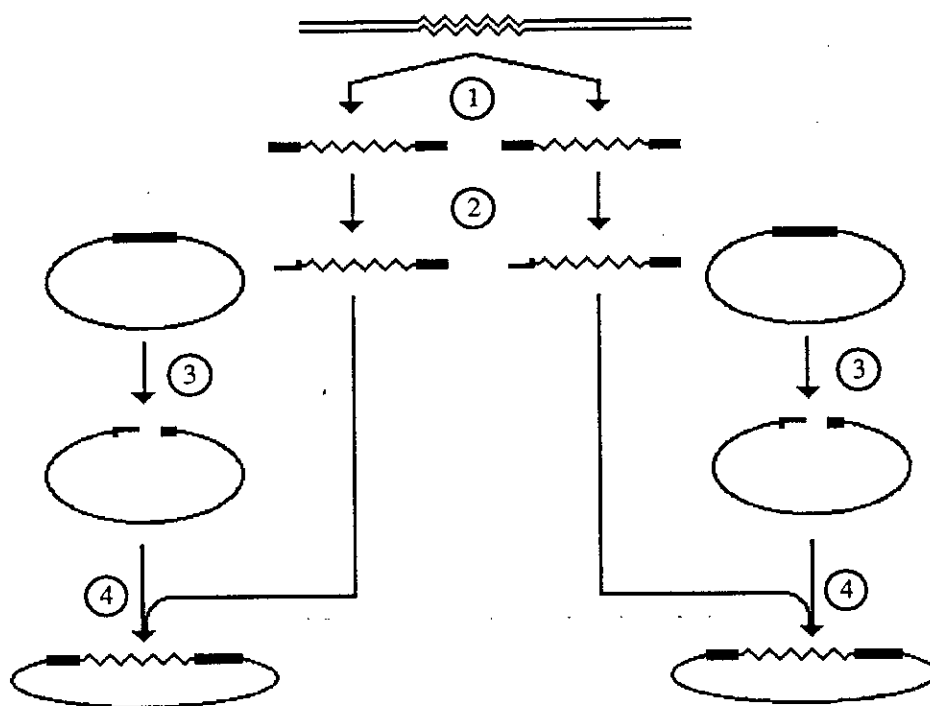


Figure. 7.5. The strategies for subcloning the *rap-1* gene is as described in the text (see Results). To confirm the changes in the genomic DNA, two PCR reactions were performed (1) with each isolates and clones. The PCR products were digested with *Pst*I (2) and individually subcloned into the pMS1S vector digested with *Pst*I and *Sma*I (3). After the ligation, the recombinant plasmids were obtained (4). The recombinant plasmids were then separately transformed, screened and sequenced.

oligonucleotides derived from the K1 allele sequence. The results are shown in Fig. 7.6. From all the sequence data available, only 21 divergent nucleotides from the K1 allele are found. Five of these are silent and result in no amino acid changes (position 264, 1005, 1653, 1926 and 2286). The other sixteen position, however, result in amino acid changes (position 187, 197, 425, 446, 802, 859, 880, 982, 990, 1019, 1108, 1204, 1351, 1427, 2155 and 2198). No deletion or addition of nucleotide was found. All of these nucleotide changes occur preferably at the first, second and third base of a codon, respectively.

The pattern of nucleotide variations in *rap-1* gene show some similarity among different isolates of *P. falciparum*. Seven variations are found more than once. Nucleotide change at position 1019 is found in all strains studied and is also present in the Honduras I/CDC clone (Howard, 1992). The nucleotide changes at 187th, 425th, 802th, 990th 1180th and 1351th also found at least twice. This suggested that the nucleotide changes in these positions do not occur randomly. Among the alleles studied, it seems that only one of two nucleotides will occupy a given position. This implies that any given position one of two amino acids will be found. The only exception is the nucleotide at position 990 where three types of codon changes were found. However, it still shows dimorphic variation at the amino acid level. The amino acid variations in the different *rap-1* alleles are shown in Fig. 7.7..

There are several features should be noted (i) all proline and cysteine residues in the K1 allele are conserved in all isolates studied. (ii) No amino acid change is found in the KSSSPS motifs and its related repeats. (iii) The identified immunodominant epitopes of the monoclonal antibodies studied in this thesis and the sequence surrounding the adjacent cleavage site are also conserved. (iv) There is no amino acid change in the putative signal peptide sequence at the N-terminus.

Most variations are found to occur in the middle and toward the N terminus of the gene as shown in Fig. 7.8. The majority of non-silent nucleotide variations

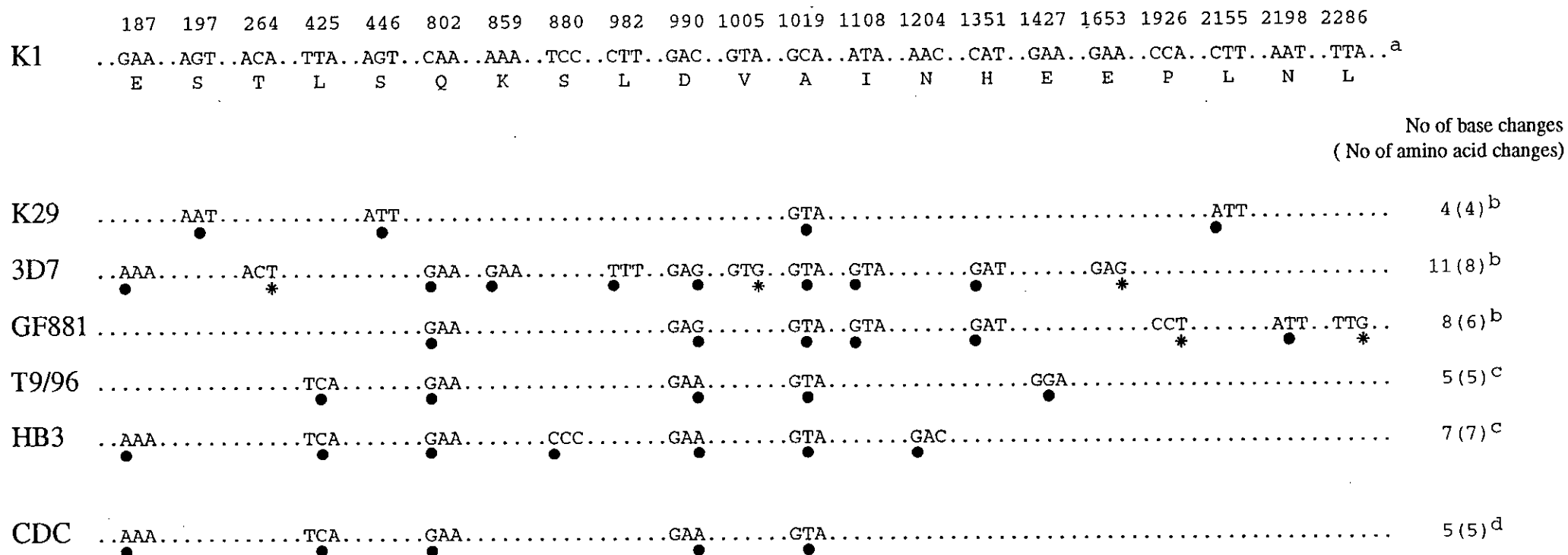


Figure 7.6. Schematic diagram shows nucleotide changes among different strains of *P. falciparum* from different geographic sources. The numbers on the top represent the mutation position. Most of the base changes result in amino acid changes (●) but some of them are silent mutation (*).

a) from Ridley *et al*, 1990a.

b) sequences were generated from PCR products and were confirmed by sequencing two independent PCR reactions.

c) only one PCR product was sequenced.

d) from Howard, 1992.

	63	66	142	149	268	287	294	328	330	340	370	402	451	476	719	733	
K1	E....S....L....S....Q....K....S....L....D....A....I....N....H....E....L....N....	a															
K29	N.....I.....V.....I.....	b															
3D7	K.....E....E.....F....E....V....V.....D.....	b															
GF881	E.....E....V....V.....D.....I....	b															
T9/96	S.....E.....E....V.....G.....	c															
HB3	K.....S.....E.....P.....E....V.....D.....	c															
CDC	K.....S.....E.....E....V.....	d															

Figure 7.7. Schematic diagram shows amino acids changes of RAP-1 protein among different strains of *P. falciparum* from different geographic sources. The numbers on the top represent the amino acid numbers. The silence mutations (compared to the K1 sequence) are excluded.

a) from Ridley *et al*, 1990a.

b) sequences were generated from PCR products and were confirmed by sequencing two independent PCR reactions.

c) only one PCR product was sequenced.

d) from Howard, 1992.

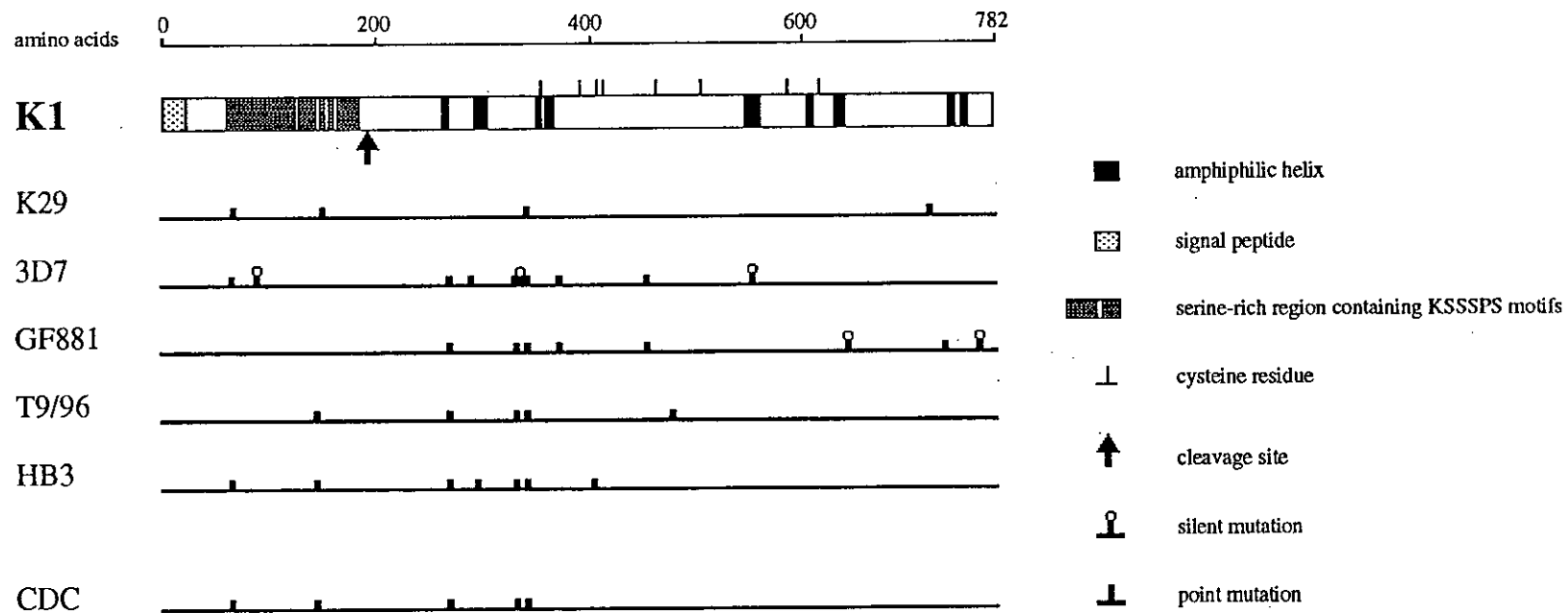


Figure 7.8. Schematic diagram shows amino acid variation in different strains in comparison with the structural diagram of RAP-1 protein in the K1 isolate.

cluster in the middle of the *rap-1* gene. The nucleotide variations at the C terminus are mostly silent or result in conserved amino acid changes.

The isolate which is the most diverse from the K1 isolate is 3D7. K29 has the closest sequence to the K1 isolate. Silent nucleotide variations are found only in the isolates which are the most diverse from the K1 isolate, namely 3D7 and GF881 (Fig. 7.6). The alignment of variation points between different isolates show some similarity in the T9/96 and Honduras I/CDC clone (Fig. 7.6).

7.3. Discussion.

The accumulative data presented here demonstrates that the *rap-1* gene exists in all studied isolates and confirms that the *rap-1* gene is highly conserved among different isolates and clones regardless of their origins (see below). Some antigens and isoenzymes of these isolates and clones have previously been characterized and demonstrated variation in several phenotypes, for example reactivity against monoclonal antibodies (McBride *et al*, 1982, McBride *et al*, 1985 and Walliker *et al*, 1987), differences in protein mobility on 2-dimensional gel electrophoresis (Fenton, 1987) or differences in the forms of adenosine deaminase isoenzyme (Walliker *et al*, 1987 and Thaithong *et al*, 1984).

The conservation of the RAP-1 protein and other rhoptry antigens such as RAP-2 (Saul *et al*, 1992), RhopH3 (Brown and Coppel, 1991) and the apical-associated protein AMA-1 or PF83 (Thomas *et al*, 1990) supports the idea that rhoptry proteins may play an important role in the parasite.

From the sequence analysis, the data suggested that between 99.5 to 99.8% of nucleotide sequences or approximately 99 to 99.5% of protein sequences of *rap-1* allele are conserved when compared with the K1 allele (Fig. 7.6. and Fig. 7.7.). Another protein which possibly associates with rhoptries, AMA-1/PF83, shows approximately 4% variation in both the amino acid and DNA level (Thomas *et al*,

1990). This protein not only has a cluster of amino acid changes at the middle of the gene and toward the N-terminus but also shows the highest frequency of amino acid variation in the middle region of the gene, similar to *rap-1*. Analysis of another rhoptry protein which associates with the RAP-1 protein, namely RAP-2, showed that about 99.7% of amino acid residues are conserved (Saul *et al*, 1992).

Because the fact that the use of K1 allele as a reference in the comparison may distort the overall image of *rap-1* gene variations, a consensus sequence of the *rap-1* gene was deduced and compared with other *rap-1* alleles. The result is shown in Fig. 7.9.. The *rap-1* gene appears more conserved when it is compared with consensus sequence with between 99.4 to 99.7% of the nucleotide sequence conserved.

Most of the positions where amino acid variations are observed, the consensus amino acid residues, 14 of 16, have preferences toward the higher frequency amino acids (of nonrepetitive coding regions) found in *Plasmodium falciparum* as observed by Saul and Battistutta (Saul and Battistutta, 1988). The same result was also observed at the nucleotide level. Fifteen of twenty-one codon change positions have consensus codons which are found at a higher frequency than their variants.

It is known that proline and cysteine residues are important for the structural determination of a protein. Proline is known to interrupt the helical structure while two cysteine residues are able to form a disulfide bond in a protein molecule. These amino acid residues are well conserved among different *rap-1* alleles. Only one nucleotide change occurs at a proline codon at the 642nd amino acid but there is no change at the amino acid level. This suggests that these amino acids may be particularly important for the overall structure and function of the RAP-1 protein as suggested by Ridley and his collaborates (Ridley *et al*, 1991).

No correlation between diversity of RAP-1 gene and geographical origin can be drawn as the sample size is too small. However, the different alleles can be divided into three broad groups, K1 group (K1 and K29), 3D7 group (3D7 and GF881), CDC group (CDC, HB3 and T9/96). This classification based on the

	63	66	142	149	268	287	294	328	330	340	370	402	451	476	719	733	
Consensus	E....S....L....S....E....K....S....L....E....V....I....N....H....E....L....N....																
K1	Q.....D....A.....																a
K29	N.....I....Q.....D.....I.....																b
3D7	K.....E.....F.....V.....D.....																b
GF881	V.....D.....I.....																b
T9/96	S.....G.....																c
HB3	K.....S.....P.....D.....																c
CDC	K.....S.....																d

Figure 7.9. Schematic diagram shows amino acids changes of RAP-1 protein among different strains of *P. falciparum* from different geographic sources compared with consensus sequence. The numbers on the top represent the amino acid numbers. The silence mutations (compared to the consensus sequence) are excluded.

a) from Ridley *et al*, 1990a.

b) sequences were generated from PCR products and were confirmed by sequencing two independent PCR reactions.

c) only one PCR product was sequenced.

d) from Howard, 1992.

similarity of the amino acid variations, comparison with the consensus sequence (Fig. 7.9.). It must be noted that the T9/96 clone which was originally isolated from another province in Thailand has no amino acid change in common with K1 and K29 alleles. On the other hand, the sequence of *rap-1* alleles from Thailand isolate and clones, K1, K29 and T9/96, are similar to each other compared with those from different origins, 3D7 and GF881 (Fig. 7.7.). The low level of sequence diversity among CDC, HB3, and K1 alleles which originated from different geographical sources was also observed (Fig. 7.7.). This partly suggested that the amino acid changes in closely related alleles may similar to each other but this has to be confirmed by further study of *rap-1* alleles.

The sequence data received from the HB3 and Honduras I/CDC clone both of which are derived from H1 from Honduras (Walliker *et al*, 1987 and Howard, 1992) are similar, but not identical, to each other. There are two unique nucleotide changes at position 880 and 1024 which only occurred in the HB3 clone (Fig. 7.6.). This may be because there are multiple populations in the H1 isolate which is a common occurrence in field isolates (Thaithong *et al*, 1984). Alternatively, mutations could have been generated during prolonged culture.

In short, the results in this chapter indicate that the *rap-1* alleles in different isolates and clones studied are highly conserved, but limited variations are observed. The proline and cysteine residues, in particular, are conserved. All the major epitopes of monoclonal antibodies tested and the proteolytic cleavage site region are also highly conserved. Together with the inhibitory effect of 2.13-group monoclonal antibodies, it leads to the conclusion that the immunodominant region near the cleavage site, TLTPLEELYP₂₁₀, and its flanking regions may play crucial role in RAP-1 function and structure.

Chapter 8

Conclusion.

8.1. Conclusion.

The epitope mapping studies of the inhibitory monoclonal antibodies reveal information about the possible structure-function relationships of the RAP-1 protein and the immunogenicity. Because all the inhibitory monoclonal antibodies recognized a linear epitope, TLTPLEELYP₂₁₀, near to the cleavage site (Between A₁₉₀ and D₁₉₁), it suggests that the cleavage of the RAP-1 protein may be important for its functions, e.g. for activation. Activity regulation of protein function by means of proteolytic cleavage was also found in other proteins, for example human cathepsin S, a lysosomal cysteine protease (Wiederanders *et al*, 1992) and several aspartic protease (Blair and Semler, 1991) etc.

Pulse-chase labelling studies previously showed that the RAP-1 protein is synthesized at mid-trophozoite stage and processed into 65 kDa protein at the time of schizont rupture (Clark *et al*, 1987). Other studies carried out in Dr. J. McBride's laboratory demonstrated that the 65 kDa protein is only associated with parasitised erythrocytes and not with merozoites (Harnyuttanakorn *et al*, 1992). These lead to the conclusion that the 65 kDa form of the RAP-1 protein may play an important role in the releasing of malaria parasites from the red blood cells and the cleavage of the RAP-1 to the 65 kDa form may be a crucial step to the protein function. The amphiphilic sequences on the RAP-1 protein may allow for membrane interaction leading to disruption of the erythrocyte membrane (Ridley *et al*, 1990a).

The immunodominant of the epitope TLTPLEELYP₂₁₀ in mice immunized with the native form of the RAP-1 protein is striking. Information about the RAP-1 protein immunogenicity in different animals is obtained from the comparison of the rabbit polyclonal sera and the human sera. Although an antigenicity of a protein is not

an intrinsic property of the protein and depends largely on immunized animals, the 'inhibitory epitope' region on the RAP-1 protein is recognized by both rabbit and human immune sera pool which had arisen from exposure to the native form of the RAP-1 protein. This result suggests the possibility of using the RAP-1 protein to stimulate an immune response to the 'inhibitory epitope' in order to achieve protective immunity in humans.

Different forms of the RAP-1 protein, native and non-native forms, show similar but not identical immunogenicity in mice. While all inhibitory monoclonal antibodies, obtained from immunization with native form of the RAP-1 protein, recognized the 'inhibitory epitope', the expressed RAP-1 protein (non-native form) elicits immune response against 3 different epitopes. Most of these monoclonal antibodies recognized the EEFEYD₂₄₈ peptide which suggests that this epitope may be highly immunodominant in the non-native form. However, two of these monoclonal antibodies also recognized the NTLTPLEELY₂₀₉ peptide. This suggests that the 'inhibitory epitope' is immunogenic in both native and non-native forms. The use of expressed protein which represents only a part of the RAP-1 protein in immunization also suggests that it is possible to design the malaria vaccine based on the RAP-1 protein as a small expressed protein or a synthetic peptide containing the 'inhibitory epitope'.

The sequence analysis of the *rap-1* gene in different isolates and clones points out that the *rap-1* gene is a highly conserved gene. The 'inhibitory epitope' region and other identified epitopes are also conserved. No amino acid change is found at the cleavage site. This supports the hypothesis that a specific cleavage mechanism operates during the processing of the RAP-1 protein. The conservation of this protein strengthens its potential as a malaria vaccine and a target gene for *P. falciparum* screening in the field.

8.2. Future study.

Several topics arising from this study on the RAP-1 protein and the inhibitory monoclonal antibodies need to be studied in more detail. In particular the mechanisms by which the inhibitory monoclonal antibodies work needs to be confirmed. The inhibitory effects of monoclonal antibodies from Hoffman La Roche should also be established to assess if proximity of an epitope to the cleavage site results in monoclonal antibody inhibition. The expressed RAP-1 protein or a synthetic peptide containing the 'inhibitory epitope' should be used for vaccination trial in monkeys to study their immunogenicity and protective capabilities. Also, the immune response against this protein should be studied in the field to reveal the response to the RAP-1 protein in immune individuals.

References

- Aikawa, M. (1971) Plasmodium: The Fine structure of Malaria Parasites. *Exp. Parasitol.* 30, 284-320.
- Aikawa, M., Miller, L.H., Johnson, J. and Rabbege, J. (1978) Erythrocyte entry by malarial parasite. A moving junction between erythrocyte and parasite. *J. Cell Biol.* 77, 72-81.
- Aikawa, M., Miller, L.H., Rabbege, J.R. and Epstein, N. (1981) Freeze-Fracture Study on the Erythrocyte Membrane during Malarial Parasite Invasion. *J. Cell Biol.* 91, 55-62.
- Amit, A.G., Mariuzza, R.A., Phillips, S.E.V. and Poljak, R.J. (1986) Three-Dimensional Structure of an Antigen-Antibody Complex at 2.8°A Resolution. *Science* 233, 747-753.
- Aronson, N.E., Silverman, C., Wasserman, G.F., Kochan, J., Hall, B.T., Esser, K., Young, J.E. and Chulay, J.D. (1991) Immunization of owl monkeys with a recombinant protein containing repeated epitopes of a *Plasmodium falciparum* glycoporphin-binding protein. *Am. J. Trop. Med. Hyg.* 45, 548-559.
- Atherton, E. and Sheppard, R.C. (1989) Solid phase peptide synthesis, a practical approach. IRL Press at Oxford University Press. Oxford.
- Ballou, W.R., Hoffman, S.L., Sherwood, J.A., Hollingdale, M.R., Neva, F.A., Hockmeyer, W.T., Gordon, D.M., Schneider, I., Wirtz, R.A, Young, J.F., Wasserman,

G.F., Reeve, P., Diggs, C.L. and Chulay, J.D. (1987) Safety and efficacy of a recombinant DNA *Plasmodium falciparum* sporozoite vaccine. *Lancet* i, 1277-1281.

Bannister, L.H., Mitchell, G.H., Butcher, G.A. and Dennis, E.D. (1986) Lamellar membranes associated with rhoptries in erythrocytic merozoites of *Plasmodium knowlesi* : a clue to the mechanism of invasion. *Parasitol.* 92, 291-303.

Bannister, L.H. and Mitchell, G.H. (1989) The Fine Structure of Secretion by *Plasmodium knowlesi* Merozoites during Red Cell Invasion. *J. Protozool.* 36, 362-367.

Benjamin, D.C., Berzofsky, J.A., East, I.J., Gurd, F.R.N., Hannum, C., Leach, S.J., Margoliash, E., Michael, J.G., Miller, A., Prager, E.M., Reichlin, M., Sercarz, E.E., Smith-Gill, S.J., Todd, P.E. and Wilson, A.C. (1984) The antigenic structure of proteins: a reappraisal. *Ann. Rev. Immunol.* 2, 67-101.

Berzins, K., Perlmann, H., Wählin, B., Carlsson, J., Wahlgren, M., Udomsangpetch, R., Bjorkman, A., Patarroyo, M.E. and Perlmann, P. (1986) Rabbit and human antibodies to a repeated amino acid sequence of a *Plasmodium falciparum* antigen, Pf155, react with the native protein and inhibit merozoite invasion. *Proc. Natl. Acad. Sci. USA* 83, 1065-1069.

Blackman, M.J., Whittle, H. and Holder, A.A. (1991) Processing of the *Plasmodium falciparum* major merozoite surface protein-1: identification of a 33-kilodalton secondary processing product which is shed prior to erythrocyte invasion. *Mol. Biochem. Parasitol.* 49, 35-44.

Blackman, M.J. and Holder, A.A. (1992) Secondary processing of the *Plasmodium falciparum* merozoite surface protein-1(MSP1) by a calcium-dependent membrane-

bound serine protease: shedding of MSP1₃₃ as a noncovalently associated complex with other fragments of the MSP1. Mol. Biochem. Parasitol. 50, 307-316.

Brinkmann, U. and Brinkmann, A. (1991) Malaria and health in Africa: The present situation and epidemiological trends. Trop. Med. Parasitol. 42, 204-213.

Brown, G.V., Culvenor, J.G., Crewther, P.E., Bianco, A.E., Coppel, R.L., Saint, R.B., Stahl, H., Kemp, D.J. and Anders, R.F. (1985) Localization of the ring-infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum* in merozoites and ring-infected erythrocytes. J. Exp. Med. 162, 774-779.

Brown, H.J. and Coppel, R.L. (1991) Primary structure of a *Plasmodium falciparum* rhoptry antigen. Mol. Biochem. Parasitol. 49, 99-110.

Brown, K.N., Boyle, D.B. and Newbold, C.I. (1982) Maturation of the intracellular parasite and antigenicity. In "Malaria and the red cell." (Evered, D. and Whelan, J. edi.) Pitman Books Ltd., London. p. 24-36.

Bushell, G.R., Ingram, L.T., Fardoulis, C.A. and Cooper, J.A. (1988) An antigenic complex in the rhoptries of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 28, 105-112.

Campbell, G.H., Miller, L.H., Hudson, D., Franco, E.L. and Andrysiak, P.M. (1984) Monoclonal antibody characterization of *Plasmodium falciparum* antigens. Am. J. Trop. Med. Hyg. 33, 1051-1054.

Cerami, C., Kwakye-Berko, F. and Nussenzweig, V. (1992) Binding of malarial circumsporozoite protein to sulfatides [Gal(3-SO₄) β 1-Cer] and cholesterol-3-sulfate

and its dependence on disulfide bond formation between cysteines in region II. Mol. Biochem. Parasitol. 54, 1-12.

Chan, S. (1991) PhD thesis. University of Edinburgh.

Cheung, A., Leban, J., Shaw, A.R., Merkli, B., Stocker, J., Chizzolini, C., Sander, C. and Perrin, L.H. (1986) Immunization with synthetic peptides of a *Plasmodium falciparum* surface antigen induces antimerozoite antibodies. Proc. Natl. Acad. Sci. USA 83, 8328-8332.

Chulay, J.D., Aikawa, M., Diggs, C., Haynes, J.D. (1981a) Inhibitory effects of immune monkey serum on synchronized *Plasmodium falciparum* cultures. Am. J. Trop. Med. Hyg. 30, 12-19.

Chulay, J.D., Haynes, J.D. and Diggs, C.L. (1981b) Inhibition of *in Vitro* Growth of *Plasmodium falciparum* by Immune Sera from Monkeys. J. Infect. Dis. 144, 270-278.

Chung, C.T. and Miller, R.H. (1988) A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acids Res. 16, 3580.

Clark, J.T., Anand, R., Akoglu, T. and McBride, J.S. (1987) Identification and characterization of proteins associated with the rhoptry organelles of *Plasmodium falciparum* merozoites. Parasitol. Res. 73, 425-434.

Clyde, D.F. (1990) Immunity to falciparum and vivax malaria induced by irradiated sporozoites: a review of University of Maryland studies, 1971-5. Bull. WHO. 68 (suppl.), 9-12.

Cohen, S., McGregor, I.A. and Carrington, S. (1961) Gamma globulin and acquired immunity to human malaria. *Nature* 192, 733-737.

Collins, W.E., Anders, R.F., Pappaioanou, M., Campbell, G.H., Brown, G.V., Kemp, D.J., Coppel, R.L., Skinner, J.C., Andrysiak, P.M., Favaloro, J.M., Corcoran, L.M., Broderson, J.R., Mitchell, G.F. and Campbell, C.C. (1986) Immunization of *Aotus* monkeys with recombinant proteins of an erythrocyte surface antigen of *Plasmodium falciparum*. *Nature* 323, 259-262.

Collins, W.E., Pappaioanou, M., Anders, R.F., Campbell, G.H., Brown, G.V., Kemp, D.J., Broderson, J.R., Coppel, R.L., Skinner, J.C., Procell, P.M., Favaloro, J.M., Corcoran, L.M., Ma, N.S-F., Mitchell, G.F. and Campbell, C.C. (1988) Immunization trials with the ring-infected erythrocyte surface antigen of *Plasmodium falciparum* in owl monkeys (*Aotus vociferans*). *Am. J. Trop. Med. Hyg.* 38, 268-282.

Cooper, J.A., Cooper, L.T. and Saul, A.T. (1992) Mapping of the region predominantly recognized by antibodies to the *Plasmodium falciparum* merozoite surface antigen MSA1. *Mol. Biochem. Parasitol.* 51, 301-312.

Cooper, J.A., Ingram, L.T., Bushell, G.R., Fardoulis, C.A., Stenzel, D., Schofield, L. and Saul, A.J. (1988) The 140/130/105 kilodalton protein complex in the rhoptries of *Plasmodium falciparum* consists of discrete polypeptides. *Mol. Biochem. Parasitol.* 29, 251-260.

Coppel, R.L., Bianco, A.E., Culvenor, J.G., Crewther, P.E., Brown, G.V., Anders, R.F. and Kemp, D.J. (1987) A cDNA clone expressing a rhoptry protein of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 25, 73-81.

Cox, F.E.G. (1983) How red blood cells and malaria parasites recognize each other. *Nature* 301, 654.

Crewther, P.E., Culvenor, J.G., Silva, A., Cooper, J.A. and Anders, R.F. (1990) *Plasmodium falciparum*: Two Antigens of Similar Size Are Located in Different Compartments of the Rhoptry. *Exp. Parasitol.* 70, 193-206.

Dame, J.B., Williams, J.L., McCutchan, T.F., Weber, J.L., Wirtz, R.A., Hockmeyer, W.T., Maloy, W.L., Haynes, J.D., Schneider, I., Roberts, D., Sanders, G.S., Reddy, E.P., Diggs, C.L. and Miller, L.H. (1984) Structure of the Gene Encoding the Immunodominant Surface Antigen on the Sporozoite of the Human Malaria Parasite *Plasmodium falciparum*. *Science* 225, 593-599.

Day, K.P. and Marsh, K. (1991) Naturally acquired immunity to *Plasmodium falciparum*. *Immunology Today* 12, A68-A71.

Dluzewski, A.R., Mitchell, G.H., Fryer, P.R., Griffiths, S., Wilson, R.J.M. and Gratzer, W.B. (1992) Origins of the parasitophorous vacuole membrane of the malaria parasite, *Plasmodium falciparum*, in human red blood cells. *J. Cell Sci.* 102, 527-532.

Dubois, P., Dedet, J., Fandeur, T., Roussilhon, C., Jendoubi, M., Pauillac, S., Mercereau-Puijalon, O. and Pereira Da Silva, L. (1984) Protective immunization of the squirrel monkey against asexual blood stages of *Plasmodium falciparum* by use of parasite protein fraction. *Proc. Natl. Acad. Sci. USA* 81, 229-232.

Dvorak, J.A., Miller, L.H., Whitehouse, W.C. and Shiroishi, T. (1975) Invasion of Erythrocytes by Malaria Merozoites. *Science* 187, 748-750.

Egan, J.E., Weber, J.L., Ballou, W.R., Hollingdale, M.R., Majarian, W.R., Gordon, D.M., Maloy, W.L., Hoffman, S.L., Wirtz, R.A., Schneider, I., Woolleff, G.R., Young, J.F. and Hockmeyer, W.T. (1987) Efficacy of Murine Malaria Sporozoite Vaccines: Implications for Human Vaccine Development. *Science* 236, 453-456.

Epping, R.J., Goldstone, S.D., Ingram, L.T., Upcroft, J.A., Ramasamy, R., Cooper, J.A., Bushell, G.R., Geysen, H.M. (1988) An epitope recognised by inhibitory monoclonal antibodies that react with a 51 kilodalton merozoite surface antigen in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 28, 1-10.

Erlich, H.A. (1989) Basic methodology. In "PCR Technology: principles and applications for DNA amplification" (Erlich, H.A., edi.) Stockton Press, New York, p. 1-5.

Etzion, Z., Murray, M.C. and Perkins, M.E. (1991) Isolation and characterization of rhoptries of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 47, 51-62.

Ewing, C., Ebringer, R., Tribbick, G. and Geysen, H.M. (1990) Antibody activity in ankylosing spondylitis sera to two sites on HLA B27.1 at the MHC groove region (within sequence 65-85), and to a *Klebsiella pneumoniae* nitrogenase reductase peptide (within sequence 181-199). *J. Exp. Med.* 171, 1635-1647.

Fenton, B. (1987) Peptide digest studies of polymorphic proteins of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 24, 13-22.

Fenton, B., Clark, J.T., Khan, C.M.A., Robinson, J.V., Walliker, D., Ridley, R., Scaife, J.G. and McBride, J.S. (1991) Structural and antigenic polymorphism of the 35- to 48-

kilodalton merozoite surface antigen (MSA-2) of the malaria parasite *Plasmodium falciparum*. Mol. Cell. Biol. 11, 963-971.

Gangloff, S., M'Zoughi, R., Lett, E., Scholler, M., Baer, J., Pini, A., Ogier, J. and Klein, J. (1992) Epitope mapping of *Streptococcus mutans* SR protein and human IgG cross-reactive determinants, by using recombinant proteins and synthetic peptides. J Immunol. 148, 3249-3255.

Gelfand, D.H. (1989) *Taq* DNA polymerase. In "PCR Technology: principles and applications for DNA amplification" (Erlich, H.A., edi.) Stockton Press, New York, p. 17-22.

Geysen, H.M., Meloen, R.H. and Barteling, S.J. (1984) Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc. Natl. Acad. Sci. USA 81, 3998-4002.

Geysen, H.M., Barteling, S.J. and Meloen, R.H. (1985) Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein. Proc. Natl. Acad. Sci. USA 82, 178-182.

Geysen, H.M., Rodda, S.J., Mason, T.J., Tribbick, G. and Schoofs, P.G. (1987) Strategies for epitope analysis using peptide synthesis. J. Immunol. Methods 102, 259-274.

Good, M.F., Berzofsky, J.A., Maloy, W.L., Lunde, M.N., Moss, B., Miller, L.H., Hayashi, Y., Fujii, N. and Hockmeyer, W.T. (1987) Genetic control of the immune

response to a *Plasmodium falciparum* sporozoite vaccine and to the circumsporozoite protein. Vaccine 87. Cold Spring Harbour Laboratory. p. 81-85.

Good, M.F., Miller, L.H., Kumar, S., Quakyi, I.A., Keister, D., Adams, J.H., Moss, B., Berzofsky, J.A. and Carter, R. (1988) Limited Immunological Recognition of Critical Malaria Vaccine Candidate Antigens. Science 242, 574-577.

Gough, J.A. and Murray, N.E. (1983) Sequence diversity among related genes for recognition of specific targets in DNA molecules. J. Mol. Biol. 166, 1-19.

Grossberger, D. (1987) Minipreps of DNA from bacteriophage lambda. Nucleic Acids Res. 15, 6737.

Hall, R., McBride, J.S., Morgan, G., Tait, A., Zolg, J.W., Walliker, D. and Scaife, J. (1983) Antigens of the erythrocytic stages of the human malaria parasite *Plasmodium falciparum* detected by monoclonal antibodies. Mol. Biochem. Parasitol. 7, 247-265.

Hall, R., Hyde, J.E., Goman, M., Simmons, D.L., Hope, I.A., Mackay, M. and Scaife, J. (1984) Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria. Nature 311, 379-382.

Harlow, E. and Lane, D. (1988) Antibodies. A laboratory manual. Cold Spring Harbour Laboratory.

Harnyuttanakorn, P., McBride, J.S., Donachie, S., Heidrich, H.-G. and Ridley, R.G. (1992) Inhibitory monoclonal antibodies recognise epitopes adjacent to a proteolytic cleavage site on the RAP-1 protein of *Plasmodium falciparum*. Mol. Biol. Parasitol. 55, 177-186.

Hellebust, H., Murby, M., Abrahmsén, L., Uhlén, M. and Enfors, S. (1989) Different approaches to stabilize a recombinant fusion protein. *BIO/TECHNOLOGY* 7, 165-168.

Herrington, D.A., Clyde, D.F., Losonsky, G., Cortesia, M., Murphy, J.R., Davis, J., Bagar, S., Felix, A.M., Heimer, E.P., Gillessen, D., Nardin, E., Nussenzweig, R.S., Nussenzweig, V., Hollingdale, M.R. and Levine, M.M. (1987) Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. *Nature* 328, 257-259.

Herrington, D.A., Clyde, D.F., Davis, J., Bagar, S., Murphy, J.R., Cortese, J.F., Bank, R.S., Nardin, E., DiJohn, D., Nussenzweig, R.S., Nussenzweig, V., Torres, J.R., Murillo, J., Cortesia, M., Sturchler, D., Hollingdale, M.R. and Levine, M.M. (1990) Human studies with synthetic peptide sporozoite vaccine (NANP)₃-TT and immunization with irradiated sporozoites. *Bull. WHO* 68 (suppl.), 33-37.

Herrington, D., Davis, J., Nardin, E., Beier, M., Cortese, J., Eddy, H., Losonsky, G., Hollingdale, M., Sztein, M., Levine, M., Nussenzweig, R.S., Clyde, D. and Edelman, R. (1991) Successful immunization of humans with irradiated malaria sporozoite: humoral and cellular responses of the protected individuals. *Am. J. Trop. Med. Hyg.* 45, 539-547.

Hill, R.J., Watt, F., Wilson, C.M., Fifi, T., Underwood, P.A., Tribbick, G., Geysen, H.M. and Thomas, J.O. (1989) Bands, interbands and puffs in native *Drosophila* polytene chromosomes are recognized by a monoclonal antibody to an epitope in the carboxy-terminal tail of histone H1. *Chromosoma (Berl.)* 98, 411-421.

Holder, A.A. and Freeman, R.R. (1981) Immunization against blood-stage rodent malaria using purified parasite antigens. *Nature* 294, 361-364.

Hollingdale, M.R., Aikawa, M., Chen, G., Meis, J.F.G.M., Sakhuja, K., Sina, B. and Zhu, J. (1990) Pre-erythrocytic stage malaria parasites: non-circumsporozoite protein antigens. *Bull.WHO* 68 (suppl.), 178-180.

Holt, E.H., Nichols, M.E., Etzion, Z and Perkins, M.E. (1989) Erythrocyte invasion by two *Plasmodium falciparum* isolates differing in sialic acid dependency in the presence of glycophorin A antibodies. *Am. J. Trop. Med. Hyg.* 40, 245-251.

Hope, I.A. (1984a) PhD thesis. University of Edinburgh.

Hope, I.A., Hall, R., Simmons, D.L., Hyde, J.E. and Scaife, J.G. (1984b) Evidence for immunological cross-reaction between sporozoites and blood stages of a human malaria parasite. *Nature* 308, 191-194.

Hope, I.A., Mackay, M., Hyde, J.E., Goman, M. and Scaife, J.G. (1985) The gene for an exported antigen of the malaria parasite *Plasmodium falciparum* cloned and expressed in *Escherichia coli*. *Nucleic Acids Res.* 13, 369-379.

Howard, R.F. and Reese, R.T. (1984) Synthesis of merozoite proteins and glycoproteins during the schizogony of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 10, 319-334.

Howard, R.F., Stanley, H.A., Campbell, G.H. and Reese, R.T.(1984) Proteins responsible for a punctate fluorescence pattern in *Plasmodium falciparum* merozoites. *Am. J. Trop. Med. Hyg.* 33, 1055-1059.

Howard, R.F. (1990) The lower-molecular-weight protein complex (R_I) of the *Plasmodium falciparum* rhoptries lacks the glycolytic enzyme aldolase. Mol. Biochem. Parasitol. 42, 235-240.

Howard, R.F. (1992) The sequence of the p82 rhoptry protein is highly conserved between two *Plasmodium falciparum* isolates. Mol. Biochem. Parasitol. 51, 327-330.

Howells, R.E. (1985) The modes of action of some anti-protozoal drugs. Parasitol. 90, 687-703.

Hyde, J.E. (1990) Molecular Parasitology. Open University Press. Buckingham.

Ifediba, T. and Vanderberg, J.P. (1981) Complete *in vitro* maturation of *Plasmodium falciparum* gametocytes. Nature 294, 364-366.

James, D.M. and Gilles, H.M. (1985) Human antiparasitic drugs: pharmacology and usage. John Wiley and Son, New York. p. 144-156.

Jongwutiwes, S., Tanabe, K., Nakazawa, S., Yanagi, T. and Kanbara, H. (1992) Sequence variation in the tripeptide repeats and T cell epitopes in P190 (MSA-1) of *Plasmodium falciparum* from field isolates. Mol. Biochem. Parasitol. 51, 81-90.

Kaslow, D.C., Quakyi, I.A., Syin, C., Raum, M.G., Keister, D.B., Coligan, J.E., McCutchan, T.F. and Miller, L.H. (1988) A vaccine candidate from the sexual stage of human malaria that contains EGF-like domains. Nature 333, 74-76.

Kaslow, D.C., Good, M.F., Syin, C., Quakyi, I.A., Keister, D.B. and Miller, L.H. (1989) Molecular Analysis of Zygote/Ookinete Target Antigen of Transmission-blocking Immunity in Human and Avian Malaras. Vaccine 89. Cold Spring Harbour Laboratory. p. 319-324.

Kemp, D.J., Coppel, R.L., Cowman, A.F., Saint, R.B., Brown, G.V. and Anders, R.F. (1983) Expression of *Plasmodium falciparum* blood-stage antigens in *Escherichia coli*: Detection with antibodies from immune humans. Proc. Natl. Acad. Sci. USA 80, 3787-3791.

Khusmith, S., Charoenvit, Y., Kumar, S., Sedegah, M., Beaudoin, R.L. and Hoffman, S.L. (1991) Protection Against Malaria by Vaccination with Sporozoite Surface Protein 2 Plus CS Protein. Science 252, 715-718.

Klayman, D.L. (1985) Qinghaosu (Artemesinin): an antimalarial drug from China. Science. 228, 1049-1055.

Kleid, P.G., Yansura, D., Smalll, B., Dowbenko, D., Moore, D., Grubman, M., McKercher, P., Morgan, D.O., Robertson, B.H. and Bachrach, H.L. (1981) Cloned viral protein vaccine for foot and mouth disease. Responses in cattle and swine. Science 214, 1125-1129.

Knell, A.J. edi. (1991) Malaria: a publication of the tropical programme of the Wellcome Trust. Oxford University Press. Oxford.

Kreier, J.P. and Baker, J.R. (1987) Parasitic protozoa. Allen & Unwin (Publishers) Ltd. London.

Krotoski, W.A., Collins, W.E., Bray, R.S., Garnham, P.C.C., Cogswell, F.B., Gwadz, R.W., Killick-kendrick, R., Wolf, R., Sinden, R., Koontz, L.C. and Stanfill, P.S. (1982). Demonstration of hypnozoites in sporozoite-transmitted *Plasmodium vivax* infection in chimpanzees liver. Am. J. Trop. Med. Hyg. 31, 1291-1293.

Ladda, R., Aikawa, M. and Sprinz, H. (1969) Penetration of erythrocytes by merozoites of mammalian and avian malarial parasites. J. Parasitol. 55, 633-644.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Larkin, G.L. and Thuma, P.E. (1991) Congenital malaria in a hyperendemic area. Am. J. Trop. Med. Hyg. 45, 587-592.

Leech, J. (1991) *Plasmodium falciparum* cytoadherence. In "40th forum in immunology" Annals de Institute Pasteur. 142, 681-687.

Lindsay, S.W., Adiamah, J.H., Miller, J.E. and Armstrong, J.R.M. (1991) Pyrethroid-treated bednet effects on mosquitoes of the *Anopheles gambiae* complex in the Gambia. Med. Vet. Ento. 5, 477-484.

Lustigman, S., Anders, R.F., Brown, G.V. and Coppel, R.L. (1988) A component of an antigenic rhoptry complex of *Plasmodium falciparum* is modified after merozoite invasion. Mol. Biochem. Parasitol. 30, 217-224.

Mazier, D., Beaudoin, R.L., Mellouk, S., Druilhe, P., Texier, B., Trosper, J., Miltgen, F., Landau, I., Paul, C., Brandicourt, O., Guguen-Guillouzo, C. and Langlois, P.

(1985) Complete Development of Hepatic Stages of *Plasmodium falciparum* *in vitro*. Science 227, 440-442.

McBride, J.S., Walliker, D. and Morgan, G. (1982) Antigenic Diversity in the Human Malaria Parasite *Plasmodium falciparum*. Science 217, 254-257.

McBride, J.S., Welsby, P.D. and Walliker, D. (1984) Serotyping *Plasmodium falciparum* from acute human infections using monoclonal antibodies. Trans. R. Soc. Trop. Med. Hyg. 78, 32-34.

McBride, J.S., Newbold, C.I. and Anand, R. (1985) Polymorphism of a High Molecular Weight Schizont Antigen of the Human Malaria Parasite *Plasmodium falciparum*. J. Exp. Med. 161, 160-180.

Mendis, K.N., David, P.H. and Carter, R. (1991) Antigenic polymorphism in malaria: is it an important mechanism for immune evasion? Immunology Today 12, A34-A37.

Meshnick, S.R. (1990) Chloroquine as intercalator: a hypothesis revived. Parasitology Today. 6, 77-79.

Miller, L.H., Haynes, J.D., McAuliffe, F.M., Shiroishi, T., Durocher, J. and McGinness, M.H. (1977) Evidence for differences in erythrocyte surface receptors for the malaria parasites *Plasmodium falciparum* and *Plasmodium knowlesi*. J. Exp. Med. 146, 277-281.

Miller, L.H., Howard, R.J., Carter, R., Good, M.F., Nussenzweig, V. and Nussenzweig, R.S. (1986) Research Toward Malaria Vaccines. Science 234, 1349-1356.

Millet, P., Atkinson, C.T., Aikawa, M., Hollingdale, M.R. and Collins, W.E. (1991a) Strain specificity in the liver-stage development of *Plasmodium falciparum* in primary cultures of New World monkey hepatocytes. Am. J. Trop. Med. Hyg. 45, 236-242.

Molano, A., Segura, C., Guzman, F., Lozada, D. and Pattarroyo, M.E. (1992) In human malaria protective antibodies are directed mainly against the Lys-Glu ion pair within the Lys-Glu-Lys motif of the synthetic vaccine SPf66. Parasite Immunol. 14, 111-124.

Moore, D.V. and Lanier, J.E. (1961) Observation on two *Plasmodium falciparum* infections with an abnormal response to chloroquine. Am. J. Trop. Med. Hyg. 10, 5-9.

Moreno, A. and Patarroyo, M.E. (1989) Development of an asexual blood stage malaria vaccine. Blood 74, 537-546.

Nahlen, B.L., Lobel, H.O., Cannon, S.E. and Campbell, C.C. (1991) Reassessment of blood donor selection criteria for United States travelers to malarious areas. Transfusion (Arlington) 31, 798-804.

Napier, R.M. and Venis, M.A. (1992) Epitope mapping reveals conserved regions of an auxin-binding protein. Biochem. J. 284, 841-845.

Neurath, A.R., Strick, N. and Lee, E.S.Y. (1990) B cell epitope mapping of human immunodeficiency virus envelope glycoproteins with long (19- to 36- residue) synthetic peptides. J Gen. Virol. 71, 85-95.

Nussenzweig, R.S., Vanderberg, J.P., Most, H. and Orton, C. (1967) Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei*. Nature 216, 160-162.

Nussenzweig, R.S. and Nussenzweig, V. (1985) Development of a Sporozoite Vaccine. Parasitology Today 1, 150-152.

Nussenzweig, R.S. (1990) Recombinant proteins and synthetic peptides as malaria vaccine candidates. Can. J. Microbiol. 36, 817-820.

Ohlin, M., Broliden, P., Danielsson, L., Wahren, B. , Rosen, J., Jondal, M. and Borrebaeck, C.A.K. (1989) Human monoclonal antibodies against a recombinant HIV envelope antigen produced by primary *in vitro* immunization. Characterization and epitope mapping. Immunol. 68, 325-331.

Oka, M., Aikawa, M., Freeman, R.R., Holder, A.A. and Fine, E. (1984) Ultrastructural localization of protective antigens of *Plasmodium yoelii* merozoites by the use of monoclonal antibodies and ultrathin cryomicrotomy. Am. J. Trop. Med. Hyg. 33, 342-346.

Padlan, E.A., Silverton, E.W., Sheriff, S., Cohen, G.H., Smith-Gill, S.J. and Davies, D.R. (1989) Structure of an antibody-antigen complex: Crystal structure of the HyHEL-10 Fab-lysozyme complex. Proc. Natl. Acad. Sci. USA 86, 5938-5942.

Pancake, S.J., Holt, G.D., Mellouk, S. and Hoffman, S.L. (1992) Malaria Sporozoites and Circumsporozoite Proteins Bind Specifically to Sulfated Glycoconjugates. J. Cell Biol. 117, 1351-1357.

Papsidero, L.D., Sheu, M. and Ruscetti, F.W. (1989) Human Immunodeficiency Virus Type1-Nutralizing Monoclonal Antibodies Which React with p17 Core Protein: Characterization and Epitope Mapping. J. Virol. 63, 267-272.

Pasvol, G. and Wilson, R.J.M. (1982) The interaction of the malaria parasites with red blood cells. British Med. Bull. 38, 133-140.

Patarroyo, M.E., Romero, P., Torres, M.:L., Clavijo, P., Moreno, A., Martínez, A., Rodríguez, R., Guzman, F. and Cabezas, E. (1987) Induction of protective immunity against experimental infection with malaria using synthetic peptides. Nature 328, 629-632.

Patarroyo, M.E., Amador, R., Clavijo, P., Moreno, A., Guzman, F., Romero, P., Tascon, R., Franco, A., Murillo, L.A., Ponton, G. and Trujillo, G. (1988) A synthetic vaccine protects humans against challenge with asexual blood stages of *Plasmodium falciparum* malaria. Nature 332, 158-161.

Perkins, M.E. (1984) Surface proteins of *Plasmodium falciparum* merozoites binding to the erythrocyte receptor, glycophorin. J. Exp. Med. 160, 788-798.

Peters, W. (1985) The problem of drug resistance in malaria. Parasitol. 90, 705-715.

Peterson, M.G., Marshall, V.M., Smythe, J.A., Crewther, P.E., Lew, A., Silva, A., Anders, R.F. and Kemp, D.J. (1989) Integral Membrane Protein Located in the Apical Complex of *Plasmodium falciparum*. Mol. Cell. Biol. 9, 3151-3154.

- Piétu, G., Ribba, A, Chérel, G. and Meyer, D. (1992) Epitope mapping by cDNA expression of a monoclonal antibody which inhibits the binding of von Willebrand Factor to platelet glycoprotein IIb/IIIa. *Biochem. J.* 284, 711-715.
- Playfair, J.H.L., Taverne, J., Bate, C.A.W. and De Souza, J.B. (1990) The malaria vaccine: anti-parasite or anti-disease. *Immunology Today* 11, 25-27.
- Radford, A.J., Wood, P.R., Billman-Jacobe, H., Geysen, H.M., Mason, T.J. and Tribbick, G. (1990) Epitope mapping of the *Mycobacterium bovis* secretory protein MPB70 using overlapping peptide analysis. *J. Gen. Microbiol.* 136, 265-272.
- Ramasamy, R. and Geysen, H.M. (1990) Novel cross-reactive epitopes on asexual blood stage antigens of *Plasmodium falciparum*. *Parasite Immunol.* 12, 457-471.
- Ravetch, J.V., Young, J. and Poste, G. (1985) Molecular genetic strategies for the development of anti-malarial vaccines. *BIO/TECHNOLOGY* 3, 729-740.
- Ridley, R.G., Takács, B., Lahm, H., Delves, C.J., Goman, M., Certa, U., Matile, H., Woollett, G.R. and Scaife, J.G. (1990a) Characterization and sequence of a protective rhoptry antigen from *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 41, 125-134.
- Ridley, R.G., Takács, B., Etlinger, H. and Scaife, J.G. (1990b) A rhoptry antigen of *Plasmodium falciparum* is protective in Saimiri monkeys. *Parasitol.* 101, 187-192.
- Rieckmann, K.H. (1990) Human immunization with attenuated sporozoite. *Bull.WHO* 68 (suppl.), 13-16.

Riley, E.M., Olerup, O. and Troye-Blomberg, M. (1991) The Immune Recognition of Malaria Antigens. *Parasitology Today* 7, 5-11.

Roger, N., Dubremetz, J.F., Delplace, P., Fortier, B., Tronchin, G. and Vernes, A. (1988) Characterization of a 225 kilodalton rhoptry protein of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* 27, 135-142.

Rogers, W.O., Rogers, M.D., Hedstrom, R.C. and Hoffman, S.L. (1992) Characterization of the gene encoding sporozoite surface protein 2, a protective *Plasmodium yoelii* sporozoite antigen. *Mol. Biochem. Parasitol.* 53, 45-52.

Roitt, I., Brostoff, J. and Male, D. (1985) *Immunology*. Gower Medical Publishing Ltd. London.

Ruebush II, T.K., Campbell, G.H., Moreno, A., Patarroyo, M.E. and Collins, W.E. (1990) Immunization of owl monkeys with a combination of *Plasmodium falciparum* asexual blood-stage synthetic peptide antigens. *Am. J. Trop. Med. Hyg.* 43, 355-366.

Rüther, U. and Müller-Hill, B. (1983) Easy identification of cDNA clones. *EMBO J.* 2, 1791-1794.

Sabchareon, A., Burnouf, T., Ouattara, D., Attanath, P., Bouharoun-tayoun, H., Chantavanich, P., Foucalt, C., Chongsuphajaisiddhi, T. and Druilhe, P. (1991) Parasitologic and clinical human response to immunoglobulin administration in *falciparum* malaria. *Am. J. Trop. Med. Hyg.* 45, 297-308.

Sacci, J.B.Jr., Schriefer, M.E., Resau, J.H., Wirtz, R.A., Detolla, L.J.Jr., Markham, R.B. and Azad, A.F. (1992) Mouse model for exoerythrocytic stages of *Plasmodium falciparum* malaria parasite. Proc. Natl. Acad. Sci. USA 89, 3701-3705.

Saiki, R.K. (1989) The design and optimization of the PCR. In "PCR Technology: principles and applications for DNA amplification" (Erlich, H.A., edi.) Stockton Press, New York, p. 7-16.

Salcedo, M., Barreto, L., Rojas, M., Moya, R., Cote, J. and Patarroyo, M.E. (1991) Studies on the humoral immune response to a synthetic vaccine against *Plasmodium falciparum* malaria. Clin. exp. Immunol. 84, 122-128.

Sam-Yellowe, T.Y., Shio, H. and Perkins, M.E. (1988) Secretion of *Plasmodium falciparum* Rhoptry Protein into the Plasma Membrane of Host Erythrocytes. J. Cell Biol. 106, 1507-1513.

Sam-Yellowe, T.Y. and Perkins, M.E. (1990) Binding of *Plasmodium falciparum* rhoptry proteins to mouse erythrocytes and their possible role in invasion. Mol. Biochem. Parasitol. 39, 91-100.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Saul, A. and Battistutta, D. (1988) Codon usage in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 27, 35-42.

Saul, A., Cooper, J., Hauquitz, D., Irving, D., Cheng, Q., Stowers, A. and Limpaiboon, T. (1992) The 42-kilodalton rhoptry-associated protein of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 50, 139-150.

Scaife, J.G. (1988) The cloning of antigen genes from malarial parasites and *Leishmania* species. In "Genetic Engineering 7" (Rigby, P.W. edi.), Academic Press. London. p. 57-90.

Scherf, A., Mattei, D. and Schreiber, M. (1990) Parasite antigens expressed in *Escherichia coli*. A refined approach for epidemiological analysis. J Immunol. Methods 128, 81-87.

Schmidt, G.D. and Roberts, L.S.(1989) Foundations of parasitology. 4th edition. Times Mirror/Mosby. College publishing USA.

Schofield, L., Saul, A., Myler, P. and Kidson, C. (1982) Antigenic Differences Among Isolates of *Plasmodium falciparum* Demonstrated by monoclonal antibodies. Infect. Immun. 38, 893-897.

Schofield, L., Bushell, G.R., Cooper, J.A., Saul, A.J., Upcroft, J.A. and Kidson, C. (1986) A rhoptry antigen of *Plasmodium falciparum* contains conserved and variable epitopes recognized by inhibitory monoclonal antibodies. Mol. Biochem. Parasitol. 18, 183-195.

Schofield, L., Villaquiran, J., Ferreira, A., Schellekens, H., Nussenzweig, R. and Nussenzweig, V. (1987) γ -interferon, CD8⁺ T cells and antibodies required for immunity to malaria sporozoites. Nature. 330, 664-666.

Schofield, L. (1990) The circumsporozoite protein of *Plasmodium*: a mechanism of immune evasion by the malaria parasite? Bull.WHO 68 (suppl.), 66-73.

Sherman, I.W. (1979) Biochemistry of *Plasmodium* (malaria parasites). Microbiol. Rev. 43, 453-495.

Siddiqui, W.A. (1977) An Effective Immunization of Experimental Monkeys Against a Human Malaria Parasite, *Plasmodium falciparum*. Science 197, 388-389.

Siddiqui, W.A., Tam, L.Q., Kramer, K.J., Hui, G.S.N., Case, S.E., Yamaga, K.M., Chang, S.P., Chan, E.B.T. and Kan, S. (1987) Merozoite surface coat precursor protein completely protects Aotus monkeys against *Plasmodium falciparum* malaria. Proc. Natl. Acad. Sci. USA 84, 3014-3018.

Sinden, R.E. (1984) The biology of Plasmodium in the mosquito. Experientia 40, 1330-1043.

Sinigaglia, F., Guttinger, M., Kilgus, J., Poran, D.M., Matile, H., Etlinger, H., Trzeciak, A., Gillessen, D. and Pink, J.R.L. (1988) A malaria T-cell epitope recognized in association with most mouse and human MHC classII molecules. Nature 336, 778-780.

Sinigaglia, F. and Pink, J.R.L. (1990) A Way Round the 'Real Diffeculties' of Malaria Sporozoite Vaccine Development. Parasitology Today 6, 17-19.

Slater, A.F.G. and Cerami, A. (1992) Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. Nature. 355, 167-169.

Smith, A.M. and Benjamin, D.C. (1991) The antigenic surface of Staphylococcal nuclease. II Analysis of the N-1 Epitope by Site-Directed Mutagenesis. J. Immunol. 146, 1259-1264.

Smith, A.M., Woodward, M.P., Hershey, C.W., Hershey, E.D. and Benjamin, D.C. (1991) The antigenic surface of Staphylococcal nuclease. I Mapping Epitopes by Site-Directed Mutagenesis. J. Immunol. 146, 1254-1258.

Smythe, J.A., Coppel, R.L., Brown, G.V., Ramasamy, R., Kemp, D.J. and Anders, R.F. (1988) Identification of two integral membrane proteins of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. USA 85, 5195-5199.

Stephen, C.W. and Lane, D.P. (1992) Mutant Conformation of p53: Precise epitope mapping using a filamentous phage epitope library. J. Mol. Biol. 225, 577-583.

Suckau, D., Köhl, J., Karwath, G., Schneider, K., Casaretto, M., Bitter-Suermann, D. and Przybylski, M. (1990) Molecular epitope identification by limited proteolysis of an immobilized antigen-antibody complex and mass spectrometric peptide mapping. Proc. Natl. Acad. Sci. USA 87, 9848-9852.

Tanabe, K., Mackay, M., Goman, M. and Scaife, J.G. (1987) Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. J. Mol. Biol. 195, 273-287.

Thaithong, S., Beale, G.H., Fenton, B., McBride, J., Rosario, V., Walker, A. and Walliker, D. (1984) Clonal diversity in a single isolate of the malaria parasite *Plasmodium falciparum*. Trans. R. Soc. Trop. Med. Hyg. 78, 242-245.

- Thaithong, S., Chan, S., Songsomboon, S., Wilairat, P., Seesod, N., Sueblinwong, T., Goman, M., Ridley, R. and Beale, G. (1992) Pyrimethamine resistant mutations in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 52, 149-158.
- Thomas, W.A., Waters, A.P. and Carr, D. (1990) Analysis of variation in PF83, an erythrocytic merozoite vaccine candidate antigen of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 42, 285-288.
- Trager, W. and Jensen, J.B. (1976) Human malaria parasites in continuous culture. Science 217, 254-257.
- Van Schravendijk, M.R., Wilson, R.J.M., Newbold, C.I., (1987) Possible pitfalls in the identification of glycophorin-binding proteins of *Plasmodium falciparum*. J. Exp. Med. 166, 376-390.
- Vermuelen, A.N. (1985) Sequential Expression of antigens on sexual stages of *Plasmodium falciparum* accessible to transmission-blocking antibodies in the mosquito. J. Exp. Med. 162, 1460-1476.
- Vernes, A., Haynes, J.D., Tapchaisri, P., Williams, J.L., Dutoit, E. and Diggs, C.L. (1984) *Plasmodium falciparum* strain-specific human antibody inhibit merozoite invasion of erythrocytes. Am. J. Trop. Med. Hyg. 33, 197-203.
- Virji, M. and Heckels, J.E. (1989) Location of a Blocking Epitope on Outer-membrane Protein III of *Neisseria gonorrhoeae* by Synthetic Peptide Analysis. J. Gen. Microbiol. 135, 1895-1899.

Vreden, S.G.S., Verhave, J.P., OeHinger, T., Sauerwein, R.W. and Meuwissen, J.H.E.T. (1991) Phase I Clinical trial of a recombinant malaria vaccine consisting of the circumsporozoite repeat region of *Plasmodium falciparum* coupled to hepatitis B surface antigen. Am. J. Trop. Med. Hyg. 45, 533-538.

Wählin, B., Wahlgren, M., Perlmann, H., Berzins, K., Björkman, A., Patarroyo, M.E. and Perlmann, P. (1984) Human antibodies to a Mr 155,000 *Plasmodium falciparum* antigen efficiently inhibit merozoite invasion. Proc. Natl. Acad. Sci. USA 81, 7912-7916.

Walliker, D., Quakyi, I.A., Wellems, T.E., McCutchan, T.F. Szarfman, A., London, W.T., Corcoran, L.M., Burkot, T.R. and Carter, R. (1987) Genetic Analysis of the Human Malaria Parasite *Plasmodium falciparum*. Science 236, 1661-1666.

Walter, R.D. (1986) Altered dihydrofolate reductase in pyrimethamine-resistant *Plasmodium falciparum*. Mol. Biochem. Parasitol. 19, 61-66.

Ware, J., Toomey, J.R. and Stafford, D.W. (1988) Localization of a factor VIII-inhibiting antibody epitope to a region between residues 338 and 362 of factor VIII heavy chain. Proc. Natl. Acad. Sci. USA 85, 3165-3169

Wiederanders, B., Brömme, D., Kirschke, H., Figura, K.V., Schmidt, B. and Peters, C. (1992) Phylogenetic conservation of cysteine proteinases: cloning and expression of a cDNA coding for human cathepsin S. J. Biol. Chem. 267, 13708-13713.

Wilson, R.J. (1982) How the malarial parasite enters the red blood cell. Nature 295, 368-369.

Wongsrichanalai, C., Webster, H.K., Wimonwattrawatee, T., Sookto, P., Chuanak, N., Thimasarn, K. and Wernsdorfer, W.H. (1992) Emergence of multidrug-resistant *Plasmodium falciparum* in Thailand: *in vitro* tracking. Am. J. Trop. Med. Hyg. 47, 112-116.

Wyler, D.J. (1990) Modern Parasite Biology. Cellular, immunological and molecular aspects. W.H. Freeman and Company. USA.

Young, M.D., Contacos, P.G., Sticher, J.E. and Millar, J.W. (1963) Drug resistance in *Plasmodium falciparum* from Thailand. Am. J. Trop. Med. Hyg. 12, 305-314.

Appendix

Some works in this thesis were published in:

Harnyuttanakorn, P., McBride, J.S., Donachie, S., Heidrich, H.-G. and Ridley, R.G. (1992) Inhibitory monoclonal antibodies recognise epitopes adjacent to a proteolytic cleavage site on the RAP-1 protein of *Plasmodium falciparum*. Mol. Biol. Parasitol. 55, 177-186.

MOLBIO 01817

Inhibitory monoclonal antibodies recognise epitopes adjacent to a proteolytic cleavage site on the RAP-1 protein of *Plasmodium falciparum*

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(Received 24 March 1992; accepted 1 July 1992)

The low-molecular-weight rhoptry-associated protein (RAP) complex of *Plasmodium falciparum* consists of at least two gene products, RAP-1 and RAP-2, and has the ability to immunise *Saimiri* monkeys against experimental *P. falciparum* infection. Several monoclonal antibodies specifically recognise this complex and in this study we show that purified immunoglobulin derived from these monoclonals is capable of inhibiting parasite growth in vitro. It has previously been shown that RAP-1 initially appears as an 80-kDa protein (p80) in early schizogony and is processed to a 65-kDa protein (p65) in late schizogony. Several of the inhibitory monoclonals recognise both the 80- and 65-kDa proteins by Western blot analysis suggesting that they recognise linear epitopes on RAP-1. We have mapped these epitopes by testing the reactivity of the monoclonals against fragments of the *rap-1* gene expressed as β -galactosidase fusion proteins and subsequently against synthetic peptides. All of the epitopes map to a region 10–20 amino acids C-terminal to the proteolytic cleavage site for the processing of p80 to p65 at amino acid 190. We also show that the 65-kDa protein is not present in purified merozoites, suggesting that its generation is associated with merozoite release rather than erythrocyte invasion. These results are discussed with respect to possible inhibitory mechanisms for the monoclonals.

Key words: Malaria; *Plasmodium falciparum*; Rhoptry; Inhibitory monoclonal antibody; RAP-1; Epitope mapping

Introduction

Merozoite release from *P. falciparum* infected red blood cells and their subsequent invasion into fresh red blood cells is a complicated process believed to involve the co-ordinated action of many proteins. Several groups of parasite antigens have been implicated in this process and have been identified as vaccine candidates, including proteins of the

merozoite surface [1,2], the parasitophorous vacuole [3], parasitophorous vacuole membrane [4], dense granules [5,6] and the rhoptry organelles [2,7]. The function of most of these antigens is poorly understood, though sequence homologies have indicated that the parasitophorous vacuolar protein SERA is a cysteine protease [8] and biochemical evidence has shown that another antigen of uncertain location [9] contains a phospholipase C activated serine protease [10]. In the absence of a defined function for a given antigen one may still determine regions of functional significance on a protein by defining epitopes recognised by monoclonal antibodies which inhibit parasite growth. This approach has recently focused attention on the C-terminal region of the merozoite surface protein MSP-1

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Abbreviations: mAb, monoclonal antibody; IPTG, isopropyl- β -thiogalactopyranoside; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; LB, Luria broth.

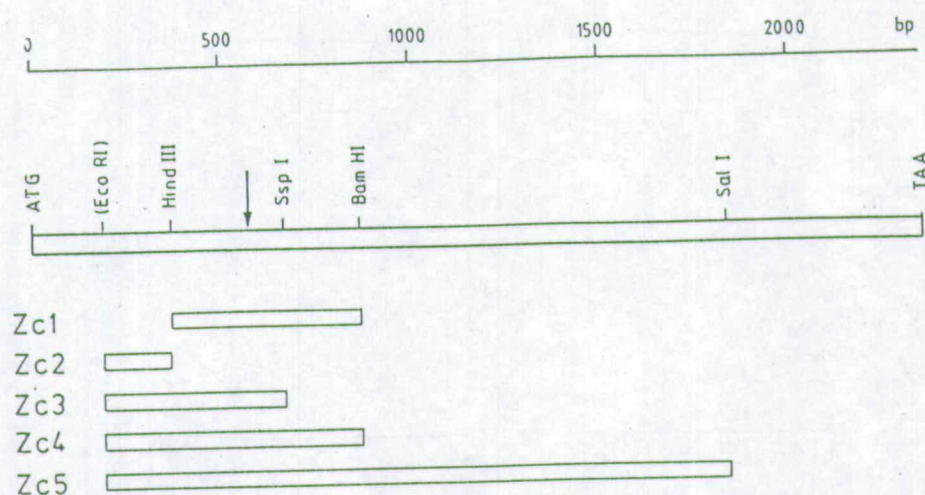


Fig. 1. Recombinant *rap-1* gene expression constructs used for preliminary mapping of epitopes. The restriction enzyme sites used to generate appropriate DNA fragments are given relative to their position within the coding region of the *rap-1* gene. These fragments were ligated into the polylinker cloning site of a pMS expression vector [28] and expressed as β -galactosidase fusion proteins. The region encoding the proteolytic cleavage site is arrowed.

IPTG. The fragments subcloned into the pMS plasmid were termed *lacZ* constructs (Zc1-Zc5) and are shown in Fig. 1. Their positions relative to the *rap-1* gene are presented. It should be noted that the *Eco*RI site indicated in the figure, by which many of the fragments were ligated into the pMS vector, is not present in the *rap-1* gene itself, but comes from an *Eco*RI linker present in the λ gt11 expression clone, λ rap1.1, which was isolated in the initial cloning of the gene [12]. In cases where there was no compatible site in the vector for subcloning, fragments were end-filled using Klenow and blunt-end ligated into pMS. All constructs were confirmed by DNA sequencing [29].

Further derivatives of a clone containing the *Eco*RI-*Bam*HI fragment (Fig. 1, construct Zc4) were obtained as a set of 'nested deletions'. The construct was linearised with *Bam*HI and subjected to exonuclease III digestion using the Erase-a-Base kit (Promega Biotech). Products which had deletions from the *Bam*HI site to the vicinity of the *rap-1* gene encoding amino acid residues 190-230 were selected. The ragged ends were filled by *S*I nuclease and Klenow according to the manufacturers instructions and then ligated to generate a functional plasmid expressing truncated forms of the *rap-1* gene. All vectors were transformed into *E. coli* strain NM522 [30] for expression.

Epitope expression by recombinants. Bacteria containing recombinant plasmids were grown to mid-log phase and *lacZ* gene expression induced by addition of IPTG to 1 mM. After 2 h, cells were harvested, resuspended in water and 1 vol. of 2 $\times$ Laemmli loading buffer added [31]. Samples were subjected to SDS-PAGE, blotted to a Hybond-C membrane (Amersham) and probed for reactivity with a variety of antibodies using an alkaline phosphatase conjugated second antibody system (Protolot components; Promega Biotech).

Peptide epitope mapping. Linear epitopes were accurately mapped by testing the reactivity of antibodies to overlapping peptides synthesised on a 96-pin plastic support in an epitope scanning kit (Cambridge Research Biochemicals) using the Geysen technique [32].

Results

Monoclonal antibody inhibition of parasite growth. The results of inhibition assays carried out using a variety of monoclonal antibodies recognising the RAP complex are shown in Fig. 2. All RAP mAbs showed a marked inhibitory effect over the control antibody. The antibody concentrations required to inhibit parasite growth were comparable to those observed previously for other

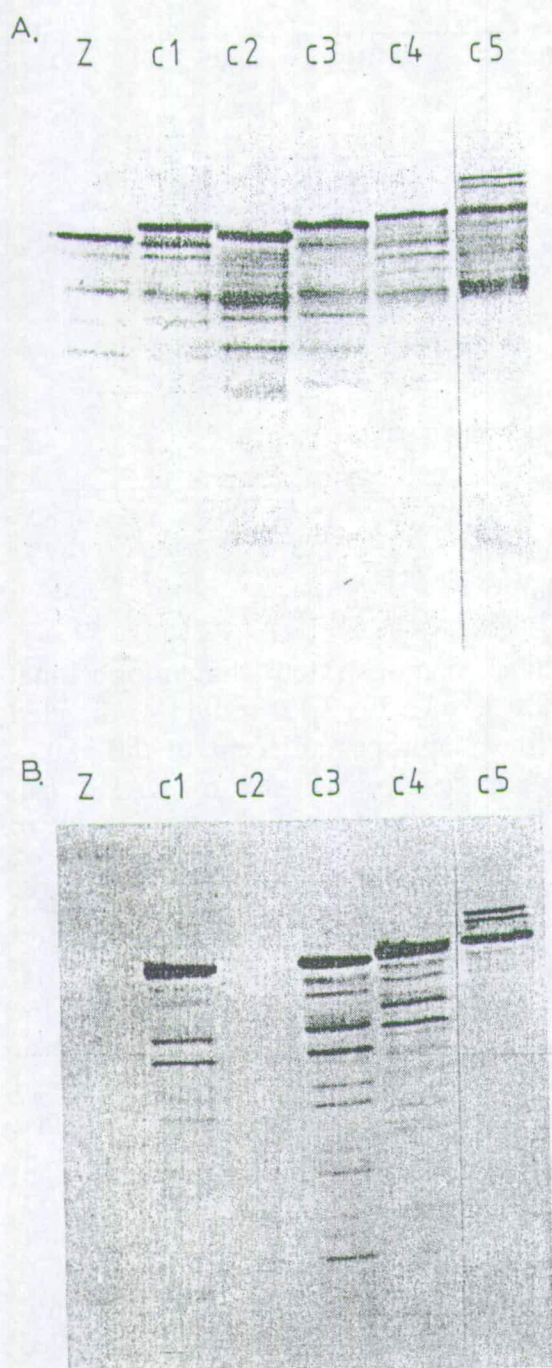


Fig. 3. Monoclonal antibody reactivities against fusion constructs Zc1-Zc5. Bacterial extracts containing protein after induction of *lacZ* expression, either from the original pMS expression vector which produces β -galactosidase (lane Z) or the RAP-1 fusion constructs Zc1-Zc5 (lanes c1-c5) were separated by SDS-PAGE, blotted on to nitrocellulose membranes and probed with antisera. (A) Control experiment using rabbit polyclonal sera raised against β -galactosidase. (B) Results obtained using mAb 2.13. Identical results were obtained for mAbs 2.15, 2.29, 7.12, 8.1 and 11.4.

recognised by mAb 2.13 is encoded by all of the constructs except Zc2, suggesting that the epitope is encoded within the *Hind*III-*Ssp*I

fragment of the *rap-1* gene (Fig. 1). Identical results were obtained for all the mAbs tested (2.13, 2.15, 2.29, 7.12, 8.1 and 11.4) indicating that the respective target epitopes were all encoded within this same region.

Next, exonuclease-derived constructs from the expression clone Zc4 (Fig. 1) were made and tested in a similar manner. The deleted RAP-1 sequences covering the region of interest for several of these constructs are given in Table I, together with the reactivity of the fusion proteins with monoclonal antibodies. The proteolytic cleavage site determining the p80 $\rightarrow$ p65 transition is shown for reference. Once again the epitopes could not be distinguished since all the mAbs reacted positively with the same constructs. From sequences of these constructs it was deduced that the mAbs recognised epitopes which are lost with the removal of amino acid residues E₂₀₇ to T₂₁₁. Thus the epitopes are no further than 20 amino acids downstream of the proteolytic cleavage site at D₁₉₁.

The epitopes were finally defined by the Geysen epitope mapping technique [32] using synthetic peptides covering residues A₁₉₀ to S₂₂₂. Overlapping hexapeptides covering this region did not react with any of the mAbs (not shown). The procedure was therefore repeated using overlapping decapeptides. From results shown in Fig. 4 we deduced that the monoclonals 2.13, 2.15, 2.29 and 7.12 recognised the nonapeptide LTPLEELYP while mAb 8.1 recognised an 8-amino acid epitope within this nonapeptide, namely LTPLEELY. The mAb 11.4 did not recognise any of the decapeptides. It is possible that the antibody recognised an epitope larger than 10 amino acids or an epitope that is determined by a secondary structure which could be present in the β -galactosidase fusion proteins but was absent in the peptides.

To summarise, the monoclonals all recognise an epitope within approximately 10 to 20 residues C-terminal to the proteolytic cleavage site at D₁₉₁ giving rise to the p80 $\rightarrow$ p65 transition. Even mAb 11.4, whose epitope has not been definitively determined must recognise an epitope in this vicinity (Table I).

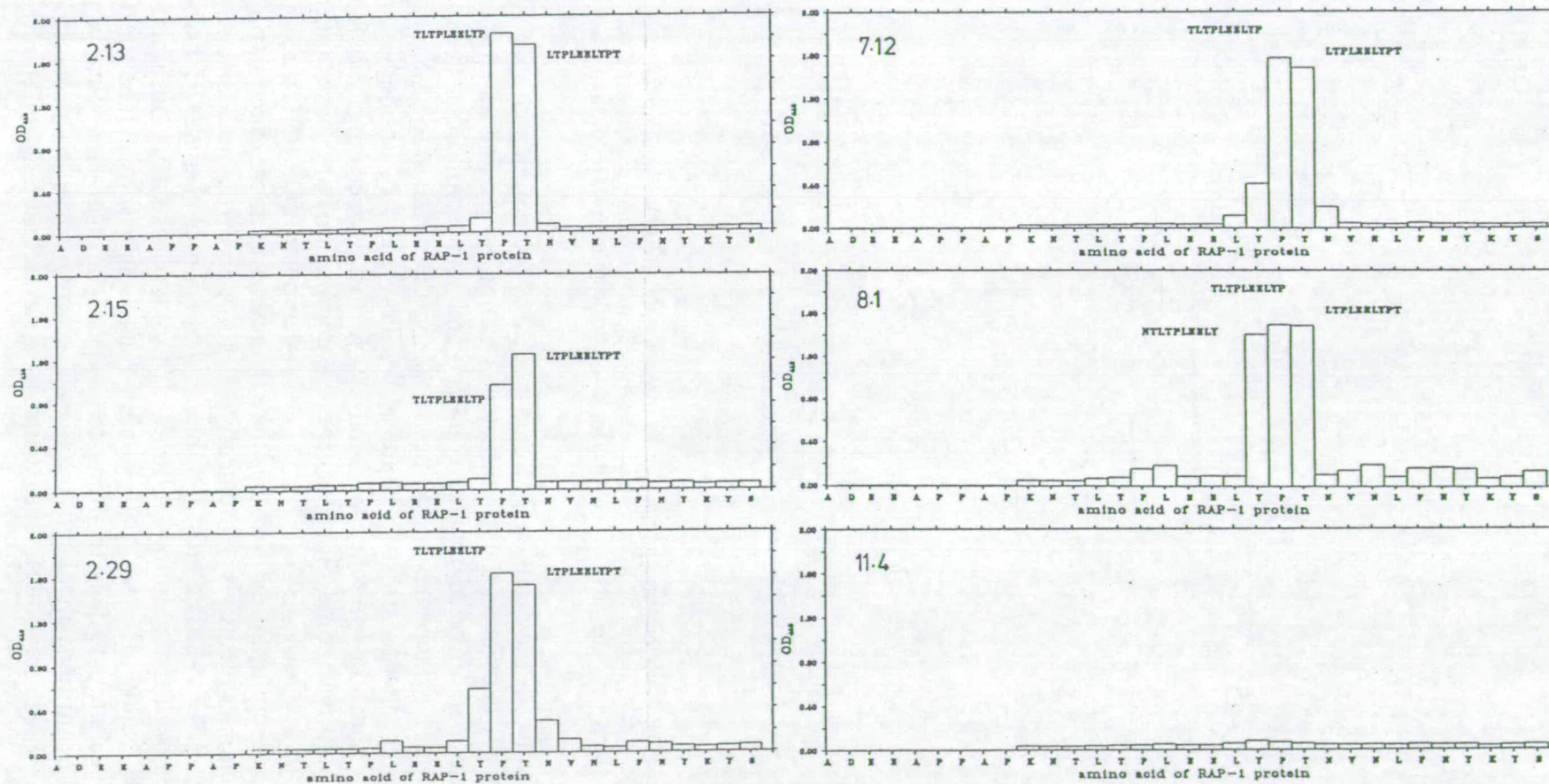


Fig. 4. Peptide mapping of monoclonal antibody epitopes. Decapeptides overlapping by one residue and covering the RAP-1 protein from residues 190-221 were prepared by the Geysen technique [32] and tested for antibody reactivity by ELISA. The sequences of peptides recognised by the antibodies are indicated.

merozoite release is not new. An electron microscopy study has shown rhoptries releasing material inside infected erythrocytes and, in some cases, merozoites within mature schizonts were observed with one 'full' rhoptry and one 'empty' rhoptry. It should be noted that only a fraction of the 80-kDa RAP-1 is ever processed to the 65-kDa product in schizonts. Much of the 80-kDa RAP-1 is carried to the newly invaded ring stages where the 65-kDa fragment is not detectable [14,16].

It is tempting to speculate that the 80-kDa protein performs a different function to its 65-kDa fragment. One could postulate that the 65-kDa form is associated with merozoite release while the 80-kDa form in freed merozoites contributes subsequently to erythrocyte invasion or to the formation of parasitophorous vacuole membrane around the new ring form [14,19]. Another alternative that is not ruled out is that the 80-kDa protein is active in both merozoite release and erythrocyte invasion and that the 65-kDa protein is a residual by-product generated after the 80-kDa protein has been released from the rhoptries and performed its merozoite release function. In this case the processing could serve to inactivate the 80-kDa protein once its task has been done. In either case the function of RAP-1 would be controlled by its proteolytic processing. We have previously presented evidence that RAP-1 may be capable of autocatalytic cleavage [12]. Self-cleavage as a means of regulating protein activity is not uncommon and is exhibited by regulatory proteins such as the *E. coli* S.O.S. response protein, Lex A, by several aspartic acid proteases and by viral polyproteins (see ref. 33 for review). Further work on the p80 → p65 cleavage of RAP-1 should clarify these aspects of RAP-1 function.

Other results from this study deserve comment. It is quite surprising that six monoclonals derived from four different mice all recognise the same region of RAP-1 and that five actually recognise the same linear peptide sequence. This suggests that the peptide represents a RAP-1 epitope which is immunodominant in vivo. The immunodomi-

nance of the LTPLEELYP epitope requires that we reinterpret previous work showing that all of our RAP monoclonals recognised all of over 80 isolates of *P. falciparum* [14]. Although this led us to propose that the RAP antigen was highly conserved, we were looking at the conservation of essentially only two epitopes, one conformational and one linear. We are now cloning the *rap-1* gene from a variety of *P. falciparum* isolates to check its degree of sequence conservation.

Acknowledgements

This work was supported by F. Hoffman-La Roche Ltd., the MRC and the Wellcome Trust. One of us (P.H.) holds a Thai Government Studentship. We thank Mike Goman, Mohammed Shahabuddin, Richard Moon and Chris Delves for their advice and support and Frank Johnson and Graham Brown for photography. This work was initiated under the guidance of John Scaife to whom we owe a tremendous debt of gratitude and to whose memory this article is dedicated.

References

- 1 Hall, R., Hyde, J.E., Goman, M., Simmons, D.L., Hope, I.A., Mackay, M., Scaife, J.G., Merkli, B., Richle, R. and Stocker, J. (1984) Major surface antigen of a human malaria parasite cloned and expressed in bacteria. *Nature* 311, 379–382.
- 2 Siddiqui, W.A., Tam, L.Q., Kramer, K.J., Hui, G.S.N., Case, S.E., Yamage, K.M., Chang, S.P., Chan, E.B.T. and Kan, S.-C. (1987) Merozoite surface coat precursor completely protects *Aotus* monkeys against *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA* 84, 3014–3018.
- 3 Knapp, B., Hundt, E., Nau, U. and Küpper, H. (1989) Molecular cloning, genomic structure and localization of a blood stage antigen of *Plasmodium falciparum* characterised by a serine stretch. *Mol. Biochem. Parasitol.* 32, 73–84.
- 4 Caspers, P., Etlinger, H., Matile, H., Pink, J.R., Stüber, D. and Takacs, B. (1991) A *Plasmodium falciparum* vaccine candidate which contains epitopes from the circumsporozoite protein and a blood stage antigen. *Mol. Biochem. Parasitol.* 47, 143–150.
- 5 Collins, W.E., Anders, R.F., Pappaioanou, M., Campbell, G.H., Brown, G.V., Kemp, D.J., Coppel, R.L., Skinner, J.C., Andrysiak, P.M., Favoloro, J.M., Corcoran, L.M., Broaderson, J.R., Mitchell, G.F. and Campbell, C.C. (1986) Immunization of *Aotus* monkeys