Glucose transport in malaria infected erythrocytes

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Glucose Transport in Malaria Infected Erythrocytes

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the degree of PhD

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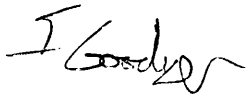

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Abbreviations.

3-O-MG	3-O-methyl-D-glucose
Å	Angstrom
AIM	Artificial Intracellular Medium
amp	Ampicillin
ATB-BMPA	2-N-(2-azi-1,1,1-trifluoroethyl)-benzoyl-bis(D-mannose-4-yloxy)-2-propylamine
ATP	Adenosine 5'-triphosphate
BCA	Bicinchoninic Acid
bp	Base pairs
BiP	Immunoglobulin Binding Protein
BSA	Bovine serum albumin
CAD	Cinnamic acid derivative
cDNA	Complementary deoxyribonucleic acid
Ci	Curie
CSP	Circumsporozoite protein
cytB	Cytochalasin B
dATP	2'-Deoxyadenosine 5'-triphosphate
dCTP	2'-Deoxycytidine 5'-triphosphate
ddATP	2' ,3' -Dideoxyadenosine 5'-triphosphate
ddCTP	2' ,3' -Dideoxycytidine 5'-triphosphate
ddGTP	2' ,3' -Dideoxyguanosine 5'-triphosphate
ddNTP	2' ,3' -Dideoxynucleotide 5'-triphosphate
ddTTP	2' ,3' -Dideoxythymidine 5'-triphosphate
DEPC	Diethyl pyrocarbonate
DFO	Desferrioxamine
dGTP	2'-Deoxyguanosine 5'-triphosphate
DIDS	4,4'-diisothiocyano-2,2'-stilbenedisulphonic acid
DNA	2'-Deoxyribonucleic acid
DNase	Pancreatic deoxyribonuclease
DNDS	4,4'-dinitro-2,2'-stilbenedisulphonic acid
dNTP	2' -Deoxynucleotide 5'-triphosphate
DTNB	5, 5'-Dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
dTTP	2'-Deoxythymidine 5'-triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FDNB	1-fluoro-2,4-dinitrobenzene
G6PDH	Glucose 6-phosphate dehydrogenase
H_2-DIDS	4,4'-diisothiocyano-2,2'-dihydrostilbenedisulphonic acid
IAA	Isoamyl Alcohol
IPTG	Isopropyl-β-D-thiogalactoside
kb	1000 bases
kD	1000 Daltons
Km	Michaelis constant
MA-DFO	N-methylanthranilic-desferrioxamine
mRNA	messenger RNA
M.W.	Molecular Weight
NADH	Nicotinamide adenine dinucleotide
NBMPR	Nitrobenzylthioinosine
NBTGR	Nitrobenzylthioguanosine
P.	*Plasmodium*
PAGE	Polyacrylamide-gel electrophoresis
PBS	Phosphate buffered saline
PC	Personal Computer
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
PFGE	Pulsed-field gel electrophoresis
PfHRP	*Plasmodium falciparum* Histidine Rich Protein
pH	= -log [Proton]
PM	Plasma membrane
PNK	Polynucleotide Kinase
PVM	Parasitophorous vacuolar membrane
RACE	Rapid Amplification of cDNA Ends
RNA	Ribonucleic acid
RNase	Pancreatic ribonuclease
rNTP	ribonucleoside triphosphate
SDS	Sodium dodecyl sulphate
SEM	Standard Error of Means
SNF	Sucrose non-fermenting
SSC	0.15 M sodium chloride and 15 mM sodium citrate
TBE	Tris base-boric acid-EDTA
TBP	TATA-binding protein
TE	Tris-EDTA
TEA	Triethylamine
TEMED	N ,N ,N',N' tetramethylethylene diamine
TM	Transmembrane
Tris	Tris(hydroxymethyl)-aminomethane
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YAC	Yeast Artificial Chromosome

Abstract

A mature *Plasmodium falciparum* parasite inside a human red blood cell consumes large amounts of glucose. This work investigates the mechanisms by which the parasites obtain the glucose required for their survival. Two different approaches are employed: a molecular biological approach and a kinetic analysis. Using molecular biological techniques, especially PCR, an attempt was made to obtain the malarial glucose transporter sequence. Although unsuccessful, this produced many novel DNA sequences from *Plasmodia* and confirmed the sequence of a portion of malarial enolase. The kinetic approach showed that glucose enters the parasite by passive diffusion, that the red blood cell transporter is not rate-limiting and that the malaria induced pore does not play a significant role in D-glucose transport. The kinetic approach also indicated that L-glucose and D-glucose enter infected cells by different mechanisms and could not rule out entry of some D-glucose through the recently discovered parasitophorous duct.

1. Introduction

1.1. Malaria

Malaria has an immense impact on humans. It is the biggest killer in the history of man (Philips, 1983), presently killing more people than any other single cause. It is no coincidence that countries with a high prevalence of malaria are the poorest in the world. There are between 250 and 500 million new cases of malaria each year and malaria currently kills 1-2.5 million people annually (Cox, 1991). Recently, research into new treatments for malaria has become even more important because in certain regions of the world the traditional antimalarial drugs (like quinine and chloroquine) are no longer effective.

1.1.1. The Cause of Malaria

Malaria is caused by a single-celled eukaryote protozoan. There are many species of *Plasmodium*, four of which (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) infect humans. Almost all the deaths from malaria are attributed to *Plasmodium falciparum*. Although common, *Plasmodium vivax* produces a milder form of the disease with frequent relapses. The other two human malarias are rare and not life threatening. In mice there are at least four common species of malaria *P. yoelii*, *P. knowlesi*, *P. vinckei* and *P. chabaudi*.

1.1.2. The Life Cycle of Malaria

All species of *Plasmodium* share a complex life cycle, which is described for *Plasmodium falciparum* in figure 1. The *Plasmodium* in its sporozoite form (11μm long, 1μm thick) is released from the salivary gland of the insect into the host's blood stream when the mosquito takes a blood meal. The sporozoites are carried in the blood to the liver where they invade the hepatocyte. The parasite, which is surrounded by a vacuolar membrane, grows and divides mitotically to become a pre-erythrocytic schizont cell. Each nucleus buds off with part of the cytoplasm to form a merozoite. Rupture of the liver cell releases thousands of merozoites into the bloodstream and ends the incubation period of the parasite (Philips, 1983).

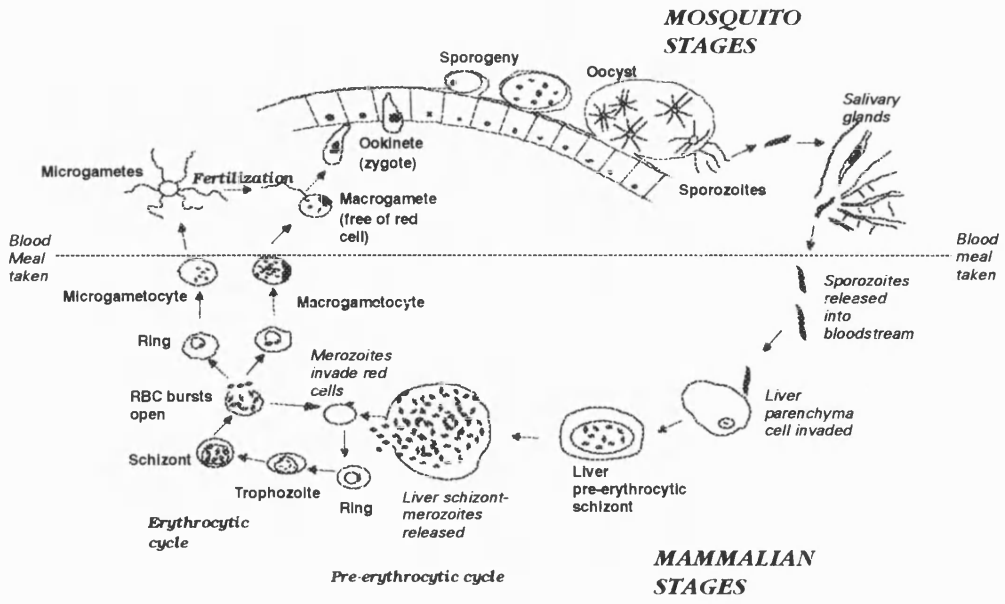

Figure 1. The life cycle of *Plasmodium falciparum*, the malarial parasite. Redrawn from R. S. Philips, Malaria.

The merozoites must now survive for a short time free in the bloodstream where they are exposed to the full wrath of the host's immune system. The merozoites rapidly bind to an erythrocyte and cause the host cell to invaginate. The merozoites probably bind to glycophorins in the red blood cell membrane (Pasvol and Jungery, 1983). The invasion process takes less than a minute (Philips, 1983) after which the parasite is almost completely enclosed inside the erythrocyte.

1.1.2.1. The Erythrocytic Stage

In the red blood cell the merozoite rapidly transforms into a ring-stage parasite. The parasite continues to grow and a food vacuole develops. After 24 hours the parasite has grown in size, takes up Giemsa stain more readily and is known as a trophozoite. The host's erythrocyte cytoplasm is rapidly ingested by the trophozoite and partially digested. The haemoglobin is degraded to the waste product haemozoin and the amino acids that are released are incorporated into the parasite's proteins (Goldberg and Slater, 1992). The trophozoite further enlarges and then divides mitotically forming schizont stage parasites. As in the liver, the nuclei bud off with cytoplasm to

form a segmented schizont. The host's erythrocyte bursts open releasing merozoites into the blood stream. The released merozoites can then infect further erythrocytes. This describes the asexual cycle that takes about 48 hours to complete for *Plasmodium falciparum*.

Within the red blood cell some ring stage parasites do not develop into trophozoites and undergo mitotic division. Instead they transform into micro- or macrogametocytes, starting the sexual cycle of reproduction. There is no genetic reorganisation within the gametocytes. *Plasmodium* is haploid throughout all the stages of development in the human (Philips, 1983).

1.1.2.2. The Mosquito Stages

When the mosquito takes a blood meal from a malaria infected person, merozoites and gametocytes in erythrocytes are taken into the insect's gut. In the midgut the gametes lose their red cell membranes. Microgametes divide mitotically three times to give 8 nuclei that go on to form 8 flagella (Philips, 1983). These swim through the blood meal. The macrogametocyte becomes primed for fertilisation and then fertilisation occurs within minutes. Over the next 12-18 hours the oocyte develops into an ookinete. Within 24 hours the ookinete crosses the epithelium of the gut-wall where it enlarges and develops into an oocyst. Development into the oocyst takes 10-12 days. Although the exact timing of meiosis is uncertain it is believed to be during this stage of the life cycle (Philips, 1983). The oocyst enlarges further and goes on to form sporozoites. Each oocyst produces thousands of sporozoites and each mosquito may have hundreds of developing oocysts in its midgut. The sporozoites enter into the haemocoel and are carried to the lumen of the salivary gland. The next time the mosquito takes a blood meal the sporozoites are released into the bloodstream of another person.

1.1.3. The History of Malaria

Although today cases of malaria are rare in Britain, it has not always been this way. The marshy lowlands in Britain were prime breeding sites for the mosquitoes and during the 16th and 17th centuries 'swamp fever' was rife in Britain (Dobson, 1989).

Probably the earliest known drug against malaria was quinine. In Peru, during the 17th century people with malaria were given the bark of a local tree (a natural source of quinine). In 1629, this treatment was administered to the wife of the Viceroy of Peru, the Countess of Chinchon, with successful results. It was not until the 19th century that quinine was extracted from this "Cinchona bark" and found to be the active component.

The causative agent of malaria and its mode of infection were not fully understood until the middle of this century. Early work was performed by Meckel in 1847 who noticed black granules in the spleens of patients who had died of malaria. These black deposits are now known to be haemozoin a degradation product of the parasite's metabolism of haemoglobin (Goldberg and Slater, 1992). Thirty years later Kelsch noted that these black deposits in the blood, were most noticeable just before the patient became feverish. In 1878 Lavaran, working in Algeria, saw clear cells, the free forms of the parasite, in the blood of infected patients (Philips, 1983).

The insect vector for the malaria parasite was not known to be the female *Anopheles* mosquito (*A. gambia*) until the 1890's. It had been noticed for a long time that mosquitoes flourished together with malaria. In 1895 Ross discovered the purplish/black haemozoin pigment in the mosquito. The link was made conclusively by Manson at the end of the 19th century who used live mosquitoes, which had been allowed to suck blood from malaria patients, in Italy. These mosquitoes were then allowed to bite healthy volunteers in London. The experiment was 'successful' and the volunteers contracted malaria (Thomson, 1958). It was not until 1947 that Garnham discovered that the parasite first infected human liver cells before infecting the erythrocytes (Philips, 1983).

Although nowadays cases of malaria in the United States, Israel, Cyprus and most of Western Europe are very rare, malaria is still a major killer especially in tropical Africa, Asia and Central and South America. The disease has proven hard to eradicate mainly because both the parasite and its mosquito host have become resistant to all the drugs so far used to kill them. For example in 1956 the World Health Organisation (WHO)

tried to rid the world of malaria by attempting to eliminate the insect vector using DTT. After early success the mosquitoes became resistant and have now returned to their original levels.

Over the last decade approximately 1800 cases of malaria have been reported in Britain each year. In the vast majority of cases the person acquired the disease abroad and returned to Britain before becoming ill. Small numbers are acquired congenitally (transplacentally) from their infected but often asymptomatic mothers (1-2 cases annually), by blood transfusion (although this has now virtually been eradicated by taking a travel history of donors and by testing donors serologically), or from infected mosquitoes that have successfully stowed away on aeroplanes. Two such cases have been reported in Britain in the last twenty years (Dobson, 1989).

1.1.4. The Pathology and Diagnosis of Malaria

The incubation period (i.e. the time between infection and any symptoms being seen) for malaria is 8-14 days. The first symptoms are usually gastrointestinal upset and headaches followed by fever when the erythrocytes burst and the merozoites are released. The fevers occur cyclically every 48 hours and each lasts for about 12 hours.

One of the long term effects of malaria on the host is anaemia. The red blood cells are lysed directly by the parasite, and both infected and uninfected erythrocytes are destroyed by phagocytes in the liver and spleen. Another long term danger is from blocked capillaries in the brain and other tissues. This is caused by the parasites, at the trophozoite stage, adhering to the walls of the capillary. Together with the destruction of red blood cells this can limit the oxygen supply to the brain, leading to convulsions and other nervous disorders. The spleen and liver of malarial patients are often enlarged.

1.1.5. *Plasmodium* Ultrastructure

The ultrastructure of *Plasmodium falciparum* has been studied in detail (Langreth *et al*, 1978). Figure 2 shows a mature *Plasmodium falciparum* trophozoite within a human red blood cell. Knobs (K) are found on the surface of erythrocytes infected by many strains of *Plasmodium falciparum*. These structures are produced by a malarial

protein that is transported to the erythrocyte membrane (section 1.1.6.). The knob protein is believed to be involved in the adhesion on malaria infected erythrocytes to epithelial cells (Smith *et al*, 1992; Chishti *et al*, 1992). The Maurer's clefts (M) are lipid based structures produced by the malarial parasite. It is thought that these vesicles are probably involved in the transport of proteins from the malarial parasite to the erythrocyte membrane (section 1.1.6.).

The malarial parasite is bounded almost completely by two membranes: the parasitophorous plasma membrane (PVM) and the parasite's plasma membrane (PM). The outer of the two membranes, the PVM, is derived from the erythrocyte. It has been suggested that the parasite is completely enclosed in these membranes and that any nutrients required by the parasite must cross all three membranes (Izumo *et al*, 1989). Recently, however, this has been called into question. A parasitophorous duct (Pouvelle *et al*, 1991) which joins the erythrocyte plasma membrane and the PVM has been reported (section 4.1.1.3.), and it has also been suggested by many workers that at certain regions the PVM and the PM fuse into a single lipid bilayer (Gormley *et al*, 1992; Elmendorf and Haldar, 1993).

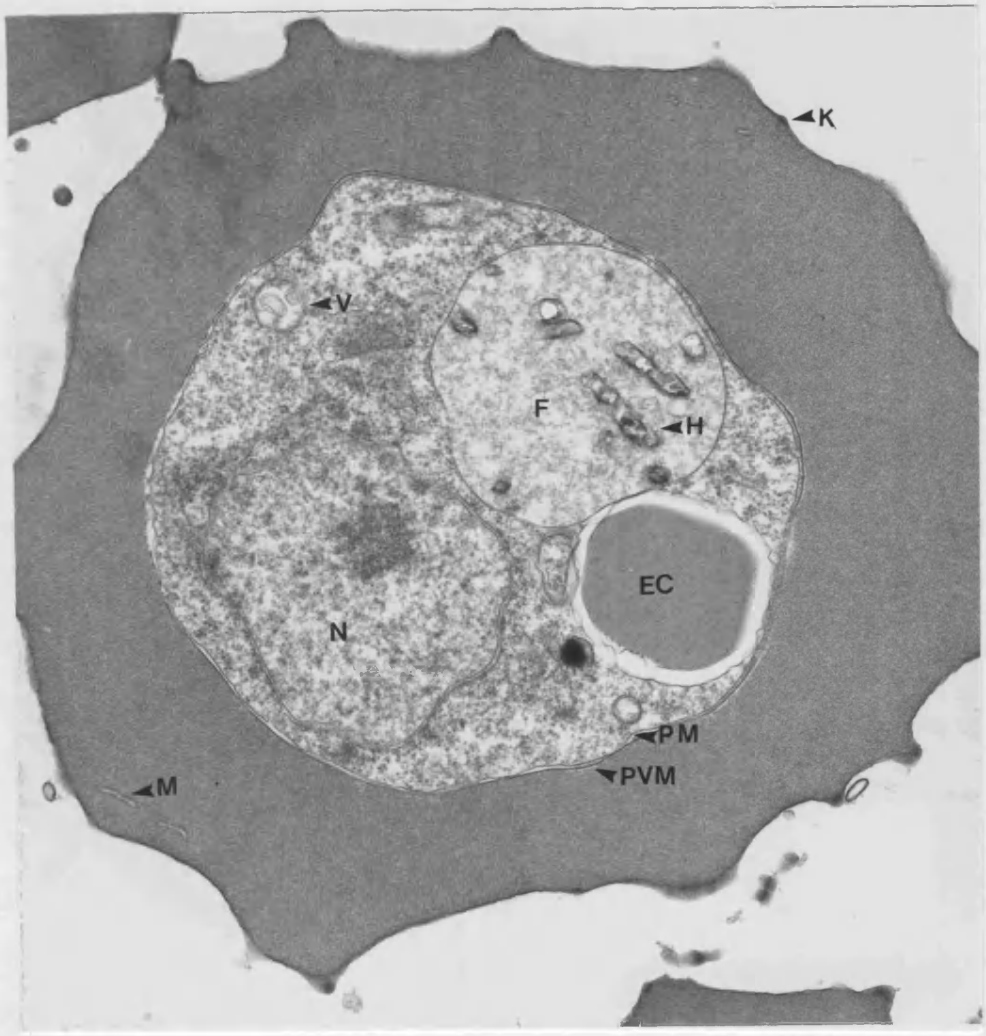

Figure 2. A transmission electron micrograph of a human red blood cell infected with a *P. falciparum* trophozoite. The print is a magnification of 25200X (1mm on the figure is equivalent to 27 nanometers). Many of the common features of the malaria infected cell are highlighted. K: Knobs, M: Maurer's cleft, PVM: Parasite Vacuolar Membrane, PM: Parasite's plasma membrane, F: Feeding vacuole, H: Haemozoin Pigment, EC: Erythrocyte cytoplasm N: Nucleus, V: Vacuoles. This electron micrograph was kindly donated by T. Schneider and T. F. Taraschi, Thomas Jefferson University, Philadelphia.

The parasite has a typical eukaryotic nucleus (N) and other organelles that are surrounded by a double membrane (V). One of these organelles may be the parasite's mitochondrion.

1.1.5.1. The feeding vacuole and haemozoin

The parasite ingests portions of the erythrocyte cytoplasm (EC) and carries it to the feeding vacuole (FV). Inside the feeding vacuole the haemoglobin is degraded with the help of a highly specific aspartic protease. This enzyme, haemoglobinase, cleaves the haemoglobin at a specific site in the hinge region opening the structure to further proteolytic attack (Goldberg and Slater, 1992). When ferrous haem is released from globin it oxidises to a toxic ferric form. To prevent damage from haem the parasite converts the compound to haemozoin (H). Haemozoin can be synthesised by heating haemoglobin to 80°C under highly acidic conditions (Goldberg and Slater, 1992). It is believed that it is comprised of a polymer of haems linked by an unusual iron-carboxylate bond (Goldberg and Slater, 1992). The parasite achieves this conversion by using an enzyme known as haem polymerase, and chloroquine and quinine inhibit the polymerisation process (Slater, 1993). Although parasite proteins associate with haemozoin, their removal does not alter the properties of the pigment, which accumulates in the host's spleen. It has been suggested that human macrophages become unresponsive following digestion of haemozoin (Goldberg and Slater, 1992; Arese *et al*, 1993).

1.1.6. Trafficking in Malaria Infected Erythrocytes

During its development within the red blood cell, the malarial parasite alters the erythrocyte in many ways. The parasite changes the lipid composition (Hsiao *et al*, 1991) and inserts some of its proteins (Gormley *et al*, 1992; Elmendorf and Haldar, 1993) into the erythrocyte membrane. It is thought that there are several mechanisms to transport specific malaria proteins to the surface of the infected cell (Gormley *et al*, 1992). Three different membranous structures have been described in parasitised cells. These structures have been described as long slender unit membrane clefts (Maurer's clefts - figure 2), circular unit membranes with electron dense contents, and vesicles with multiple membranous whorls (Gormley *et al*, 1992).

It is believed that the PVM does not simply surround the parasite. Protrusions are seen appearing from the PVM and these often bud-off forming vesicles (Elford and Ferguson, 1993). The term 'tubovesicular membrane system' (TVM) has been used to

describe these structures and it has been proposed that they have similarities to the Golgi apparatus (Elmendorf and Haldar, 1993). The TVM contains proteins with significant homology to Golgi house-keeping proteins namely BiP, ERD2 and thiamine pyrophosphatase.

The export of a number of parasite proteins has been be tracked using specific antibodies and confocal immunofluorescence (Gormley *et al*, 1992). The 300kd protein MESA (also known as PfEMP2) has been shown to be associated with electron dense vesicles that bud off the PVM. A histidine-rich protein (known as PfHRP1) has been shown to be associated with the knobs on the erythrocyte membrane. During the export of this protein it has been localised in the electron dense vesicles and membranous whorls found in the red blood cell cytoplasm. Another histidine-rich protein (known as PfHRP2) is excreted into the bloodstream from malaria infected erythrocytes. This protein appears to cross the erythrocyte as an aggregate that does not contain lipid (Gormley *et al*, 1992).

Elmendorf and Haldar (1993) suggested that the malarial parasite secretes proteins, like MESA, into the vacuolar space surrounding the parasite and that a second round of secretion occurs through the tubovesicular system. It has also been suggested that in certain positions the PVM and the PM fuse to form a single lipid bilayer (Gormley *et al*, 1992). In this model PfHRP1 would be secreted from the parasite at a region where these two regions have fused and carried in membranous vesicles to the erythrocyte membrane.

1.1.7. Carbohydrate Metabolism in *Plasmodium falciparum* Infected Erythrocytes

Plasmodium does not contain stores of carbohydrate and is totally dependent on blood glucose for its energy supply (Sherman, 1979). Virtually all the glucose utilised by *Plasmodium* is converted to lactate by glycolysis. All the glycolytic enzymes have been shown to increase in activity in infected erythrocytes (Roth *et al*, 1988) and glycolytic rates 30-50 times that of the uninfected cell have been reported (Shakespeare *et al*, 1979). Many of the glycolytic enzymes have been cloned from malarial parasites including lactate dehydrogenase (Bzik and Fox, 1993), 3-phospho-

glycerate kinase (Hicks *et al*, 1991), aldolase (Knapp *et al*, 1990), triose phosphate isomerase (Ranie *et al*, 1992) and enolase (Read *et al*, 1993 and figure 17).

There has been some debate about whether *Plasmodium falciparum* possesses an active pentose phosphate pathway. On theoretical grounds, it seemed probable that the malaria parasite would have an absolute requirement for the pentose phosphate pathway if only to supply pentoses for DNA synthesis. Human erythrocytes are resistant to *Plasmodium falciparum* when they are deficient in glucose-6-phosphate dehydrogenase (G6PDH), the first enzyme of the pentose phosphate pathway, suggesting that they require the host's enzyme for survival. For many years it was not thought that *Plasmodium falciparum* had endogenous G6PDH activity but this has now been detected (Ling and Wilson, 1988) and it has been cloned and sequenced (O'Brien *et al*, 1993).

Although *Plasmodium falciparum* contains a mitochondrion it is acristate and is not thought to possess an active citric acid cycle (Sherman, 1979).

1.1.8. The Genetics of *Plasmodium falciparum*

Plasmodium falciparum has 14 chromosomes as determined by pulse field gel electrophoresis (PFGE) and by electron microscopy (Carle and Olson, 1984; Schwartz and Cantor, 1984; Chu *et al*, 1986). The largest chromosome is 3.4Mb and the smallest 640kb (Foote and Kemp, 1989). Structurally the chromosomes are typical of lower eukaryotes. Malarial parasites do not contain a sex chromosome as the asexual parasites can develop into either a microgametocyte or a macrogametocyte. The sizes of the chromosomes vary between different *P. falciparum* isolates sometimes by several hundred kilobases. The size differences are seen both in fresh and cultured strains indicating that variations arise during mitosis as well as meiosis. The variation is almost entirely in the sub-telomeric regions and occurs in wild populations taken directly from a patient, suggesting that they have little effect on the parasite. The size variation is thought to be due to several factors (Foote and Kemp, 1989). These include:

i) Chromosomal breakage and healing in sub-telomeric regions.

ii) Frequent crossover events during meiosis. The highly repetitive nature of these regions facilitates the transfer of DNA from one chromosome to another by recombination during meiosis.

iii) Amplification and suppression of regions of the genome under selective pressure. Many of the most antigenic proteins in *Plasmodium falciparum* are encoded in regions near the ends of the chromosomes. These proteins contain many repeats making them highly antigenic. It is possible that their location in highly labile areas of the genome allows them to change quickly and evade the host's immune system. In some cases these proteins can be deleted altogether. For example the gene encoding the knob-associated histidine-rich protein is completely missing from the D10 isolate and partially deleted in other isolates (Foote and Kemp, 1989).

The major problems in the study of *Plasmodium falciparum* genetics are:

i) Recombination is difficult. To obtain a genetic cross the two strains must be cloned, induced to produce gametes *in vitro*, fed to mosquitoes, passaged through a chimpanzee and cloned and cultured again. With recent advances in culturing of mosquito stages *in vitro* (Trager *et al*, 1992) the ability to produce genetic crosses may be improved, but are still troublesome.

ii) The chromosomes are not visible by light microscopy which makes *in situ* hybridisation impossible. This is because of the comparatively small genomic size, $3x10^7$ bases, of *Plasmodium falciparum* and because the chromosomes do not condense in the same way that human chromosomes do during DNA replication.

iii) The genome is AT-rich (82%) which makes fragments of malarial DNA unstable in *Escherichia coli*. Cosmid libraries of *P. falciparum* DNA grown in *E. coli* are unstable undergoing spontaneous deletions and rearrangements.

Despite these problems much progress in mapping the malarial genome has been made in the past few years. The success of this technique has mainly been due to the use of yeast artificial chromosomes (YAC) (Burke *et al*, 1987) and PFGE (Schwartz and Cantor, 1984) that can separate chromosome sized pieces of DNA on a 1.5% agarose gel. Large pieces of DNA can be made to behave like a yeast chromosome by attaching a centromere, and a telomere to each end. This DNA can then be propagated in *Saccharomyces cerevisiae*. It has been shown that large pieces of malarial DNA (≈200kb) are stable in YACs, probably due to yeast also being AT-rich (Triglia and Kemp, 1991). A potential problem with YACs is that only one copy of the cloned DNA is present per yeast cell. This has recently been overcome by a method that amplifies YAC chromosomes so that there are up to 20 copies per cell (Smith *et al*, 1990).

Traditionally small genomes are mapped using a 'bottom up approach'. Overlapping cosmids (30-45kb) are screened with genetic markers and pieced together to obtain a map of each chromosome. As malarial DNA is not stable in *E. coli* this approach is impractical for *Plasmodium* DNA but the genome is now being mapped using YACs. Another advantage of the YAC system is that the ends of YACs can be cloned (as a 2-5kb fragment in *E. coli*) and used for rescreening to find overlapping YACs (Riley *et al*, 1990).

There are many reasons why a high density map of *Plasmodium falciparum* would be advantageous (Triglia *et al*, 1992), including the identification of specific genes involved in various processes like drug resistance and cytoadherence.

1.2. The Facilitative Sugar Transporters

Glucose is an essential energy source for most cells. For this reason, and because the lipid bilayer is impermeable to sugars, most cells require a specific glucose transport protein. Sugar transport proteins have recently been cloned from a variety of organisms, ranging from mammals through to protozoa, algae, yeasts and prokaryotes. Most of these proteins are believed to have a common structure because of their low, but significant, sequence similarity to the mammalian erythrocyte glucose transporter known as HepG2 or GLUT1 (Mueckler *et al*, 1985). Table 1 lists the members of the facilitative sugar transporter superfamily. These are discussed in more detail in section 1.2.3.

Members of the superfamily transport a range of hexoses including glucose, fructose, lactose and galactose. Two members of the family transport quinate, a six-membered ring compound (figure 3) (Hawkins *et al*, 1988; Geever *et al*, 1989; Lamb *et al*, 1990) and some transport the pentoses xylose and arabinose. The mechanism of transport also differs across the superfamily. For example some, including the mammalian transporters, allow passive diffusion, whilst others, including three transporters from *E. coli*, actively transport the sugar across the membrane in conjunction with a proton. This may be an essential adaptation. The red blood cell finds itself permanently bathed in approximately 5mM glucose and so does not need to transport glucose against a concentration gradient. Organisms such as *E. coli* find the external glucose concentration highly variable and need to accumulate sugar when the external concentration is low.

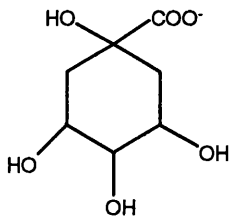

Figure 3. The structure of quinate (1,3,4,5-tetrahydroxy-hexahydrobenzoate)

Name	*Species*	*Substrate*
GLUT1 (HEPG2)	Human, rat, mouse, rabbit, chicken, pig, cow	D-glucose
GLUT2	Human, rat, mouse	D-glucose
GLUT3	Human, mouse, chicken	D-glucose
GLUT4	Human, rat, mouse	D-glucose
GLUT5	Human, rat	Fructose
GLUT7	Rat	D-glucose
SNF3	*Saccharomyces cerevisiae*	D-glucose
HXT1	*Saccharomyces cerevisiae*	D-glucose
HXT2	*Saccharomyces cerevisiae*	D-glucose
HXT3	*Saccharomyces cerevisiae*	D-glucose
HXT4	*Saccharomyces cerevisiae*	D-glucose
MAL6T	*Saccharomyces cerevisiae*	Maltose
GAL2	*Saccharomyces cerevisiae*	D-galactose
RAG1	*Kluyveromyces lactis*	D-glucose
LACP	*Kluyveromyces lactis*	Lactose
	Synechocystis PCC6803	D-glucose/H^+
AraE	*Escherichia coli*	L-Arabinose/H^+
XylE	*Escherichia coli*	D-Xylose/H^+
GalP	*Escherichia coli*	D-galactose/H^+ & D-glucose/H^+
	Zymomonas mobilis	D-glucose
STP1	*Arabidopsis thaliana*	D-glucose/H^+
	Chlorella kessleri	D-glucose/H^+
THT1	*Trypanosoma brucei brucei*	D-glucose, Fructose
THT2	*Trypanosoma brucei brucei*	D-glucose
SGTP1	*Schistosome*	Glucose ??
SGTP2	*Schistosome*	Glucose ??
SGTP4	*Schistosome*	Glucose ??
Pro1	*Leishmania enriettii*	D-glucose/H^+
Pro1-cp1	*Leishmania enriettii*	D-glucose/H^+
D1	*Leishmania donovani*	D-glucose/H^+
D2	*Leishmania donovani*	D-glucose/H^+
qa-y	*Neurospora crassa*	Quinate
QUTD	*Aspergillus nidulans*	Quinate

Table 1. Members of the facilitative sugar transporter superfamily. These are discussed in more detail in section 1.2.3.

1.2.1. The Asymmetric Kinetics of the Erythrocyte Transporter

The human erythrocyte transporter is believed to exist in two mutually exclusive forms: an inward (T_i) and an outward facing conformation (T_o). This is known as the two state carrier model (Barnett *et al*, 1975) which is depicted in figure 4.

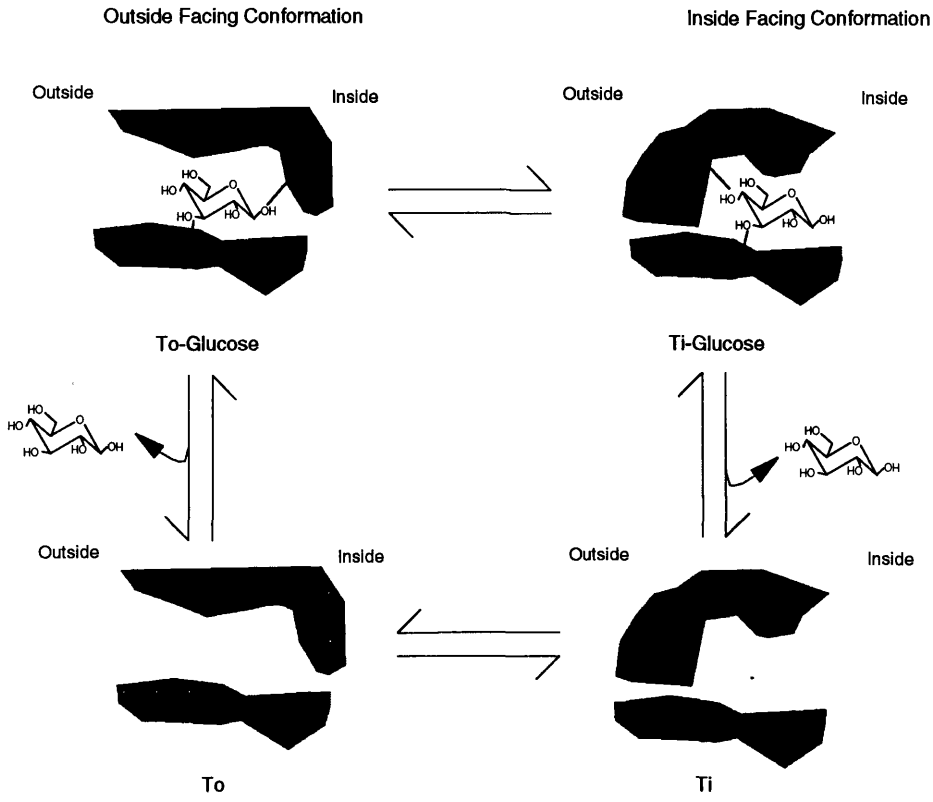

Figure 4. The two state carrier model for the human erythrocyte glucose transporter. Glucose can bind to either the inward or outward conformations.

The following experimental data on the kinetics of red blood cell glucose transport support the two state model:

i) Sugar influx and efflux can have different kinetic constants

The erythrocyte protein is regulated by phosphorylation (Carruthers, 1986). The unphosphorylated carrier is symmetric, while the phosphorylated transporter has a Km for influx of approximately 2mM and a Km for efflux of approximately 25mM (Widdas, 1980). In the two carrier model the internal and external binding sites would be expected to be constructed of two different regions of protein and similar affinities would be purely coincidental.

ii) Sugar exchange is faster than sugar flux

The rate of glucose/glucose exchange is faster than the maximal uptake or efflux rates (Widdas, 1980). For a two-state carrier model this would imply that the transporter

can change between the inward and outward facing conformations faster when the sugar is bound and that the transition in the absence of sugar is rate limiting.

iii) Differential inhibition of influx and efflux.

Experiments using 4,6-O-ethylidene-α-D-glucopyranose (ethylidene glucose) showed that the glucose analogue entered red blood cells by direct diffusion through the membrane and did not enter through the glucose transporter (Baker and Widdas, 1973b). However, ethylidene glucose on the outside of the cell is a potent non-competitive inhibitor of glucose efflux (Baker and Widdas, 1973a). The two-state carrier model accounts for this by suggesting that ethylidene glucose binds to the outward facing binding site, locking the transporter into the outward-facing conformation. When the cells were preloaded with ethylidene glucose it was found to be a poor inhibitor of uptake and did not cause the transient accumulation of glucose (Baker and Widdas, 1973a). These results are consistent with ethylidene glucose binding with a high affinity to the outward-facing binding site and a low affinity to the inward-facing site.

The effects of alkyl sugar derivatives on glucose influx and efflux have also been studied (Barnett *et al*, 1973a). Like ethylidene glucose, these compounds enter the red blood cell by simple diffusion through the lipid bilayer and are not transported by the facilitative carrier. When the sugar was derivitised in the C1 position (e.g. n-Propyl-β-D-glucopyranose) the analogue inhibited on the inside of the cells but was a poor inhibitor on the periplasmic face. By contrast, sugars derivitised in the C6 position (e.g. 6-O-propyl-D-galactose, 6-O-pentyl-D-galactose, 6-O-benzyl-D-galactose, 6-O-methyl-D-glucose, 6-O-propyl-D-glucose and 6-O-pentyl-D-glucose) were found to behave like ethylidene glucose. They were good inhibitors at the external site and poor inhibitors at the internal site (Barnett *et al*, 1973a, 1975).

From the experiments with glucose derivatives it has been suggested that glucose binding on the inside and outside of the cell involves different regions of the glucose molecule (Barnett *et al*, 1975). The glucose molecule approaches the outside binding site with the C1 end facing the transporter. The protein undergoes a conformational

change to the internal conformation by closing around the C4/C6 end and opening around the C1 end (figure 4). On leaving the cell the glucose molecule approaches with the C4/C6 end leading (Barnett *et al*, 1975).

Cytochalasin B is a specific inhibitor of the internal binding site (Taverna and Langdon, 1973; Lin and Spudich, 1974) and maintains the transporter in the inward-facing conformation. Kinetic analysis suggests that cytochalasin B is a competitive inhibitor of substrate efflux and a non-competitive inhibitor of substrate influx. By contrast the photolabel bis-mannose compound ATB-BMPA irreversibly labels the protein and locks it in the outward facing conformation (Holman and Rees, 1987).

iv) Inactivation of transporter with FDNB increases in the presence of its substrate.

Further evidence that the transport protein undergoes a conformational change when the sugar binds is provided by work using the modifying agent 1-fluoro-2,4-dinitrobenzene (FDNB). The inactivation of sugar transport in red blood cells by FDNB is faster in the presence of substrates of the protein such as D-glucose, 2-deoxy-D-glucose or mannose (Krupka, 1971). Maltose is not transported and protects the transporter from labelling (Krupka, 1971). The inhibitor phloretin similarly protects the transporter from labelling but its glucoside analogue phlorizin exposes the transporter to faster modification (Krupka, 1971). The C1, C2 and C3 alkyl substitutes of glucose enhance FDNB labelling whereas the C4 and C6 alkyl substitutes protect or have little effect on the rate of transporter labelling (Barnett *et al*, 1975). This suggests that FDNB labels a form of the transporter that binds sugars on the inside of the cell and is further evidence for the two-state carrier model.

1.2.2. Structure of Sugar Transporters

All the members of the facilitative sugar transporter superfamily are believed to have a common structure. Most are about 500 amino acids in length and have a molecular weight of between 45-55kD. Many however, migrate at around 40kD on SDS polyacrylamide gels. The proteins often appear as a diffuse band on such gels due to the glycosylation variations on the protein.

An unusual feature of this family of membrane proteins is their lack of an N-terminal signal sequence to direct the protein to the membrane. Instead of being inserted into the membrane cotranslationally, like most mammalian membrane proteins, they are inserted post-translationally into microsomes. This property has meant that it has been possible to insert the human erythrocyte transporters into the membrane of *Escherichia coli* and to obtain a functional protein (Sarkar *et al*, 1988).

A 2-dimensional model of the human erythrocyte transporter is depicted in figure 5. All the superfamily members are believed to span the lipid bilayer twelve times. The N and C termini of the protein are positioned in the cytoplasm. A large and highly hydrophilic region between TM6 and TM7, is predicted to contain a large proportion of α-helix and to be cytoplasmic (Henderson, 1991). Models suggest that about 80% of the protein is α-helical, with most of the amino acids residing within the lipid bilayer (Baldwin and Henderson, 1989). Despite this 80% of the residues are accessible to deuterium exchange suggesting that most of the protein molecule is accessible to water (Gould and Bell, 1990). This is consistent with the hypothesis that the transmembrane regions form a pore through which glucose can travel.

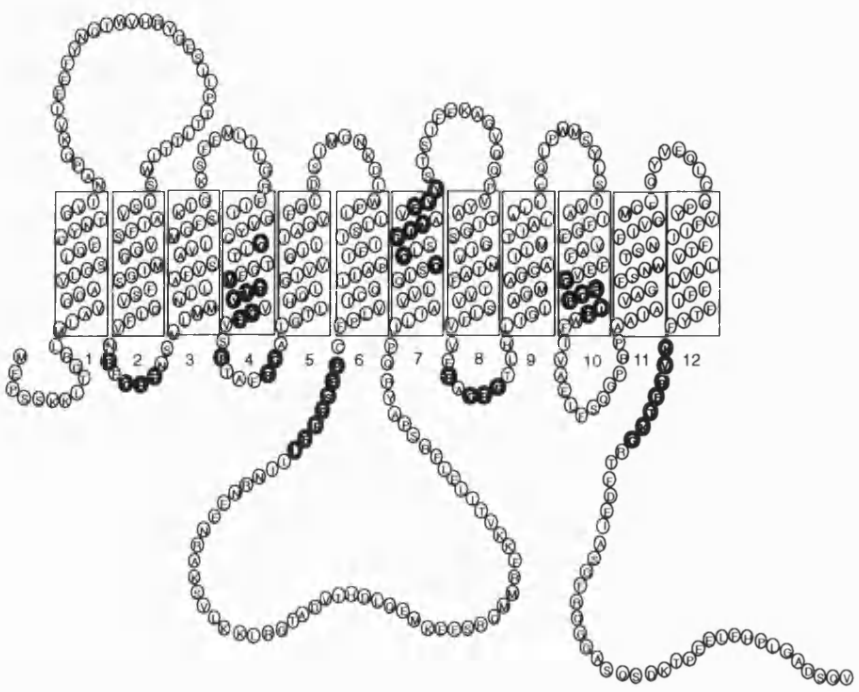

Figure 5. Schematic view of the human GLUT1 glucose transporter showing the twelve predicted transmembrane regions. Residues that are conserved within the facilitative sugar transporter superfamily are shown.

The N terminus varies in length greatly between members of the superfamily; from 10 residues in the human liver transporter to over 90 in the yeast SNF3 transporter. The region connecting transmembranes 1 and 2 is also of a highly variable length ranging from 19-66 residues. Except for the region between TM1 and TM2, and the region between TM6 and TM7, all the transmembrane regions are usually joined by short loops between 7 and 15 residues long.

Some regions of the protein are conserved throughout the superfamily. Variations in the amino acids in these regions are rare, and changes that occur are conservative substitutions. The regions of conservation are shown in figure 5.

There is a conserved region that begins in transmembrane 4 and stretches through to the start of transmembrane 5. The sequence is G-----VPMY-GE--P---RG and its importance is at present unknown.

It has been suggested that another conserved motif PESPRFL, which is located just after TM6, may serve a role in the recognition of substrates (Henderson, 1990)

In transmembrane 7 is the sequence motif QQ--GIN--FYY, which is highly conserved in all members of the superfamily. If the first glutamate molecule is mutated to a leucine in the erythrocyte transporter it loses the ability to bind the externally binding, impermeant, photolabel ATB-BMPA and the rate of glucose transport is reduced by half (Hashiramoto *et al*, 1992). It is believed that this region is part of the substrate's external binding site. The amphipathic nature of this membrane spanning region has led to the proposal that this highly conserved region may form part of the channel across the membrane.

The structural motif R-GRR, which is found in two places between TM2-3 and between TM8-9 is also very well conserved. This motif is strongly predicted to form a β-turn and is also found in other transporters that have 12 membrane spans. For example the motif is found twice in similar positions in the otherwise non-homologous citrate/H^+ symporter, lactose/H^+ and glucuronide transporters from *Escherichia coli* (Maiden *et al*, 1987; Henderson, 1991). It has been suggested (Henderson, 1990) that the protein is composed of two structurally independent domains: the C terminal and the N terminal halves. The 6 transmembrane regions in each half would fold together to produce a bilobal protein. The presence of this sequence in the same position in both halves of the protein supports this hypothesis. The presence of this feature in mammalian, yeast and bacteria sequences means that this duplication must have occurred very early in the evolution of the gene (Szkutnicka *et al*, 1989). When the *Escherichia coli* lactose/H^+ transporter (Lac Permease) is expressed in two separate halves, TM1-6 and TM7-12, the two proteins combine to produce a functional protein whereas neither half is active on its own (Kaback *et al*, 1990). Similar results have been obtained when the two halves of the human erythrocyte transporter were expressed in Baculovirus. When expressed independently the C-terminal and the N-terminal halves failed to bind the glucose transporter specific photolabel ATB-BMPA; when expressed together labelling was detected (Cope, 1993).

In transmembrane 10 of the mammalian transporters is the triple proline motif, GPGPIP, which confers considerable flexibility to this transmembrane spanning domain and is believed to have a role in changing the protein between its two forms the internal and external binding types (Gould and Bell, 1990). It is also believed that the internal facing, competitive inhibitor cytochalasin B binds near this region (Holman and Rees, 1987) in the human erythrocyte transporter. This suggests that this region may form part of the internal substrate binding site.

At the end of the protein, just after transmembrane 12 is found the motif KVPETKG, which is found in a similar position in many transmembrane superfamilies including both the muscarinic and nicotinic acetylcholine receptors, the calcium transporting ATPase from endoplasmic and sarcoplasmic reticulum, and the arsenical pump from *Escherichia coli*.

In rat GLUT1 mutants where the C-terminal 37 amino acids are deleted the transporter appears to be locked into the inward facing form that can bind cytochalasin B but not ATB-BMPA. It has therefore been suggested that the C-terminus may be involved in switching the protein between its two conformations (Oka *et al*, 1990)

1.2.3. Superfamily Members

1.2.3.1. The Mammalian Transporters

In humans there are at least 6 functionally different facilitative sugar transporters and one pseudogene. All the transporters are expressed in highly controlled ways, with very specific tissue distribution. Each transporter has different properties and fills a particular niche.

1.2.3.1.1. Mammalian GLUT1

The human GLUT1 transporter is the best studied member of this superfamily. It is located in red blood cells, the brain, blood barrier tissues (e.g. blood/brain, placenta, retina) and in insulin sensitive muscle and fat cells. Human erythrocytes are an extremely rich source of this protein (5% of membrane protein is GLUT1). Human GLUT1 is the only member of this superfamily to have been purified (Baldwin *et al*,

1982; Baldwin and Lienhard, 1989). The purification led to an N-terminal sequence, and to the cloning of the protein (Mueckler *et al*, 1985). The sequence revealed a site, between TM1 and TM2, for the attachment of an asparagine linked oligosaccharide. Since then the analogous rat, mouse, rabbit, chicken and pig sequences have been determined. All these proteins are highly homologous to the human sequence. For example the rat protein has 98% identity to the human GLUT1 at the amino acid level. This suggests that all the regions of the protein have a function so that none can diverge significantly.

As mentioned earlier this transporter shows asymmetric kinetics. The Km_{influx} is ten times lower than Km_{efflux}, cytochalasin B binds to the internal binding site, ATB-BMPA binds to the external site, and many deoxy and fluoro analogues of glucose have differing specificities for the internal and external binding sites (Barnett *et al*, 1973b). The discrepancy in Km values means the transporter acts as a unidirectional transporter when the extracellular concentration is low and the intracellular demand for glucose is high. This may explain the increased expression of GLUT1 in cultured cells where the external glucose concentration is often low (Gould and Holman, 1993).

The specificity of the transporter has been investigated. It is 2-deoxy-D-glucose > 6-deoxy-D-glucose ≈ D-glucose > D-mannose > D-galactose ≈ 2-deoxy-D-galactose > D-xylose > L-arabinose > D-fucose >> L-fucose > L-rhamnose >> L-glucose (Baldwin and Henderson, 1989).

1.2.3.1.2. Mammalian GLUT2

The low levels of GLUT1 mRNA in liver cells and the different properties of glucose transport in liver suggested that another protein was responsible for glucose transport into liver cells. The liver transporter, known as GLUT2 was cloned (Fukumoto *et al*, 1988) and shown to be highly homologous to the erythrocyte transporter. The transporter has also been localised in the pancreatic β-cells suggesting that it has a role in the glucose stimulated release of insulin from these tissues. It is also localised in the kidney and intestine where it is believed to act in conjunction with the Na^+/glucose symport protein in the unidirectional transport of glucose in these tissues.

Kinetically GLUT2 has a high Km ~42mM for 3-O-methyl-glucose and a high Kd for cytochalasin B ~10^{-6}M when compared to the erythrocyte transporter (Gould and Bell, 1990). The high Km means that at physiological concentrations the glucose flux is proportional to the concentration of glucose and hence transporter saturation does not occur and is not rate limiting. Along with the transporter's high transport capacity this makes this transporter ideal for the rapid glucose efflux from the liver following gluconeogenesis or the degradation of glycogen.

The equivalent rat (Thorens *et al*, 1988) and mouse (Asano *et al*, 1989; Suzue *et al*, 1989) sequences have been determined. The rat protein has 82% identity to the human sequence.

1.2.3.1.3. Mammalian GLUT3

Mammalian GLUT3 protein is localised in tissues that have a high demand for glucose namely the brain, nerves and the heart. This isoform may act in conjunction with GLUT1 in these tissues to help these tissues meet their high energy demands. The comparatively low km for 3-O-MG (10mM) makes this isoform essential in times of hypoglycaemia or high glucose demand when blood glucose levels are low (Gould *et al*, 1991). Tissues expressing this isoform will receive glucose in preference to tissues expressing other isoforms with higher Km's (e.g. the liver) when glucose is scarce.

The GLUT3 protein has been cloned from human (Kayano *et al*, 1988), mouse (Nagamatsu *et al*, 1991) and chicken (White *et al*, 1991) sources. All show very high degrees of homology to each other.

1.2.3.1.4. Mammalian GLUT4

The GLUT4 protein is the main glucose transporter found in the brown and white fat cells and cardiac and skeletal muscle. This isoform is the insulin regulatable glucose transporter. Stimulation of these cells with insulin produces a 30 fold increase in the rate of glucose transport. Labelling studies with the impermeant photolabel ATB-BMPA show that there is a 20 fold increase in GLUT4 at the cell surface after insulin

stimulation (Holman *et al*, 1990). The stimulation is achieved by the movement of GLUT4 protein to the plasma membrane from stores sequestered in light microsomes (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). It is believed that insulin increases the rate of exocytosis and fusion of the microsome fraction with the plasma membrane releasing these transporters into the membrane.

The human (Fukumoto *et al*, 1989), rat (James *et al*, 1989) and mouse (Kaestner *et al*, 1989) genes have been cloned and sequenced. All three proteins are highly homologous. For example, the rat and human sequences are 95% identical at the amino acid level.

1.2.3.1.5. Mammalian GLUT5

Mammalian GLUT5 has been found to be a high affinity fructose transporter (Burant *et al*, 1992). The cloned protein was expressed in *Xenopus laevis* oocytes and found to have a Km of 6mM for fructose. This isoform appears to be insensitive to cytochalasin B (Burant *et al*, 1992).

Normally, GLUT5 is located in the small intestine where it clears fructose from the gut, and in testes, spermatozoa, muscle, brain and adipose tissue where its function is to supply fructose (Burant *et al*, 1992). The human (Kayano *et al*, 1990) and rat (Rand *et al*, 1993) sequences have been determined. The human protein consists of 501 amino acids and has significant homology to the mammalian glucose transporters.

1.2.3.1.6. Mammalian GLUT6

GLUT6 is a pseudogene, found in humans, whose mRNA is found in most tissues (Kayano *et al*, 1990). The sequence has 80% identity to the human GLUT3 sequence but does not contain introns. It probably resulted from the reinsertion of a GLUT3 mRNA transcript into the genome by a viral reverse transcription event during evolution (Kayano *et al*, 1990). Due to the lack of evolutionary pressure to maintain a functional protein, the sequence has mutated extensively and now contains several stop codons and frame shifts. It is very unlikely to produce a full length or functional transporter (Kayano *et al*, 1990).

1.2.3.1.7. Mammalian GLUT7

It is believed that GLUT7 is responsible for the transport of glucose across the ER membrane into the cytosol (Waddel *et al*, 1992). When the body needs glucose the liver produces it either by the degradation of glycogen or by synthesis from pyruvate (gluconeogenesis). The final step in both these processes, the removal of the PO_4 from glucose-6-phosphate, is accomplished by the multi-component enzyme glucose-6-phosphatase. This enzyme resides in the endoplasmic reticulum and the synthesised glucose is made in the lumen of the ER.

The rat GLUT7 gene has been cloned and found to consist of 528 amino acids (Waddel *et al*, 1992). GLUT7 is 68% identical to GLUT2 at the amino acid level with very high identity in transmembrane regions 1,2,3,4,9,10. Lack of base drift in the 3rd position suggests its origin is from GLUT2.

One difference between GLUT2 and GLUT7 is that the sequence motif KKMKND is appended to the end of the C terminus of GLUT7. This sequence is believed to mark the transporter for retention in the endoplasmic reticulum (Waddel *et al*, 1992).

1.2.3.2. The Yeast Members of the Superfamily

A series of glucose transporters from yeast have been identified. Five genes (*HXT1*, *HXT2*, *HXT3*, *HXT4* and *SNF3*) have been shown to transport glucose in *Saccharomyces cerevisiae*. Four other genes in the facilitative sugar transporter superfamily have also been isolated from yeast: the maltose permease from *Saccharomyces carlsbergensis*, a galactose transporter from *Saccharomyces cerevisiae*, a lactose permease from *Kluyveromyces lactis* and a glucose/galactose permease from *Kluyveromyces lactis*.

1.2.3.2.1. The *Saccharomyces cerevisiae SNF3* Gene

Mutants lacking the *SNF3* gene are unable to use sucrose (SNF ≡ Sucrose Non-Fermenting) as a carbon source. Normally sucrose is hydrolysed to glucose and fructose in the periplasmic space and then the monosaccharides transported by the

SNF3 gene. The protein has a high affinity (Km ~ 1mM) for glucose and can also transport fructose (Bisson and Fraenkel, 1983). The low Km for glucose allows *Saccharomyces* to grow on low levels of glucose (Bisson and Fraenkel, 1983).

The protein has been cloned (Celenza *et al*, 1988) and has been localised to the left arm of chromosome IV (Marshall-Carlson *et al*, 1990). This protein is much longer than the other members of the superfamily. It consists of 884 amino acids and has a molecular weight of 97kDaltons. It contains an additional 85 amino acids at the N-terminus and an additional 303 amino acids at the C-terminus. Mutations where the C terminal 246 amino acids were removed have a greatly impaired ability to transport glucose (Marshall-Carlson *et al*, 1990). It is believed that this region is important in the protein's interaction with hexose kinases (Bisson and Fraenkel, 1983).

1.2.3.2.2. The *HXT* Family of Glucose Transporters from *Saccharomyces cerevisiae*

The four *HXT* genes so far identified as glucose transporters in *Saccharomyces* are differentially expressed. For example *HXT1* appears to be expressed during exponential growth whereas *HXT3* appears to be maximally expressed after entry into stationary phase. All the glucose transporters from *Saccharomyces* are very similar in structure. For example, the inferred amino acid sequence of *HXT3* is 87% identical to *HXT1*, 64% identical to *HXT2* and 32% identical to *SNF3* (Ko *et al*, 1993). Expression of any one of these genes allows the yeast to grow on >1% glucose (w/v). Overexpression of *HXT3* in Δ*snf3* mutants allows the cells to grow on low glucose medium (Ko *et al*, 1993).

HXT2 was first identified by its ability to restore growth to mutants that were deficient in the *SNF3* gene. It has been cloned (Kruckeberg and Bisson, 1990) and found to consist of 541 amino acids. Genetic and biochemical analysis has revealed that both this gene and SNF3 are required for wild-type levels of high-affinity glucose transport to occur (Kruckeberg and Bisson, 1990).

None of the *HXT* genes are essential for growth even in *SNF3*⁻ mutants. Mutants lacking all five genes are unable to grow on high concentrations of glucose (5% w/v) but mutants containing one functional gene are viable on high glucose medium. The mutants lacking all five genes are able to grow on low concentrations of glucose (0.5% w/v) suggesting a 6th glucose transporter that is itself glucose repressible (Ko *et al*, 1993).

1.2.3.2.3. The *Saccharomyces carlsbergensis* Maltose/H^+ Symporter Gene *MAL6T*

The presence of any one of five unlinked MAL loci (*MAL1*, *MAL2*, *MAL3*, *MAL4* and *MAL6*) confers the ability of the cells to grow on maltose. Each locus codes for three genes: a maltase protein (*MAL6S*) which is an α-glucosidase, a maltose permease gene (*MAL6T*) and a protein (*MALR*) whose function is to regulate the expression of *MALS* and *MALT* (Hong and Marmur, 1986). The maltose permease protein is in the superfamily of hexose transporting proteins.

1.2.3.2.4. The *Saccharomyces cerevisiae Gal2* Gene

The primary sequence of the *gal2* gene from *Saccharomyces cerevisiae* has been determined (Szkutnicka *et al*, 1989). The protein consists of 574 amino acids and has a molecular weight of 63,789. The *HXT2* gene has 65% identity at the amino acid level. Like other members of the superfamily this protein lacks a signal sequence for directing the protein to the plasma-membrane. The protein has a substrate site for cyclic AMP-dependent protein kinase that appears to be a way of inactivating the transporter (Szkutnicka *et al*, 1989).

1.2.3.2.5. The *RAG1* Glucose Transporter from *Kluyveromyces lactis*

The *RAG1* gene has been sequenced and encodes a protein containing 567 amino acids (Goffrini *et al*, 1990). This protein has 73% identity to the *S. cerevisiae GAL2* protein over a 500bp stretch (Goffrini *et al*, 1990) and only 29% identity with *S. cerevisiae SNF3* (Goffrini *et al*, 1990). Despite this, *RAG1* does not appear to be a galactose transporter. The protein cannot complement a *gal2* mutation in *S. cerevisiae* and Δ

rag1 mutants assimilate galactose normally. *RAG1* can complement a *snf3* mutation indicating that this protein is likely to be a glucose transporter (Goffrini *et al*, 1990).

1.2.3.2.6. The Lactose Permease of *Kluyveromyces lactis*

The *Kluyveromyces lactis* lactose permease gene is located on a 4.7kbase mRNA that codes for two genes (Chang and Dickson, 1988). The first open reading frame encodes a 587aa protein that has been shown to be lactose permease. It is not believed that the second open reading frame is involved in lactose transport (Chang and Dickson, 1988).

As with other members of the superfamily the *Kluyveromyces lactis* lactose permease does not contain an N-terminal signal sequence and does not show significant homology to lactose permease from *Escherichia coli* (Chang and Dickson, 1988).

1.2.3.3. Prokaryotic Members of the Superfamily

Currently five genes homologous to the mammalian and yeast facilitative transporters have been isolated from prokaryotes. These include the glucose transporters from the Gram negative bacteria *Zymomonas mobilis* and the cyanobacteria *Synechocystis* and the xylose, arabinose and galactose/glucose transporters from *Escherichia coli*.

1.2.3.3.1. The *Synechocystis* Glucose Transporter

A glucose transporter has been isolated from the cyanobacterium *Synechocystis* strain PCC 6803. It was cloned by complementation in mutants that could not transport glucose and was found to be homologous to the mammalian transporters (Schmetterer, 1990). When the gene was interrupted the mutants again lost their ability to transport glucose.

Fructose is toxic to *Synechocystis* and it has been noted that mutants that lack the ability to transport glucose (and lack a functional glucose transporter) are resistant to fructose. This very strongly suggests that the transporter also transports fructose albeit with reduced affinity (Flores and Schmetterer, 1986).

1.2.3.3.2. The *Escherichia coli araE* Gene

The pentose L-arabinose is transported into *E. coli* by two mechanisms, one of which is a proton symporter. The protein encoding the symport protein has been cloned (Maiden *et al*, 1988), consists of 472 residues, has a molecular weight of 51,683, is commonly known as *araE* and is a member of this superfamily.

The proton symporter has the following specificity 6-deoxy-D-galactose ≈ L-arabinose > 6-deoxy-D-glucose > D-xylose >> D-galactose ≈ D-glucose (Baldwin and Henderson, 1989). The uptake of sugar is inhibited by cytochalasin B (Kd ≈ 10^{-6}M) and can be photolabelled under appropriate conditions. The sugar substrates protect the protein from cytochalasin B labelling (Baldwin and Henderson, 1989).

1.2.3.3.3. The *Escherichia coli XylE* Gene

Escherichia coli possess a xylose-proton symporter protein known as *xylE*. This protein has been cloned (Davis and Henderson, 1987), and was found to consist of 491 residues and to have a molecular weight of 53, 607.

Kinetically it has been found that cytochalasin B is not able to inhibit xylose transport by this protein. The *xylE* protein is believed to be a proton symporter because it is uncoupler sensitive and xylose transport is accompanied by an intracellular pH change (Davis and Henderson, 1987).

1.2.3.3.4. The *GalP* Gene of *Escherichia coli*

The *galP* locus in *Escherichia coli* has traditionally been associated with the ability of the bacteria to utilise galactose. Despite this, the specificity of the *galP* protein is similar to mammalian GLUT1. The specificity is D-glucose > 2-deoxy-D-glucose > D-galactose > 6-deoxy-D-glucose > D-talose > 2-deoxy-D-galactose > D-mannose > D-fucose > D-xylose >> L-glucose ≈ L-arabinose (Baldwin and Henderson, 1989). Cytochalasin B inhibits transport with a Kd of ~10^{-6}M and cytochalasin B binding is inhibited by D-glucose, D-galactose and 2-deoxy-D-glucose (Cairns, *et al*, 1991). The

protein migrates at 38,000kD on an SDS polyacrylamide gel and has homology to the *Escherichia coli* arabinose and xylose symporters (Baldwin and Henderson, 1989).

1.2.3.3.5. The *Zymomonas mobilis* Facilitative Glucose Transporter

Zymomonas mobilis is a Gram negative bacterium that, during evolution, has become highly specialised for growth in plant saps with a high sugar content. The glucose transporter from *Z. mobilis* is located in an operon with the first three enzymes of the pentose phosphate pathway glucokinase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydratase (Barnell *et al*, 1990). The pentose phosphate enzymes were cloned by complementation in *E. coli* and the glucose transporter sequence determined purely by its proximity to these enzymes on the operon (Barnell *et al*, 1990). The *Z. mobilis* glucose transporter is 473 amino acids long and kinetically it has been confirmed that it is a facilitative transporter with a Km of ~10mM (DiMarco and Romano, 1985).

1.2.3.4. Other Members of the Superfamily

1.2.3.4.1. The *Arabidopsis thaliana* D-glucose/H+ Symporter

A glucose transporting protein that has homology to the mammalian transporters has been cloned from the higher plant *Arabidopsis thaliana* (Sauer *et al*, 1990b). It is interrupted by three introns, consists of 522 amino acids and has a M.W. of 57,581 Daltons. When expressed in *Schizosaccharomyces pombe*, the protein caused the accumulation of 3-O-methyl-glucose, D-glucose and galactose (Sauer *et al*, 1990b). This suggests that the transport of glucose may be an active process in *Arabidopsis*.

1.2.3.4.2. The *Chlorella kessleri* D-Glucose/H^+ Symporter

The transport of D-glucose into *Chlorella kessleri* has been conclusively shown to be linked to the transport of a proton (Komor and Tanner, 1974). Addition of sugars caused a transient proton uptake under both respiratory and photosynthetic conditions. The non-metabolisable glucose analogue 6-deoxy-D-glucose was also used and it was found that its uptake was associated with proton flux (Komor and Tanner, 1974). Transport of sugars was found to be reduced considerably under anaerobic conditions,

in the dark, and in the presence of uncouplers. For example 50μM carbonylcyamide-p-trifluoromethyloxyphenyl-hydrazone totally destroyed transport. Sodium and potassium ions were unable to substitute for H^+ in the transport system (Komor and Tanner, 1974).

The transporter has been characterised kinetically. The Km values for D-glucose, 6-deoxy-D-glucose and 3-O-methyl-glucose are $1.5x10^{-5}$M, $2.7x10^{-4}$M and $1.0x10^{-3}$M respectively (Sauer *et al*, 1990a).

The gene responsible for D-glucose/H+ transport in *Chlorella* has been isolated and sequenced (Sauer and Tanner, 1989). The isolated gene has been expressed in *Schizosaccharomyces pombe* (Sauer *et al*, 1990a). The transformed cells accumulated 3-O-methyl-D-glucose up to 10 fold whereas wild-type *S. pombe* only equilibrate the sugar (Sauer *et al*, 1990a).

1.2.3.4.3. The D-Glucose Transporter from *Trypanosoma brucei brucei*

In trypanosomes two tandemly arranged multigene families of glucose transporters have been isolated (Bringaud and Baltz, 1993). The first, known as *THT1*, has been expressed in *Xenopus* oocytes and found to have properties identical to those found in the bloodstream form of the parasite. The second, THT2, is expressed differentially in the procyclic form of the parasite (Bringaud and Baltz, 1993). Both *THT1* and THT2 have homology to the mammalian facilitative transporters and both sequences are virtually identical over the second half of the protein (Bringaud and Baltz, 1992, 1993).

In the human host, trypanosomes are totally dependent on glucose for their energy supply and they have developed a unique organelle (the glycosome) to help it to maintain a fast turnover of glucose. In the bloodstream form *THT1* is insensitive to the inhibitors cytochalasin B and phloretin (Eisenthal *et al*, 1989). It is, however, inhibited by phlorizin that is traditionally considered an inhibitor of the mammalian glucose/Na^+ symporter (Koepsell and Madrala, 1987) and ineffective against this superfamily of

transporters. *THT1* appears to be kinetically symmetrical with a Km of ~4mM for 6-deoxy-D-glucose (Eisenthal *et al*, 1989).

The metabolism of glucose in trypanosomes appears to be poorly regulated. It appears that both glucose transport and glycolysis are unregulated and are working at full capacity (Ter Kuile, 1993). The transport of glucose across the plasma membrane appears to be rate-limiting. The bloodstream form of *T. brucei* transports glucose by way of a facilitative carrier (Eisenthal *et al*, 1989).

Both 1-deoxy-D-glucose and 1-fluoro-β-D-glucose exist exclusively in pyranose ring forms and both interact efficiently with *THT1*. This suggests that the transporter accepts glucose in a pyranose form (Eisenthal *et al*, 1989). Interestingly it appears as though the same transporter transports fructose, with a high affinity (Km ~ 2.5mM), in the furanose ring form (Fry *et al*, 1993).

1.2.3.4.4. The *Leishmania* D-glucose Transporters

Although both are kinetoplastids, *Leishmania* and *Trypanosoma* encounter widely differing conditions in their respective hosts. *Trypanosoma brucei* inhabits the midgut of the tsetse fly and the mammalian bloodstream; *Leishmania* the gut of the sandfly and the lysosomal vesicle of the human macrophage. Trypanosomes, in the human blood stream, are in a well-regulated system where the pH, temperature and glucose concentration are constant allowing the parasite to have a glucose metabolism that is uncontrolled (Ter Kuile, 1993). The lysosome of the human macrophage, by contrast, is acidic (pH4.0) and a much more hostile environment than the bloodstream. For this reason *Leishmania* must control metabolism carefully. *Leishmania* controls the internal pH and the electrochemical gradient across its plasma membrane allowing it to withstand large extracellular pH changes (Ter Kuile, 1993).

It has been reported (Zilberstein and Dwyer, 1985) that *Leishmania* transports glucose in conjunction with a proton. The evidence for this included the accumulation of 2-deoxy-D-glucose in untreated cells, the uptake of glucose in response to an artificial proton gradient and an increased flow of protons, in energy depleted cells, on the

addition of D-glucose. Recently it has been proposed that these conclusions are erroneous and D-glucose enters the parasites through a facilitative transporter (Ter Kuile, 1993). The formation of 6-phospho-2-deoxy-D-glucose explains the accumulation of the sugar and the stoicheometry of 2-deoxy-D-glucose/H^+ uptake was found to be about 10:1 once the phosphorylation of the analogue was taken into account (Ter Kuile, 1993). It has been suggested that D-glucose is passively transported across the plasma membrane and actively transported into an organelle. This would explain the D-glucose induced proton flux (Ter Kuile, 1993). Unlike the trypanosome protein the transport of glucose into *Leishmania* is inhibited by cytochalasin B and phloretin (Zilberstein and Dwyer, 1985).

Two isoforms, in the facilitative sugar superfamily have been isolated from *Leishmania enriettii* (Cairns, *et al*, 1989; Stack *et al*, 1990). These proteins are encoded in a tandem repeat of genes. All the genes appear to be virtually identical (known as *pro1*) except for the first. The first sequence is identical to the others except for the N-terminal cytoplasmic region (Stack *et al*, 1990), which contains 132 amino acids. These amino acids are completely different to the 48 amino acids found at the extracellular amino terminus of the other genes in the tandem repeat (Stack *et al*, 1990). The mRNAs encoding both of these isoforms are found in the promastigote (insect) form of the parasite (Stack *et al*, 1990).

Two other genes in the facilitative sugar superfamily have been isolated from *Leishmania donovani* (Langford *et al*, 1992). The two genes are known as D1 and D2. The three genes *Pro1*, D1 and D2 are located on different chromosomes in *Leishmania donovani* (Langford *et al*, 1992). The gene, D1 is very similar to the other members of the superfamily containing all the conserved regions and having 33% homology to the *E. coli* arabinose transporter *araE* (Langford *et al*, 1992). The D1 gene appears to be expressed equally abundantly in both the promastigote and amastigote (macrophage) stages of the life-cycle (Langford *et al*, 1992). One unusual feature of D1 is the very short N-terminus and long C-terminus of this protein. The first transmembrane region is predicted to begin with the third amino acid. The D2 protein is most similar to *Pro1* (44% identity) and both lack some of the conserved

regions. D2, like *Pro1*, is expressed predominantly in the insect form of the parasite (Langford *et al*, 1992).

1.2.3.4.5. The Sugar Transporters of *Schistosome*

Recently 3 genes with homology to the other members of this superfamily have been isolated from the parasitic worm *Schistosome*. These three genes *SGTP1*, *SGTP2* and *SGTP4* were cloned using the polymerase chain reaction and oligonucleotides raised against conserved regions in the superfamily primary structure (Skelly, 1993).

1.2.3.4.6. The *Neurospora crassa* Quinate Transporter

The *qa* gene cluster consists of five structural and two regulatory genes involved in the metabolism of quinate (figure 3) as a carbon source (Geever *et al*, 1989). The genes are co-ordinately induced by quinate and repressed by glucose (Geever *et al*, 1989). One of the genes in this cluster *qa-y* is the quinate transporter that has significant homology to human GLUT1 and other members of the superfamily (Geever *et al*, 1989).

1.2.3.4.7. The *Aspergillus nidulans* Quinate Transporter

Like *Neurospora*, *Aspergillus nidulans* can use quinate as a sole carbon source and the six genes required for quinate metabolism are linked on chromosome VIII (Hawkins *et al*, 1988). On this gene cluster is located the *QUTD* quinate permease gene that has homology to the facilitative sugar transporters.

2. Materials and Methods

2.1. *Plasmodium* Manipulation - Materials and Solutions

2.1.1. Solutions and General Laboratory Chemicals

All the solutions used were either purchased from Gibco-BRL, Merck-BDH or Sigma and prepared using ultra pure water (Milli-Q) and detergent free glass and plastic ware. All sterile filtering was performed with a 0.22μm filter. Unwanted cells were soaked in a 10% sodium hypochlorite solution for 12 hours before disposal.

2.1.2. Malarial Species and Strains

Plasmodium falciparum strain IT04 was obtained from Dr C. Newbold, John Radcliffe Hospital, Oxford. *Plasmodium yoelii* (strain YM) was obtained from Wellcome Research, Beckenham.

2.1.3. Culture Media and Plastic-Ware

Gentamicin (10mg/ml), L-Glutamine (200mM) and 1X RPMI-1640 Culture Medium (with HEPES, Minus Glutamine) were purchased from Gibco.

Incomplete medium was prepared by adding 1ml of 10mg/ml gentamicin to a 500ml bottle of RPMI-1640 medium.

Human A Rhesus+ red blood cells were obtained weekly from the local hospital and stored in 25ml aliquots with an equal volume of incomplete medium. The cells were washed before use with incomplete medium and the Buffy coat (the upper portion of packed cells), which contained the white blood cells, was removed. Human serum (A Rhesus+) was obtained from the transfusion service and stored at -20°C until ready to use.

Culture medium ('complete' medium) was prepared by adding the following chemicals to a 500ml bottle of RPMI-1640 medium: 50ml human serum, 5ml of 12.5μg/ml hypoxanthine\10% glucose solution, 1ml of 10mg/ml gentamicin, and 5ml 200mM L-glutamine (Geary *et al*, 1985). When the complete medium had been made it was

stored at -20°C until ready to use. Once thawed the medium was used within two weeks.

Disposable transfer pipettes, 1ml and 10ml Pasteur pipettes, 15 and 50ml sterile tubes and 75cm^2 culture flasks were obtained from Falcon.

Percoll® was obtained from Pharmacia.

Giemsa stain was purchased from BDH. It was filtered and diluted 10 fold with distilled water before use.

2.2. Culturing and Manipulation of *Plasmodium falciparum*

Plasmodium falciparum was maintained in continuous culture by an adaptation of the 'candle jar method' (Haynes *et al*, 1976; Trager and Jensen, 1976; Jensen and Trager, 1977) and is described below.

2.2.1. Microscopic Examination of *Plasmodium falciparum*

The culture was removed from the incubator and 0.5ml placed into an Eppendorf tube. The 0.5ml aliquot was microfuged for 10 seconds; the rest of the culture was returned to the incubator. The supernatant was discarded and the pelletted cells resuspended in the small amount of fluid that remained using a disposable plastic pipette. A drop of this suspension was placed in the centre of a glass microscope slide and smeared across it using a second slide. When the slide was dry, it was rinsed briefly with methanol, to fix the cells, and then placed in Giemsa's stain. After 2 minutes (or longer) the slide was removed from the stain, rinsed under the tap and blotted dry. The slide was examined using oil immersion. The malaria stained purple/blue whereas the red blood cells were pink/green. The parasites were counted distinguishing the different stages.

2.2.2. Changing Medium

The culture was removed from the incubator and gently swirled to return the settled cells to suspension. The culture was poured into a 50ml sterile tube and centrifuged at 500g in the bench centrifuge for 5 minutes. The supernatant was decanted and replaced with fresh, prewarmed (37°C) culture medium. The cells were resuspended

and returned to the 75cm^2 culture flask. The culture was gassed with 3% CO_2, 5% O_2 and 92% N_2 by inserting a pipette attached to the gas line into the culture flask.

Routinely the cells were maintained in 50ml of culture medium and 0.5ml of blood in 250ml (75cm^2) culture flasks. If there were more than 10% trophozoites and schizonts in the culture it was split into two or four and fresh blood added to maintain the 1% haematocrit.

2.2.3. CRYOPRESERVATION OF ERYTHROCYTES INFECTED WITH *P. FALCIPARUM*

2.2.3.1. Freezing cells in Sorbitol

The cells were pelleted by centrifugation at 500g for 5 minutes, the medium was aspirated and the cells gently resuspended in the small amount of supernatant that was remaining. An equal volume of sorbitol freezing solution (3% w/v sorbitol, 0.65% w/v NaCl and 28% v/v glycerol) was slowly added using a 1ml syringe and a 25 gauge needle. Care was taken to ensure that the freezing solution was prewarmed to 37°C as glycerol cannot penetrate the cells at 20°C (Mutetwa and James, 1985). The sample was transferred to NUNC freezing tubes and frozen rapidly by plunging into liquid nitrogen.

2.2.3.2. Thawing of deep frozen cells.

The vial was removed from the liquid nitrogen and placed at 37°C to thaw quickly. Once the cells had thawed, the sample was placed in a 50ml Falcon tube and a 1/5 volume of 12% NaCl (prewarmed to 37°C) added. The 12% NaCl was added dropwise through a 1ml syringe and 25 gauge needle with continuous agitation. The sample was allowed to stand for five minutes before adding 10ml of prewarmed (37°C) 1.6% saline. The solution was added slowly using a 21 gauge needle. The sample was then centrifuged at 500g for 5 minutes in the bench centrifuge, the supernatant removed, and 10ml of prewarmed 0.9% NaCl/0.2% glucose added dropwise to the pellet using a 21 gauge needle. After centrifugation at 1 500rpm for 5 minutes the supernatant was removed and the cells washed with incomplete medium. The cells were centrifuged and resuspended in 50ml of 37°C culture medium. More red blood cells were added to produce a 1% haematocrit. The cells were cultured as 10 x 5ml cultures in 25ml culture flasks for between one and five days at 37°C until they started

to grow with a 48 hour life cycle when the cells were transferred to 75cm^2 culture flasks (section 2.2.2.).

2.2.4. Concentration of Erythrocytes Infected with Mature Stage Parasites by Gelatine Flotation

In the early part of the project the cells were purified using Plasmagel™ (Laboratoire Roger Bellon, 159 Avenue A. Peretti, 92200 Neuilly Sur Seine, France) (Pasvol *et al*, 1978) but this compound became unavailable and was replaced with a 'home-made' substitute that behaved in a way indistinguishable from Plasmagel (Goodyer *et al*, 1993). This was an adaptation of a previously described method (Jensen, 1978).

A 6% w/v gelatine stock was made and diluted to produce the Plasmagel substitute. The stock solution was made by dissolving 1.5g of 150 bloom bovine gelatine (Sigma) in 25ml of warm (37°C) 0.7% NaCl, 0.2% $CaCl_2.2H_2O$ and 25mM HEPES. The pH was adjusted to 7.4 with NaOH, filtered through Whatman's No. 1 filter paper and autoclaved. The stock was diluted to 1% w/v gelatine with incomplete medium. Both Plasmagel and our substitute were stored at 4°C until ready to use (Goodyer *et al*, 1993).

Whether Plasmagel or our gelatine solution was used the concentration method was the same. The cells were removed from the incubator and centrifuged in a 50ml Falcon tube for 5 minutes at 500g to pellet the cells. The culture medium was removed and the cells gently resuspended in the small amount of supernatant remaining (≈0.5ml). The gelatine solution was warmed to 37°C and placed in 1ml aliquots in 15ml polypropylene tubes. The concentrated cell suspension (≈1ml) was mixed with the 1ml of gelatine solution. After incubation at 37°C for 15-30 minutes, the uninfected and ring infected erythrocytes settled below a layer of erythrocytes infected with mature stage (trophozoite and schizont) parasites. The upper layer was carefully removed into a clean tube using a plastic pipette and washed with incomplete medium before use. Care was taken not to wait too long before removing the upper layer as the infected cells settled into the lower layer with time lowering the yield. A Giemsa stained slide of the cells was prepared (section 2.2.1.) to determine parasitaemia. After purification, typically, 80-95% of the erythrocytes present contained a mature stage

parasite (Goodyer *et al*, 1993). The lower layer, containing mainly uninfected red blood cells and ring stage parasites, were often reintroduced into culture after washing with incomplete medium.

2.2.5. Synchronisation of *P. falciparum* Cultures with Sorbitol

The cells were synchronised using a 5% sorbitol in incomplete medium (Lambros and Vanderberg, 1979) that was sterile filtered and stored at 4°C until ready to use. The cells were removed from the incubator and centrifuged in the bench centrifuge at 500g for 5 minutes. As much of the medium as possible was removed from the cells which were then resuspended in 5ml of the 37°C sorbitol solution. The cells were incubated at 37°C for 5 minutes and then centrifuged again to pellet the cells. The supernatant was removed and the cells again resuspended in 5ml of the sorbitol solution. After a further 5 minutes incubation at 37°C, the cells were pelletted by centrifugation at 500g, washed with incomplete medium and reintroduced into culture (section 2.2.2.). A Giemsa stained slide (section 2.2.1.) of the synchronised cells revealed that the mature stage (trophozoite and schizont) parasites had lysed leaving only uninfected red blood cells and those containing rings intact.

2.2.6. Release of Parasites From the Erythrocyte Plasma Membrane using Saponin

A solution of 0.15% saponin in pH7.4 PBS was prepared (Stauber and Walker, 1946). The cells were pelleted by centrifugation in a bench centrifuge at 500g for 5 minutes, the medium was carefully removed, and the cells resuspended in 5ml of the 0.15% saponin solution. The red blood cells lysed immediately. After centrifugation at 500g for 10 minutes the supernatant was removed and replaced with another 5ml of the 0.15% saponin solution. After centrifugation at 500g for 10 minutes, the released parasites, which were still surrounded by the parasitic vacuolar membrane, were washed twice in pH7.4 PBS before use.

2.2.7. Release of Free Parasites by N_2-Cavitation

The mature *P. falciparum* parasites were concentrated by gelatine flotation (section 2.2.4.), washed twice in modified PBS (140mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 5mM sodium phosphate and 5mM glucose, pH 7.2) (Mikkelsen *et al*, 1986)

and then resuspended to 5% haemocrit in modified PBS. The cells were placed in the Parr bomb. Air was flushed from the bomb, and the cells subjected to 30 atmospheres of pressure for 30 minutes at room temperature. The cells were not stirred during this time as this appeared to lyse the mature parasites and cause excess clumping. The cells were then released through the valve at the bottom of the bomb removing the pressure rapidly. The cells were released into a siliconised tube (section 2.4.7.) containing a $1/10^{th}$ volume of modified PBS containing 10mM EGTA to prevent clumping. The cells were centrifuged at 500g for 10 minutes and resuspended in artificial intracellular medium (AIM). The AIM was a high potassium artificial intracellular buffer which consisted of 120mM KCl, 20mM NaCl, 10mM Pipes, 1mM $MgCl_2$, and 5mM glucose pH 6.7 (Mikkelsen *et al*, 1986)

2.2.8. Purification of Free *P. falciparum* Parasites on a Percoll Gradient

The Percoll® (Pharmacia) was diluted to 90% with 10X AIM (Mikkelsen *et al*, 1986) and then diluted further to 35% with 1X AIM. The Percoll® (8ml) was placed in the bottom of a 10ml polycarbonate ultracentrifuge tube (Beckman) and centrifuged at 30 000g for 30 minutes in a 70.1Ti rotor. The cells (resuspended in 2ml of AIM) were carefully layered on top of the gradient and then centrifuged at 10 000g for 10 minutes. The free parasites failed to enter the Percoll® gradient whereas the red blood cells banded towards the bottom of the tube. The released parasites were carefully removed into a Siliconised tube (section 2.4.7.) and washed in AIM twice to remove all traces of Percoll®.

2.2.9. Fluorescence of Free Parasites with Rhodamine 123

Intact viable malarial parasites maintain a potential across their membrane and hence retain the fluorescent dye Rhodamine 123 (Tanabe, 1983). Free parasites in AIM buffer, at a protein concentration of 0.1mg/ml, were equilibrated for 15 minutes at 37° C with 1.0µg/ml Rhodamine 123. After a 10 fold dilution with AIM the cells were centrifuged at 500g for 10 minutes, resuspended in AIM and examined immediately under a fluorescent microscope (Mikkelsen *et al*, 1986).

2.2.10. MAINTENANCE OF *PLASMODIUM YOELII* IN MICE

Mice (CD1 and BalbC strains) were infected with *Plasmodium yoelii* malaria parasites by inter-peritoneal injection of 100µl of infected mouse blood. The course of the infection was followed by removing a drop of blood from the animals, by tail bleeding, followed by examination of Giemsa stained smears (section 2.2.1.). When approximately 90% of the animal's erythrocytes were infected it was anaesthetised and its blood removed by cardiac puncture. It usually took 4-6 days for the animals to contract malaria. Spare infected mouse blood was mixed with 35% v/v glycerol and plunged into liquid nitrogen. To infect more mice this blood was thawed quickly by placing at 37°C and then injecting 100µl into uninfected mice.

2.2.11. REMOVAL OF WHITE BLOOD CELLS FROM *P. YOELII* INFECTED MOUSE BLOOD

Many methods are available for the removal and separation of white blood cells from red blood cells but the only effective way of separating *P. yoelii* infected erythrocytes from uninfected ones is to use cellulose (Homewood and Neame, 1976). This is because mature *P. yoelii* infected red blood cells have a similar density to white blood cells and thus co-purify on Percoll gradients (Baggaley and Atkinson, 1972).

Siliconised glass wool (section 2.4.7.) was placed at the bottom of a PDQ column and dry CF12 cellulose fibre (Whatman) placed on top until the column was full. The cellulose was then packed by sealing the top of the column (using a thumb) and applying suction to the bottom (Williams and Richards, 1973). More cellulose was added until the cellulose would not pack any further. The infected mouse blood was washed in PBS, resuspended in 50ml of PBS and placed in a 50ml syringe that was attached to the top of the column. The cells were slowly pushed through the column; the red blood cells came through the column while the white blood cells remained in the cellulose (figure 6).

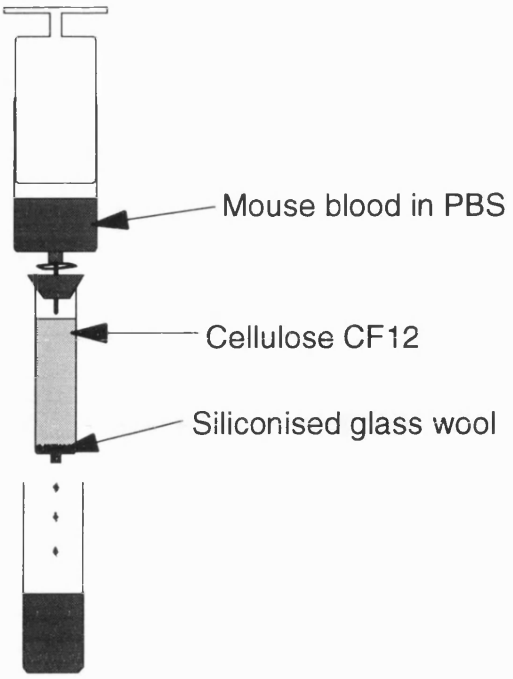

Figure 6. Cellulose column used to separate white blood cells from malaria infected mouse erythrocytes.

Complete removal of white blood cells was confirmed by examination of a Giemsa stained slide (section 2.2.1.).

2.2.12. Purification of Mature *P. yoelii* Parasites using Percoll® Gradients

Plasmodium yoelii infected mouse blood contains all the intraerythrocytic stages of the malarial life cycle at any one time, i.e. it is unsynchronised. To purify red blood cells infected with mature stage parasites from uninfected red blood cells and those infected with ring stage parasites, a Percoll® gradient was used (Kutner *et al*, 1985). Percoll® was diluted to 90% with 10X PBS containing 50mM D-glucose. Aliquots of this diluted Percoll® was further diluted to 80%, 70% and 60% with 1X PBS containing 5mM D-glucose. The 90% Percoll® (4ml) was placed in the bottom of a 15ml Corex tube and then 3ml of 80% Percoll® carefully layered on top. On this, 3ml of 70% and 2ml of 60% Percoll® were layered. The cells (in 3ml of PBS containing 5mM glucose) were then carefully layered on the Percoll® and centrifuged in a SS34 rotor at 10 000g for 20 minutes (Kutner *et al*, 1985). The cells band throughout the Percoll on the basis of density. The cells infected with mature stage parasites, which were closer to

the top of the gradient, were carefully removed and washed twice with 30ml of PBS containing 5mM glucose and examined under oil immersion (section 2.2.1.).

2.3. Molecular Biology Materials and Solutions

2.3.1. Vectors, Enzymes and General Laboratory Chemicals

Restriction endonucleases, DNA modifying enzymes, lambda DNA and pUC18 vector were obtained from either Pharmacia LKB or Northumbria Biologicals. General laboratory chemicals were obtained either from Merck BDH or Sigma. Radioactive nucleotides, [^{32}P]-α-dATP (5000 Ci/mmol), [^{32}P]-γ-ATP (3000 Ci/mmol) and [^{35}S]-α-dATP (1000 Ci/mmol), were obtained from NEN Ltd., Stevenage. The "Sequenase®" DNA sequencing kit were bought from United States Biochemical Corporation, X-gal was obtained from NBL, and IPTG and agarose from Sigma.

2.3.2. *Escherichia coli* Strains

The following three strains of *E. coli* were used during the project.

E. coli TG1	K12 Δ(*lac*, *pro*) *sup*E, *thi*, *hsd*D5/F'*tra*D36, *pro* AB$^+$, *lac*I^q, *lac*ZΔM15
E. coli DH5α	F$^-$ φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *hsd*R17(r_k^-, m_k^+) *deo*R *thi*-1 *sus*E44 λ$^-$ *gyr*A96 *rel*A1
E. coli XL1-Blue	*sup*E 44, *hsd*R17, *rec*A1, *end*A1, *gyr*A46, *thi*, *rel*A1 *lac*$^-$, F' [*pro*AB$^+$, *lac*I^q, *lac*ZΔM15, *Tn*10(*tet*^{R})]

2.3.3. Media

All the solutions were made using distilled and filtered (Milli-Q) water. *Escherichia coli* cultures were manipulated using good microbiological practice. Sterilisation of equipment and cell cultures was achieved by treating with 2% Hycolin solution. The following broths and agar solutions were used for growth of *E. coli*.

LB	1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl
LB AMP Plates	1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% Bacto-Agar. The solution was autoclaved and allowed to cool to 55°C before 100μg/ml ampicillin was added and the solution was poured into Petri dishes.
SOB broth	2% Bacto-tryptone, 0.5% yeast extract, 0.05% NaCl
SOC broth	2% Bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 20mM D-glucose, 10mM $MgSO_4$, 10mM $MgCl_2$
DYT broth	1.6% Bacto-tryptone, 1% yeast extract, 0.5% NaCl

All the media were prepared with distilled water and sterilised by autoclaving at 20 lb/in^2 for 30 minutes.

2.3.4. Buffers and Solutions

The following solutions were prepared using Milli-Q filtered water.

TE buffer	10mM Tris-HCl, 1mM EDTA, pH7.5
10X TBE	0.89M Tris borate, 20mM EDTA, pH8.0
50X TAE	1M Tris acetate, 50mM EDTA pH8.0
20X SSC	3M NaCl, 0.3M sodium citrate, pH7.0
100X Denhardt's	2% w/v Bovine serum albumin, 2% w/v ficoll™ (type 400, Pharmacia), 2% w/v PVP (polyvinylpyrollidone).
Salmon sperm DNA (Sigma Type III sodium salt)	10mg/ml solution in water. Sonication was required to get the salmon sperm DNA to dissolve.

A number of stock solutions were also prepared and used to make the buffers required for restriction analysis and other manipulations.

2.3.5. SequaGel™ Acrylamide Stock Solutions

The SequaGel™ (National Diagnostics) solutions were used whenever a polyacrylamide gel was made. The kit comes with 3 solutions which are mixed in the required proportions to provide the correct percentage gel. The three solutions are:

SequaGel Concentrate	23.75% acrylamide, 1.25% methylene bisacrylamide, 8.3M urea
SequaGel Diluent	8.3M urea
SequaGel 10X Buffer	8.3M urea, 1.0M Tris-Borate, 20mM EDTA pH8.3

2.4. General DNA Methods

2.4.1. Determination of DNA Concentration and Purity

The concentration of nucleic acids was determined by placing the sample (1-50μg) in a quartz cuvette and measuring the absorbance at 260nm (A_{260}). For double stranded

DNA one absorbance unit is equivalent to 50μg/ml and for single stranded DNA it is equivalent to 33μg/ml of nucleic acid. For single stranded RNA one absorbance unit is equivalent to 40μg/ml of nucleic acid.

The purity of the nucleic acid was determined by measuring the A_{260}/A_{280} ratio of the sample. Proteins absorb maximally at 280nm whereas nucleic acids absorb most at 260 nanometres. A high A_{260}/A_{280} ratio indicates a pure nucleic acid sample. A spectrum of the sample was also taken between 320nm and 240nm. A pure sample has a profile that peaks at 260nm and falls off on each side. If the spectrum does not do this or if the A_{260}/A_{280} ratio is less than 1.6 for single stranded, or 1.8 for double stranded nucleic acid, the sample is unlikely to be pure.

2.4.2. Agarose Gel Electrophoresis

The agarose was dissolved in TBE or TAE by heating to almost boiling in a microwave. When cooled to 55°C, ethidium bromide was added, to a final concentration of 0.5μg/ml, and the solution poured into the mould. The cast had been sealed with masking tape to prevent the agarose solution from leaking. A comb was placed in position so that it was 2mm from the bottom of the gel case and 10mm from the edge of the mould and the gel left to set. The concentration of agarose depended on the type of DNA that was to be separated (table 2).

Agarose concentration %w/v	Type of DNA to separate	Size of DNA
0.3%	Genomic DNA	>20kb
0.7%	Large vectors, digested genomic DNA	20kb - 5kb
1%	Vectors, PCR products, large fragments	5kb - 50bases
2%	Small PCR products, small fragments	<500bases

Table 2. The concentration of agarose used for the separation of DNA depends on the size of the nucleic acid.

Once the gel had set, the comb and masking tape were removed and the gel placed in a gel tank that contained TBE or TAE and 0.5μg/ml ethidium bromide. The samples were loaded into the wells after the addition of a $1/10^{th}$ volume of loading dye (50% glycerol, 0.05% xylene cyanol, 0.05% bromophenol blue). To size the DNA fragments HindIII or PstI digested λ DNA (which produce fragments of known size) was loaded

alongside the DNA samples. The gels were run at 100-200 volts routinely or at 25V for preparative purposes. The DNA migrated towards the anode.

Once the samples had separated the gel was removed from the tank and placed on a UV transilluminator. The DNA bands were fluorescent due to the presence of ethidium bromide. A record of the gel was then made by photographing using Polaroid 660 film.

2.4.3. Isolation of DNA Fragments from Agarose Gels by 'Freeze Squeezing'

The DNA band, visualised under UV (section 2.4.2.), was excised from the gel using a razor blade. Excess agarose was trimmed off and then the slice of agarose was placed in a 0.5ml Eppendorf that had a hole in the bottom (made with a 25 gauge needle) and which was plugged with siliconised glass wool (section 2.4.7.). The tube was plunged into liquid nitrogen to freeze rapidly, placed in a 1.5ml Eppendorf tube and centrifuged at 10 000g for 10 minutes in a microfuge at room temperature. After centrifugation the DNA had passed into the 1.5ml Eppendorf tube; the agarose remained above the glass wool in the smaller tube (Tautz and Renz, 1983).

2.4.4. Isolation of DNA Fragments from Agarose Gels Using the 'GeneCleaning®II Kit'

The GeneClean protocol was used to purify DNA from agarose gels. The method relies on a specially formulated silica matrix called Glassmilk or GeneClean beads. These beads bind DNA in high salt and low temperature and release the DNA in low salt and higher temperatures. The DNA sample was electrophoresed on an agarose gel (section 2.4.2.) using TAE buffer. After the DNA had migrated, it was visualised under UV (section 2.4.2.), and the agarose containing the sample excised with a clean razor blade. The agarose was weighed and added to three volumes of 6M NaI solution. The sample was heated to 55°C for the minimum time required to melt the agarose, the sample cooled on ice, and 10µl of the GeneClean beads added. After 30 minutes on ice, when the DNA had bound to the beads, the sample was pulsed in the microfuge and the NaI solution removed. The beads were washed three times with 1ml of NEW wash (supplied) by adding the NEW wash and then vortexing the sample

until the beads were fully resuspended. The sample was then pulsed in the microfuge and the wash buffer removed. After the last wash the remaining NEW wash was carefully removed following centrifugation using a small white Gilson tip. The beads were dried by rotary evaporation to remove any traces of ethanol, and then resuspended in 10-50µl of water or TE buffer. After heating to 55°C for 5 minutes the sample was microfuged and the liquid containing the DNA removed to a fresh tube. The elution was then repeated with a further 10µl of water or TE buffer to collect the DNA trapped in the bead's bed volume. Typically 50-80% of the DNA was recovered by this method and was found to be pure enough to sequence or subclone.

2.4.5. Preparation of Dialysis Tubing

The tubing was cut into 20cm strips, boiled for 10 minutes in 2% w/v Na_2CO_3 and 1mM EDTA, and rinsed with distilled water. The tubing was then boiled with 1mM EDTA and stored at 4°C in this buffer. Care was taken to ensure that the dialysis tubing remained submerged during storage and was only handled with gloves. The tubing was rinsed with distilled water before use.

2.4.6. Cutting DNA with Restriction Enzymes

The restriction digestion of DNA was performed in 0.5ml or 1.5ml Eppendorf tubes using the buffer conditions given for the particular enzyme. An appropriate amount of enzyme was used. One unit of enzyme totally digests 1.0µg of DNA in 1 hour at the enzyme's optimal temperature. The restriction enzymes were stored in buffers that contained glycerol. Care was taken to ensure that the concentration of glycerol did not become too high as glycerol inhibits enzyme activity. The volume of enzyme stock was never greater than $1/10^{th}$ of the final volume of the reaction.

2.4.7. Silanation of Plastic Tubes, Tips and Glass Wool

Plasticware was siliconised to make the surface hydrophobic and to prevent loss of material. This was used especially for free parasites (section 2.2.7.) and for RNA samples (section 2.4.31.). Glass plates were treated to prevent acrylamide adhering to them during DNA sequencing (section 2.4.26.4.). Dry plasticware and glassware were rinsed thoroughly in 2% dimethyl-dichloro-silane in 1,1,1-trichloroethane (BDH) and

then in ethanol. The plasticware was then washed in distilled water and allowed to dry in a 55°C oven. Glass plates were washed with water and dried using a towel.

2.4.8. AUTORADIOGRAPHY

Radioactive gels and blots were placed against Fuji RX-100 film in a cassette. When weak ^{32}P samples were used, the film was pre-flashed, an intensifying screen was used and the cassette was stored at -70°C in the chest freezer. After the required time the film was developed manually using Kodak Developer LX24 and Kodak Fix FX40.

2.4.9. EQUILIBRATION OF PHENOL

Water saturated phenol (glass distilled grade) was equilibrated using pH8.0 100mM Tris-HCl. The aqueous layer was discarded, and replaced with more 100mM Tris-HCl until the aqueous solution reached pH8.0. Distilled water was then mixed with the phenol to remove excess Tris-HCl and then discarded. The phenol was stored at -20° C, or at 4°C in the dark for up to a week. If the phenol became discoloured it was discarded.

2.4.10. PURIFICATION OF NUCLEIC ACIDS USING PHENOL

Phenol equilibrated to pH8.0 was mixed in a 1:1 ratio with the DNA sample. The two layers were mixed and then the sample centrifuged at 10 000g for 5 minutes. The upper layer, which contained the DNA sample, was removed to a fresh tube. Care was taken not to remove any of the white precipitate at the interface of the two layers. The extraction was then repeated using a mixture of phenol:chloroform:Iso Amyl Alcohol in a ratio of 24:24:1. The chloroform helps the two layers to separate completely and lower the concentration of phenol in the aqueous phase. The excess phenol was then removed by extracting with chloroform:iso amyl alcohol (24:1).

2.4.11. PRECIPITATION OF NUCLEIC ACIDS USING ETHANOL OR ISOPROPANOL

Routinely, DNA was precipitated from solutions by adding 0.1 volumes of 3M sodium acetate (pH5.5) and 2.5 volumes of absolute ethanol that had been cooled to -20°C before use. The tubes were left at -20°C for 30 minutes then the nucleic acid sedimented by centrifuging at 10 000g for 10 minutes. The supernatant was removed and the nucleic acid washed with 0.5ml of ice-cold 70% (v/v) ethanol. The sedimented

nucleic acid was dried by rotary evaporation and dissolved in water or TE buffer as required.

Precipitation of nucleic acids with isopropanol was similar to using ethanol. An equal volume of isopropanol and 0.1 volumes of 3M NaAc pH5.5 were added to the sample which was then incubated at -20°C for 30 minutes before centrifugation at 10 000g for 10 minutes. The sediment was again washed with 70% (v/v) ethanol, dried under vacuum and resuspended in water or TE buffer.

2.4.12. Preparation of G25/G50 Sephadex Resin

One gram of powdered G25 or G50 was added to 100ml of distilled water and allowed to swell for 6-8 hours. The slurry was washed five times with distilled water to remove soluble dextrans, and then the water was replaced with 20% v/v ethanol.

2.4.13. Purification of DNA from Nucleotides using a G25/G50 Sephadex® Column

Siliconised glass wool (section 2.4.7.) was placed at the bottom of a disposable 1ml plastic syringe and G25 or G50 Sephadex® was added to fill the syringe. The column was placed in a test tube and centrifuged at 500g for 5 minutes in a bench centrifuge. If the bed volume of the column was less than 1ml more resin was added and it was again centrifuged. Once the bed volume was 1ml the column was washed with 100µl of TE pH7.6 by centrifugation at 500g for 5 minutes. The column was washed twice more (with TE buffer) to remove residual ethanol from the column and then the DNA sample (in TE buffer) placed on the column. An Eppendorf tube, that had its cap removed, was placed in the test tube, the column replaced and centrifuged at 500g for 5 minutes. Free nucleotides and small oligonucleotides were trapped by the resin whereas the DNA sample was eluted into the Eppendorf tube. The G25 column excluded oligonucleotides larger than twelve bases; G50 oligonucleotides larger than sixteen bases.

2.4.14. Purification of DNA from Salts using CL6-B Columns

Sepharose CL6-B (Pharmacia Ltd.) was used to purify DNA fragments from salts, phenol, OH^- ions and pieces of DNA less than 400 bases long. A 25 gauge needle was

used to puncture the bottom of a 0.5ml Eppendorf tube. The hole was covered to a depth of 5mm with glass beads of 0.4mm diameter. The tube was then filled with CL6-B Sepharose resin (equilibrated in TE buffer pH7.6) and centrifuged at 500g for 5 minutes or until the resin was dry. The column was washed three times (with TE buffer) to remove residual ethanol from the column and then the sample (in TE buffer) placed on the column. The column was placed in a fresh 1.5ml Eppendorf tube and the sample, in 20-200µl, loaded. After centrifugation the DNA was eluted into the 1.5ml Eppendorf tube while the oligonucleotides, salts and other impurities remained in the column matrix.

2.4.15. Purification of DNA using a NENSORB™-20 Column

NENSORB™ columns (DuPont NEN) were used to purify DNA samples from nucleotides, protein, salt and agarose contamination. The NENSORB™ column was washed with 2ml of HPLC grade methanol and then with 2ml of solution A (100mM Tris-HCl pH7.5, 1mM EDTA, 10mM triethylamine). The DNA sample (up to 20µg) in 1ml of solution A was then passed through the column followed by solution A (3ml). After washing the column with 2ml of water the DNA was eluted with 200µl of 50% v/v methanol followed by 200µl of 20% v/v isopropanol and dried in a rotary evaporator. The DNA was of high purity (suitable for ligating and sequencing) after purification.

2.4.16. Radioactive Labelling of Oligonucleotides

The 5' hydroxyl of oligonucleotides was labelled using [^{32}P]-γ-ATP and T4 polynucleotide kinase. The oligonucleotide (10pmoles) was resuspended in 50mM Tris-HCl pH7.6, 10mM $MgCl_2$, 5mM DTT, 100µM EDTA, 100µCi [^{32}P]-γ-ATP and 10U of T4 polynucleotide kinase. After incubation at 37°C for 30 minutes unlabelled ATP was added (500µM final concentration) to chase the reaction and improve incorporation. The sample was incubated for a further two minutes before making up to 100µl with TE buffer and placing on a G25 spun column (section 2.4.13.) to remove unincorporated label.

2.4.17. LABELLING DNA BY RANDOM PRIMING

Nucleic acid fragments larger than 200bp were labelled using Klenow in preference to T4 Polynucleotide kinase. This method incorporates many radiolabelled ^{32}P molecules per DNA fragment. The purified DNA (100ng) was denatured by boiling for three minutes and then rapidly cooled on ice. The sample was mixed with 50ng of random 14-mer oligonucleotides and resuspended in 50mM Tris-HCl pH7.6, 10mM $MgSO_4$, 100μM DTT, 600μM dGTP, 600μM dCTP, 600μM dTTP, 100μCi of [^{32}P]-α-dATP and 5 units of Klenow. The tube was left at room temperature for 3 hours before the reaction was stopped by adding four volumes of TE buffer. Unincorporated nucleotides were removed using a 1ml G50 column (section 2.4.13.).

2.4.18. REMOVAL OF 3' TERMINAL PHOSPHATE

The terminal phosphate was removed from DNA samples using Calf Intestinal Phosphatase (CIP). The DNA sample was resuspended in 50mM Tris-HCl pH8.3, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, 1mM spermidine and 0.1U of calf intestinal phosphatase. After 30 minutes incubation at 37°C a further 0.1U of enzyme was added and the sample incubated at 56°C for 15 minutes.

2.4.19. LIGATION OF DNA FRAGMENTS

For blunt ended ligations, linearised and phosphatase treated (section 2.4.18.) plasmid DNA was mixed with the fragment to be cloned in a molar ratio of 1:3, then resuspended in 25mM Tris-HCl pH7.5, 10mM EDTA, 10mM DTT, 0.5mM ATP, 5mM $MgCl_2$, 15% PEG 8000 and 10U of T4 DNA ligase. The ligation was then allowed to proceed at 15°C for at least 24 hours.

Vectors and fragments with compatible overhanging ends (produced with the same restriction enzyme) were mixed in equal molar concentrations. The DNA mixture was then resuspended in 25mM Tris-HCl pH7.5, 10mM $MgCl_2$, 10mM DTT, 400μM ATP and 1U of T4 DNA ligase. The ligation was then allowed to proceed at room temperature for 4 hours.

2.4.20. SYNTHESIS OF OLIGONUCLEOTIDE PROBES

2.4.20.1. Synthesis of the probe

After deciding the sequence of the probe, the oligonucleotides were prepared on a Model 381A Applied Biosystems automatic DNA synthesiser. After synthesis, the matrix material, to which the oligonucleotide was bound, was removed and placed in a screw cap Eppendorf containing 1ml of concentrated ammonia solution. The sample was placed at 55°C overnight to cleave the oligonucleotide from the column and remove the protecting groups from the oligonucleotide. The oligonucleotide was dried in a rotary evaporator and then dissolved in 1ml of water. The probe was placed in a 15ml Corex tube and 0.1ml of 3M NaAc pH5.5 and 3ml of absolute ethanol were added (section 2.4.11.). The sample was left at -20°C overnight to precipitate the oligonucleotide, and then centrifuged at 10 000g for 10 minutes in a SS34 rotor using a Sorvall centrifuge. The supernatant was discarded and the nucleic acid washed with 80% ethanol. After drying, the oligonucleotide was resuspended in 1ml of water. The concentration and purity of the oligonucleotide was determined by measuring the A_{260} and A_{280} (Section 2.4.1.) of the sample. The DNA was usually at a concentration of about 150μmolar.

2.4.20.2. Checking the Quality of Artificially Synthesised Probes

To check the quality of the oligonucleotide probe, 10pmoles was end-labelled using 5μCi [^{32}P]-γ-ATP (section 2.4.16.) and electrophoresed on a 20x20cm, 0.2mm thick, 20%, denaturing polyacrylamide gel (Sequagel - section 2.3.5.). The sample was mixed with an equal volume of loading dye (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), heated at 75°C for 2 minutes and loaded onto the gel. The samples were electrophoresed at 400V (14mA) for 2-3 hours in TBE running buffer. The top plate was removed and Saran wrap™ placed over the gel and lower plate before exposing to film (section 2.4.8.). After 10 minutes the film was developed (section 2.4.8.). Correctly synthesised oligonucleotides (which were all full length) showed a single band whereas poorly synthesised probes revealed laddering on the autoradiograph.

2.4.20.3. Purifying a Poorly Synthesised Probe by Shadowing

If the oligonucleotide was a mixture of products (i.e. laddered) it was electrophoresed on a 20% polyacrylamide gel. The sample was dried and resuspended in 100µl of sterile water and 100µl of denaturing loading dye (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 60°C for 5 minutes. The sample was loaded on the gel and electrophoresed (using TBE as running buffer) at 50mA for 3-4 hours. The gel was examined under UV light (with a fluorescent plate as background) and the slowest migrating (largest) band cut out and placed in a screw topped universal bottle. The sample was gently shaken with 1ml of 0.3M NaAc and 1mM EDTA overnight to elute the DNA from the polyacrylamide. The sample was centrifuged at 500g to pellet the polyacrylamide. The supernatant was removed and the DNA precipitated by the addition of three volumes of absolute ethanol (section 2.4.11.) and incubation overnight at -20°C. The sample was centrifuged at 10 000g for 10 minutes in a Sorvall SS34 rotor. The DNA was washed with 80% ethanol, dried, and resuspended in 100µl TE buffer. The A_{260} of the sample was determined to calculate the concentration of DNA (section 2.4.1.).

2.4.21. PREPARATION OF COMPETENT *ESCHERICHIA COLI*

A single colony of *E. coli* (strain XL1-Blue or TG1) from an LB plate was inoculated into 5ml of LB buffer and incubated at 37°C overnight, with continual agitation at 200rpm. The following morning 0.5ml of the overnight culture was added to 40ml of DYT in a 250ml flask, and shaken at 37°C until the culture had an optical density at 550nm of 0.3 absorbance units. The cells were then plunged into ice cold water and centrifuged at 4°C in the bench centrifuge at 500g for 5 minutes. The cells were gently resuspended in 20ml of 50mM $CaCl_2$ ensuring that the sample remained at 4°C throughout the procedure. After 20min on ice, the cells were again centrifuged at 500g and gently resuspended in 2ml of ice cold 50mM calcium chloride. The cells remained competent for up to 48 hours as long as they were kept on ice.

When highly competent cells were required ($\approx 10^9$ transformants/µg of DNA) super competent *E. coli* (strain DH5α) were purchased from Gibco.

2.4.22. TRANSFORMATION OF *E. COLI* WITH PLASMID DNA

The competent cells (200µl) were mixed with the DNA (1 - 50ng) and placed on ice for 30 minutes. The sample was then placed at 42°C for 90 seconds to heat shock the cells before the addition of 1ml SOC broth. The tube was then placed at 37°C for one hour and the cells (2µl - 1ml) were spread onto 10cm diameter agar plates containing LB + 100µg/ml ampicillin (section 2.3.3.). For volumes greater than 200µl the cells were centrifuged at 500g for 5 minutes, the supernatant discarded and the cells resuspended to concentrate. After the plates had dried they were incubated at 37°C overnight. If the vector had a colour selectable marker 50µl of 0.1M IPTG and 50µl of 2% (w/v) X-gal, in dimethyl formamide, were spread on each plate (LB AMP) and allowed to dry before spreading the cells. When the transformation was successful colonies were visible on the plate the following morning.

2.4.23. PLASMID DNA 'MINIPREPS'

Small scale preparation of DNA was carried out using a modification of the alkaline lysis method (Birnboim and Doly, 1979). The method was used mainly to screen large numbers of colonies for the presence of the correct insert in the vector following a transformation. A single colony of the transformed *E. coli* (DH5-α, XL1-Blue or TG1) was placed in 3ml of LB containing 100µg/ml ampicillin and shaken at 37°C for no less than 6 hours. When grown, 1ml of the culture was placed in an Eppendorf tube and centrifuged at 5 000g for 5 minutes to pellet the cells. The medium was removed and the cells resuspended in 150µl of ice cold GTE (50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA). Lysozyme was not required to lyse the cells. After incubation on ice for 5 minutes, 300µl of freshly prepared 1% SDS, 200mM NaOH was added and the cells returned to ice. When the sample became clear and viscous, 200µl of 3M potassium acetate pH4.5 was added. The sample was mixed, and then placed on ice for 15 minutes. The sample was centrifuged at 4°C for 10 minutes at 10 000g in a microfuge to remove the white precipitate. The supernatant, which contained the plasmid DNA, was extracted with an equal volume of equilibrated phenol pH8.0 (section 2.4.10.). The aqueous layer was removed to a fresh Eppendorf tube and 1ml of ethanol was added. The sample was left at room temperature for 10

minutes before centrifuging at 10 000g for 10 minutes. The DNA pellet was washed with 1ml of 70% ethanol, dried by rotary evaporation and resuspended in 50µl of 20µg/ml ribonuclease A dissolved in TE buffer. A sample of the DNA (usually 3µl) was digested with appropriate restriction enzymes (section 2.4.6.) and electrophoresed on an agarose gel (section 2.4.2.) to check whether the vector contained the correct insert.

2.4.24. PLASMID DNA 'MAGIC™ MINIPREPS'

Small scale DNA preparations suitable for DNA sequencing were obtained using the magic™ miniprep method from Promega. A single colony was picked from a LB AMP plate (section 2.3.3.) and inoculated into 5ml of LB containing 100µg/ml ampicillin. The culture was incubated at 37°C with constant shaking at 200rpm for at least 6 hours. The entire culture was centrifuged at 10 000g for 5 minutes. The pellet was transferred to a microfuge tube and resuspended in 200µl of cell suspension buffer (50mM Tris-HCl pH7.5, 10mM EDTA and 100µg/ml RNAseA); 200µl of cell lysis solution (0.2M NaOH, 1% SDS) was added immediately afterwards. The tube was gently inverted until the cells had lysed and the solution turned clear. After lysis, 200µl of 3M potassium acetate pH4.5 was added. The sample was centrifuged at 12 000g for 5 minutes to pellet the cell debris. The supernatant was transferred to a clean Eppendorf tube and 1ml of DNA purification resin added. A magic miniprep minicolumn was added to the end of a syringe that had its plunger removed. The sample, containing the resin, was placed in the syringe barrel and pushed through the column using the plunger. The resin (and the bound plasmid DNA) was washed with 3ml of ice cold 0.1M NaCl, 10mM Tris-HCl pH7.4, 2.5mM EDTA, 50% v/v ethanol. The minicolumn was removed from the syringe barrel, placed on top of a 1.5ml Eppendorf tube and centrifuged at 12 000g for 20 seconds to dry the column. The column was then placed on a fresh Eppendorf tube and 50µl of hot (80°C) water was added. After 1 minute the column was again centrifuged at 12 000g for 20 seconds to elute the DNA into the Eppendorf tube. The tube was capped and the sample stored at -20°C.

2.4.25. PLASMID DNA 'MAXIPREPS'

The cells (XL1-Blue or DH5α) were grown for 16-24 hours in 200ml LB buffer containing 100μg/ml ampicillin. The cells were harvested by centrifugation at 5 000g for 10 minutes in a Sorvall GSA rotor and resuspended in 3ml of GTE (25mM Tris-HCl pH8.0, 10mM EDTA, 50mM glucose). The cells were placed on ice and 6ml of freshly prepared 0.2N NaOH/1% SDS was carefully added to lyse the cells. When the solution had turned clear and viscous, indicating cell lysis, 3.75ml of 3M KAc (pH 4.6) was added. After incubation on ice for twenty minutes the sample was centrifuged at 12 000g for 15 minutes in a SS34 rotor. The sample remained at 4°C throughout the procedure. The supernatant was removed to a fresh 50ml tube and extracted twice with an equal volume of phenol/chloroform/IAA (25:24:1) (section 2.4.10.) and then once with chloroform/IAA (24:1). Two volumes of ice cold ethanol were then added to the sample which was then incubated at 4°C for 30 minutes. After centrifugation at 10 000g for 15 minutes in a SS34 rotor the pellet was washed with 70% ethanol, dried and resuspended in 9ml of TE buffer (pH8.0). Caesium chloride (9g) and 0.6ml of 10mg/ml ethidium bromide were added to the sample which was placed in a 16x76mm quick-seal Beckman polyallomer ultracentrifuge tube, balanced to within 5mg and sealed using the 'quick-sealer'. The sample was centrifuged at 15°C for 15 hours at 200 000g in a 70.1Ti rotor in the ultracentifuge (Beckman). The Quickseal tube was removed and examined under UV light. Two bands of DNA could be seen to fluoresce in the middle of the tube with the RNA at the bottom of the tube (figure 7).

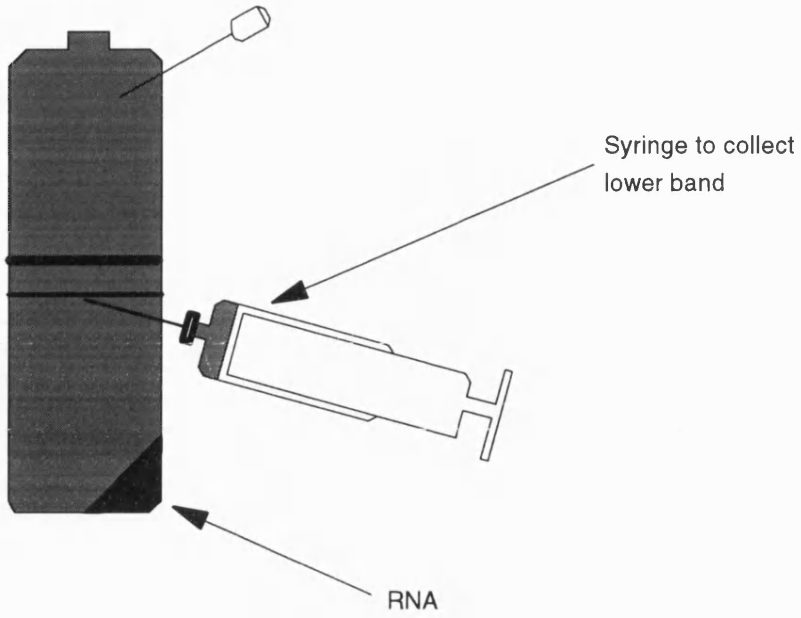

Figure 7. A caesium chloride gradient. The lower of the two bands was taken into a syringe and further purified.

The lower of the two bands was taken into a hypodermic syringe using a 19 gauge needle and mixed with water saturated butanol to remove the ethidium bromide. This butanol extraction was repeated until the sample was colourless. The DNA was then placed in dialysis tubing (section 2.4.5.) and dialysed 4 times at 4°C against 1 litre of pH8.0 TE buffer to remove caesium salt. The sample was placed in a 15ml Corex tube, and two volumes of ethanol and 0.1 volumes of 3M NaAc were added to precipitate the DNA (section 2.4.11.). The DNA sediment was washed with 70% ethanol, dried and resuspended in 1ml of pH8.0 TE buffer. The $A_{260}\backslash A_{280}$ ratio was taken to determine purity and the concentration of DNA calculated from the A_{260} (section 2.4.1.).

2.4.26. DNA Sequencing

2.4.26.1. Annealing.

The sequencing method was as recommended in the USB corporation Sequenase® kit. The kit uses a modified version of T7 DNA polymerase (known as Sequenase®) which has a low 3'→5' exonuclease activity. The double stranded DNA prepared either from a maxiprep (section 2.4.25.) or by a Magic® miniprep (section 2.4.24.) (≈5-10µg in 20 µl) was denatured using 5µl 1M NaOH and incubated at room temperature for 5 min.

The sample was then spun through a CL6-B column (section 2.4.14.) to remove all traces of alkali. Seven microlitres of the denatured DNA were made up to a final volume of 10µl with 1pmol deoxyribonucleotide primer and Sequenase® reaction buffer (final concentrations 40mM Tris-HCl pH 7.5, 25mM $MgCl_2$, 50mM NaCl). The sample was placed at 75°C for 2 minutes and then cooled slowly from 75°C to 30°C in a beaker of water. The annealed template was placed on ice and used within 4 hours.

2.4.26.2. Labelling reaction

The Sequenase® labelling mix (7.5mM each dGTP, dCTP, dTTP) was diluted 1:5 with distilled water and the Sequenase® DNA polymerase enzyme was diluted 1:8 with enzyme dilution buffer (10mM Tris-HCl pH 7.5, 5mM DTT, 0.5mg/ml BSA). The annealed template (10µl) was mixed with 1µl 0.1M DTT, 2µl diluted labelling mix, 0.5 µl [^{35}S]-α-dATP and 2µl of diluted enzyme, and incubated for 2 minutes at room temperature. On hot days the reaction was performed in a 20°C water bath.

2.4.26.3. Termination.

The composition of the termination mixes are:

ddATP - 80µM each dATP, dCTP, dGTP, dTTP, 8µM ddATP, 50mM NaCl
ddTTP - 80µM each dATP, dCTP, dGTP, dTTP, 8µM ddTTP, 50mM NaCl
ddCTP - 80µM each dATP, dCTP, dGTP, dTTP, 8µM ddCTP, 50mM NaCl
ddGTP - 80µM each dATP, dCTP, dGTP, dTTP, 8µM ddGTP, 50mM NaCl

Samples (2.5µl) of each termination mix were pipetted into separate wells of a microtitre plate and placed in a 37°C water bath. To each of the four termination mixes 3.5µl of the labelling reaction mix was added and the tubes incubated at 37°C for 5 minutes. An aliquot (4µl) of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each tube and mixed thoroughly. The samples could be stored for up to a week at -70°C before loading onto the sequencing gel.

2.4.26.4. Preparation of 6% polyacrylamide gel.

A glass plate, of dimensions 21cm x 40cm (the back plate), was soaked overnight in 2M KOH, washed with water, dried and then washed with ethanol. A smaller piece of glass, the front plate (21cm x 37cm), was washed thoroughly with water and then with ethanol. The front plate was siliconised (section 2.4.7.) to make it hydrophobic. The

back plate was coated with a mixture of 25ml ethanol, 0.6ml 10% acetic acid and 200µl of bind silane A174 (Merck BDH). After 5 minutes the mixture was removed and the plate washed with water and then ethanol. Spacers (0.35mm) were placed along the long edges of the back plate and fixed with glue. The front plate was placed on the back plate (separated by the spacers) and three edges of the plates aligned. The 6% acrylamide solution was made using the Sequagel® reagents (section 2.3.5.) by mixing 12ml of concentrate, 33ml of diluent and 5ml of buffer. The acrylamide was polymerised with 25µl of TEMED and 200µl of 16% ammonium persulphate. Before the acrylamide had set it was poured between the plates and the flat edge of a sequencing comb placed in the top of the gel so it was about 0.5cm under the smaller plate. The gel was clamped and allowed to set for 30 minutes. Once set, the outsides of the plates were cleaned and the comb carefully removed. The trough made by the comb was rinsed thoroughly in TBE buffer and the comb placed into the well with the shark's teeth 1mm into the acrylamide. The gel was placed vertically in the gel tank and clamped. Running buffer, TBE, was placed in both the upper and lower troughs of the tank and then the urea that had gathered in the wells was removed using a glass Pasteur pipette filled with TBE buffer.

2.4.26.5. Loading and electrophoresis of DNA samples.

The samples were heated to 75°C for 2 minutes to denature and then loaded onto the gel (2µl per lane). The gel was electrophoresed at 700V-1500V for the required time. Sequences close to the primer were obtained by terminating the run when the bromo-phenol blue reached the bottom of the gel (about 3 hours). For sequences further from the primer, the gel was electrophoresed for between 6 and 16 hours. Once the DNA had migrated the required distance, the gel was removed and the smaller plate carefully lifted off using a small spatula. The gel remained stuck to the larger plate which was washed for 20 minutes in 10% v/v methanol, 10% v/v acetic acid to remove the urea from the gel. The gel and large plate were rinsed in water for 10 minutes and then dried on a hot flat bed. The dry gel was then wafer thin and firmly attached to the larger plate. The gel was placed next to a piece of film and left for 16-100 hours before developing (section 2.4.8.).

2.4.26.6. Analysis of the Sequence.

The sequence was read and analysed using the Staden software package for the PC and the GCG software package on a UNIX or VAX mainframe.

2.4.27. DNA POLYMERASE I TREATMENT OF DNA

Single stranded overhanging ends were repaired using DNA polymerase I. The DNA was incubated at 37°C in 50mM Tris-HCl pH 7.2, 100mM $MgSO_4$, 0.1mM DTT, 50μg/ml BSA and 5 units of DNA polymerase I. After 5 minutes dNTPs (125μM each) were added and the sample incubated for a further 20 minutes. The sample was then purified by phenol extraction (section 2.4.10.) followed by precipitation using ethanol and NaAc (section 2.4.11.).

2.4.28. SOUTHERN BLOTTING TECHNIQUE

2.4.28.1. Transfer of DNA

The DNA samples were separated by agarose gel electrophoresis, removed from the gel tank and viewed under UV light (section 2.4.2.). A photograph of the gel was taken with a ruler next to the gel so that the migration of bands was recorded. The gel was placed in denaturing solution (1.5M NaCl, 0.5M NaOH) and gently agitated on an orbital shaker until the xylene cyanol had turned from light blue to green. The denaturing solution was then replaced with neutralising buffer (1.5M NaCl, 0.5M Tris-HCl pH7.2, 0.001M EDTA) and gently shaken until the xylene cyanol returned to its original blue colour. About 500ml of 20X SSC (3M NaCl, 0.3M sodium citrate) was poured into the transfer tank and two pieces of 3mm filter paper (Whatman) placed across the tank so that they dipped into the SSC on either side. The gel was carefully placed in the centre of the filter paper ensuring that bubbles were not trapped under the gel. The gel was then covered with the transfer membrane (GeneScreen+™ or Hybond-N™) that had been soaked in 2X SSC before use. Again care was taken to ensure that bubbles were not trapped between the membrane and the gel. The gel was surrounded in polythene so that buffer did not leak up the sides of the gel. Two more pieces of 3mm filter paper and about six centimetres of paper towel were placed on top of the filter paper to soak up the SSC as it passed through the gel and membrane. A

1kg weight was placed on top of the towels and then the apparatus was left overnight (see figure 8).

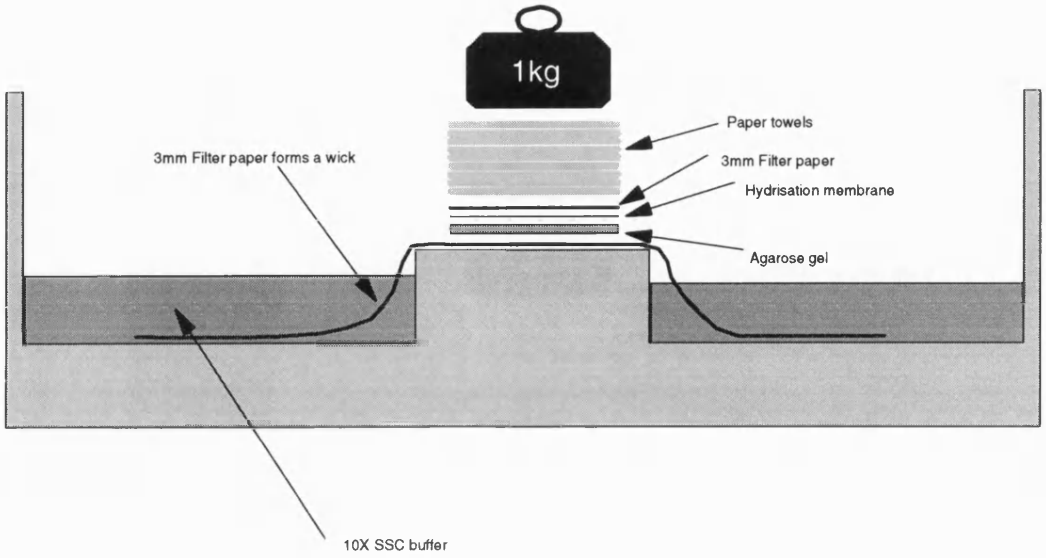

Figure 8. Apparatus used to transfer DNA from an agarose gel to a hybridisation membrane.

The blotting was checked by viewing the membrane under ultraviolet light. If the DNA had transferred, it was placed on a piece of 3mm paper that had been soaked in denaturing solution, and then about a minute later on a piece soaked in neutralisation solution. After a wash in 2X SSC, the membrane was baked at 80°C for 4 hours.

2.4.28.2. Hybridisation of the Labelled Probe to the Membrane

The membrane was placed in a perspex box containing the pre-hybridisation buffer (6X SSC, 5X Denhardt's, 0.5% SDS). One millilitre of 0.50mg/ml Salmon sperm was denatured by boiling for 5 minutes and then cooled rapidly on ice and added to the box. The box was shaken for no less than 6 hours at the hybridisation temperature before adding the radiolabelled probe and hybridising overnight. If the probe was an oligonucleotide it was 5' end-labelled (section 2.4.16.) with T4 PNK and [^{32}P]-γ-ATP; if it was a longer piece of DNA it was labelled with [^{32}P]-α-dATP by random priming (section 2.4.17.).

2.4.28.3. Washing and Autoradiography of the Labelled Membrane

The hybridisation buffer was removed from the membrane and replaced with 2X SSC, 0.1% SDS (or 6X SSC, 0.1% SDS for a less stringent wash). The membrane was washed at low temperatures (room temperature) initially, and the temperature slowly increased to raise the stringency and remove more of the label from the membrane. The wash buffer was changed regularly and the amount of radioactivity remaining on the filter monitored using a Geiger-Müller tube. Once enough non-specific radioactivity was removed, the membrane was sealed in a polythene bag (to prevent it from drying) and exposed to preflashed film (section 2.4.8.).

2.4.29. Isolation of Total Genomic DNA from *Plasmodium falciparum*

The cell pellet ($2x10^{10}$ cells in 400µl) was removed from -70°C storage and thawed on ice. The cells were resuspended in 50mM Tris-HCl pH9.0, 100mM EDTA and 200mM sodium chloride (Herrmann and Frischauf, 1987). Care was taken to ensure that the cells had fully resuspended before the sample was made 1% w/v with sodium dodecyl sulphate (Towner, 1991). Proteinase K (0.5mg/ml) was carefully dissolved and the reaction mix was incubated at 55°C for 24-48 hours. The sample was then extracted twice with an equal volume of TE equilibrated phenol pH8.0 (section 2.4.10.) and once with phenol/chloroform/IAA (25:24:1) rolling on a horizontal shaker for 1 hour each time to mix the layers gently. The sample was centrifuged at 10 000g for 10 minutes after each phenol extraction to remove any precipitated protein from the aqueous layer. The sample was then dialysed four times against one litre of 50mM Tris-HCl pH8.0, 20mM EDTA and 10mM NaCl and twice against TE buffer. The A_{260} of the DNA was determined (section 2.4.1.) and a fraction of the sample (≈100ng) was run on a 0.3% agarose gel (section 2.4.2.) at 25V overnight. If the DNA was intact a sample of it (≈1µg) was digested with 5U of EcoRI (section 2.4.6.). After 2 hours at 37°C the DNA was run on a 0.7% agarose gel (section 2.4.2.). If the DNA was digested by EcoRI further purification of the DNA was unnecessary. Any DNA samples that did not cut were transferred to a glass beaker, cooled on ice and 1/4 volume of prechilled 7.5M ammonium acetate was mixed with the sample before 0.8 volumes of cold isopropanol was layered on top (Towner, 1991). At the interface the

DNA precipitated and was spooled out on the end of a sealed glass Pasteur pipette. The DNA was washed with ice cold 70% ethanol, air dried, and allowed to dissolve in TE buffer for 1 week before the A_{260} was taken (section 2.4.1.) and the purity determined by digestion with restriction enzymes (section 2.4.6.).

2.4.30. Precautions Taken to Prevent Degradation of RNA

Wherever possible, dry chemicals were reserved for RNA use and stored in a separate cupboard to the other laboratory chemicals. All solutions were prepared using either sterile disposable plasticware, glassware that had been baked at 180°C for 8 hours, or equipment that had been treated with diethylpyrocarbonate. Water was made RNAse free by adding diethylpyrocarbonate (DEPC) to a final concentration of 0.1% (v/v). The water was stirred for 1 hour and then autoclaved. As residual traces of DEPC can modify RNA, the DEPC treated water was not allowed to come into contact with nucleic acids. Automatic pipettes (Gilson), Corex tubes, gel moulds and tanks, and other implements that would not stand baking were soaked for 3 hours in 0.1% v/v DEPC, washed with RNAse free water and allowed to air dry in a fume cupboard. Hydrogen peroxide was used to wash the work area before handling RNA samples. All solutions were made with ultra pure water (Milli-Q), placed in RNAse free bottles and autoclaved for 2 hours.

2.4.31. Extraction of Total RNA from *Plasmodium yoelii*

Cell pellets were ground in liquid nitrogen and the cold powder sprinkled at a concentration of 10^7 cells/ml into denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate pH7.0, 0.5% N-lauryl sarcosine, 0.1M β-mercaptoethanol) and transferred to a 15ml Corex tube (Chomczynski and Sacchi, 1986). A 1/10 volume of 2M sodium acetate pH4.0 was added and the sample mixed thoroughly. An equal volume of water saturated, glass distilled grade phenol (taken directly from the bottle) was then mixed with the sample followed by a $^1/_5{}^{th}$ volume of chloroform/IAA (24:1). The sample was then shaken for 10 seconds and left on ice for 15 minutes. During this time the sample separated into two layers; the brown material moved into the lower phenolic layer. The sample was centrifuged for 10 minutes at 4°C and 10 000g in a Sorvall centrifuge. The RNA, in the upper aqueous layer, was separated from the

DNA and proteins at the interface and in the lower layer, and sedimented, at -20°C, using an equal volume of isopropanol. The RNA was sedimented by centrifuging at 10 000g for 10 minutes in a SS34 rotor. The RNA was washed by re-dissolving in denaturing solution and then sedimenting by adding an equal volume of isopropanol. The sample was left for at least 1 hour at -20°C and then centrifuged at 10 000g for 10 minutes in a SS34 rotor. The supernatant was removed and the nucleic acid washed in 10ml of RNAse free 70% v/v ethanol and again centrifuged. The pellet was dried under vacuum, resuspended in RNAse free H_2O and the A_{260} (section 2.4.1.) taken to calculate the amount of RNA purified. A sample of the RNA (1µg) was electrophoresed on an RNAse free agarose gel. Good quality samples were characterised by the appearance of clear, undegraded bands of ribosomal RNA in a long streak of fluorescent material.

2.4.32. Isolation of Messenger RNA from *P. Yoelii* Using DYNA® Beads

RNAse free DYNA® beads, which can be purchased coupled covalently to oligo $(dT)_{25}$, were used to purify polyA containing RNA species (Hornes and Korsnes, 1990). The magnetic beads (1.0mg) were dispensed into a 1.5ml Eppendorf tube and washed in 2X binding buffer (20mM Tris-HCl pH7.5, 1.0M LiCl, 2mM EDTA) by resuspending in the buffer and then placing next to a magnet. The beads stuck to the side of the reaction tube while the buffer was carefully removed. The tube was then removed from the magnet and the beads resuspended in 100µl of 2X binding buffer. The RNA (75µg in 100µl) was denatured by heating to 65°C for 2 minutes, added to the beads and allowed to hybridise for 5 minutes. The tube was then placed next to the magnet and the supernatant, containing ribosomal and transfer RNA, removed leaving the mRNA bound to the beads. The beads were washed twice with 200µl of washing buffer (10mM Tris-HCl pH7.5, 0.15M LiCl, 1mM EDTA). The last traces of washing buffer were then removed before the RNA was eluted by adding 5µl of elution buffer (2mM EDTA pH7.5) and heating at 65°C for 2 minutes. The solution, containing the mRNA, was placed in a fresh tube and either used immediately or stored at -70°C with 2 volumes of ethanol.

2.4.33. First Strand cDNA Synthesis

The mRNA (≈1μg) was heated to 65°C for 3 minutes to denature. The mRNA was then resuspended in 50mM Tris-HCl (pH8.15), 6mM $MgCl_2$, 40mM KCl, 1mM DTT, 10U of RNAse inhibitor, 1.5mM dNTPs, 50ng/μl poly-dT oligonucleotide (dT_{14}) and 25U of murine reverse transcriptase C, and placed in an RNAse free Eppendorf. To check that the cDNA was correctly synthesised and that the sample had not degraded 5 μl of the sample was labelled. The labelling reaction was performed by placing 50μCi of [^{32}P]-α-dATP in an RNAse free Eppendorf tube, drying in a rotary evaporator and adding 5μl of the reaction mixture. Both the labelled and unlabelled tubes were incubated at 37°C for 1 hour. The labelled sample was electrophoresed on an alkaline agarose gel. The sample was passed down a 1ml G25 column (section 2.4.13.) to remove unincorporated radiolabel and then resuspended in 25mM NaOH, 10% v/v glycerol and 0.05% bromophenol blue. The alkaline gel was made by melting 0.8g of agarose in 80ml of 30mM NaOH and 2mM EDTA. When the agarose had cooled to 55°C it was poured onto a glass plate over which a comb had been placed. The gel was made as thin as possible and the comb was placed as close to the plate as possible without touching. The gel was poured using a 10ml glass pipette to layer the molten agarose gently onto the plate. When the gel was set it was placed in an electrophoresis tank filled with running buffer (30mM NaOH and 2mM EDTA). The sample was loaded onto the gel with 5' end-labelled (section 2.4.16.) HindIII digested λ DNA which had been resuspended in 25mM NaOH, 10% v/v glycerol and 0.05% bromophenol blue. The sample was electrophoresed at 30mA until the bromophenol blue had moved halfway. It was then dried on a flat bed gel dryer and exposed to film (section 2.4.8.) at -70°C overnight. A long streak of nucleic acids from 500-4000bp was seen indicating that large molecular weight cDNA was present.

2.4.34. Polymerase Chain Reaction Amplification of DNA

The PCR was performed in a final volume of 100μl using either a Perkin Elmer Cetus or a Techne PHC2 thermal cycler. A typical reaction mix was:

Stock solution	Final Concentration	Volume added
10X Reaction Buffer	1X	10µl
dNTP mix, 1.25mM each dNTP	0.2mM each dNTP	16µl
Primer 1, 25µM	1.25µM	5µl
Primer 2, 25µM	1.25µM	5µl
Template DNA in distilled H_2O	100 copies/µl	63.5µl
TAQ DNA polymerase	2.5 Units	0.5µl

The 10X reaction buffer was supplied with the enzyme. The PCR reaction mix was overlaid with 100µl of mineral oil and then subjected to temperature cycling. Typically, the sample was denatured by heating to 95°C for 5 minutes before the addition of the enzyme and then subjected to 45 cycles with melting at 95°C for 1.5 minutes, annealing at 45°C for 1.5 minutes and extension at 72°C for 3 minutes. The exact conditions, however, varied according to the primers used. Once the PCR reaction had finished the products were analysed using agarose gel electrophoresis (section 2.4.2.).

3. Cloning of the Malarial Glucose Transporter

3.1. Introduction

Molecular biology has revolutionised biochemistry over the last decade. Since the first molecular biology laboratories in the early eighties the cloning of genes has become both widespread and routine. There are now over 100 000 sequences deposited in the international EMBL and GENBANK databases.

This prolific growth in DNA sequence knowledge is due to the many uses that the obtained sequence can be put to. For example, obtaining the sequence of the malarial glucose transporter would:

i) allow the malarial sequence to be compared to the other cloned glucose transporters. This could allow differences in the structure of the genes to be exploited in the design of a specific drug that blocks the malarial transporter, and are ineffective against the host's transporters.

ii) facilitate the expression of the gene in a foreign host (e.g. COS cells, baculovirus or *Xenopus* oocytes). Because of the higher levels of protein produced and the absence of the red blood cell transporter, the expressed gene would be more amenable to kinetic analysis than the transporter in its native environment. The Km and Vmax, and the inhibition characteristics of the transporter could be determined.

iii) allow mutagenesis studies of the transporter that may yield important results about what function certain residues in the protein have. This is already being accomplished for the human glucose transporters where these studies are revealing which residues form the internal and external binding sites (Hashiramoto *et al*, 1992).

iv) help to gain information on the genetics of *Plasmodium*. This could help to position malaria more accurately on the phylogenetic tree, increase the accuracy of codon usage tables, and possibly provide information about control and transcription in malaria.

Many methods are available to clone genes. Traditionally, genes were cloned by screening either a cDNA or a genomic library. Briefly, either mRNA is reverse transcribed or genomic DNA digested and the nucleic acid incorporated into the λ bacteriophage. The plaques that are formed by the λ infected *E. coli* are blotted with either a gene from another organism or an oligonucleotide based on the sequence of part of the gene. The positive clones are purified and sequenced. This method is comparatively insensitive and it often takes many months before it is known whether the clone you have identified is part of your gene or a false positive. Usually this method is only successful if a gene from a closely related organism or an oligonucleotide with a high match is available.

An adaptation of this method, known as genomic blotting, has also had some success. Genomic DNA is prepared, digested with restriction enzymes and run on a low percentage agarose gel. This DNA is blotted onto nitrocellulose and probed with a gene specific sequence. The nucleic acid that hybridises is purified and used to prepare a selected library from which a clone can be isolated. This has similar limitations to screening a full genomic library and is only successful where a good gene specific probe is available.

It is also possible to clone genes by complementation in *E. coli*. This method relies on the expression of the protein inducing a phenotypic change in a mutant *E. coli* strain. This method has been successfully used to obtain the primary sequence of the *Plasmodium falciparum* glucose phosphate isomerase gene (Kaslow and Hill, 1990). Double mutant *Escherichia coli*, which had lost the ability to utilise glucose both through the pentose phosphate pathway and through glycolysis were complemented with a library of mung-bean nuclease digested malarial DNA. The bacteria that grew on glucose were found to contain a vector that was expressing the malarial glucose phosphate isomerase gene. This method has the advantage that it does not require prior knowledge of the gene sequence. It is not thought that this method is feasible for cloning the malarial glucose transporter. Firstly, although the glucose transporter superfamily do not usually contain a signal sequence and human glucose transporters

have been functionally expressed in *E. coli* (Sarkar *et al*, 1988), it is uncertain whether the malarial glucose transporter will insert correctly into the *E. coli* plasma membrane and produce a functional protein. Secondly, as at least six native proteins are known to transport glucose in *E. coli*, it would be difficult to produce a mutant lacking the ability to transport glucose.

Another method that does not rely on sequence information is functional expression. This method was used to isolate the α-subunit of the Na^+/glucose symporter from human intestine (Hediger *et al*, 1987a, b). A cDNA library was prepared in λ-zap, subdivided, and RNA made from each part of the library. This RNA was injected into *Xenopus laevis* oocytes and the fraction with the largest glucose/Na^+ transport activity further subdivided. In this way the clone containing the transporter was eventually isolated. This method would probably be successful in isolating the malarial glucose transporter also. However, this method is very labour intensive and was not feasible in our laboratory.

Recently many genes have been cloned using the polymerase chain reaction. For example the *Plasmodium falciparum* glycolytic enzyme 3-phosphoglycerate kinase has been cloned this way (Hicks *et al*, 1991) and in our laboratory a number of insect opsins, a muscarinic acetyl choline receptor from *Schistocerca gregaria* (locust) and the *Haemonchus contortus* GABA receptor have been isolated. This method is sensitive and allows a rapid screening procedure. It has been suggested that the sensitivity of the polymerase chain reaction allows genes to be isolated using oligonucleotides that would not work using a blotting technique (Hicks *et al*, 1991). A PCR product can be cloned and sequenced followed by isolation of the entire gene in a very short time.

The polymerase chain reaction amplifies DNA between two oligonucleotide primers (figure 9). Firstly the double stranded DNA is mixed with the two oligonucleotides, dNTPs and enzyme. This mixture is heated to 95°C causing the DNA template to become denatured and single stranded. The sample is then cooled allowing the primers to anneal to the single stranded DNA template. After heating the sample to the

enzyme's optimal temperature of 72°C, the TAQ polymerase extends the oligonucleotide primer making a complementary strand to the template. This product, known as a long product, will usually extend beyond the second oligonucleotide. This temperature cycling is repeated many times. During the next cycle the second oligonucleotide binds to the long product of the first round and the enzyme makes double stranded DNA until the template stops at the end of the first oligonucleotide. As more cycles are performed the proportion of long products becomes insignificant. After 30 cycles 1 molecule of template will have produced 2^{30} molecules of product.

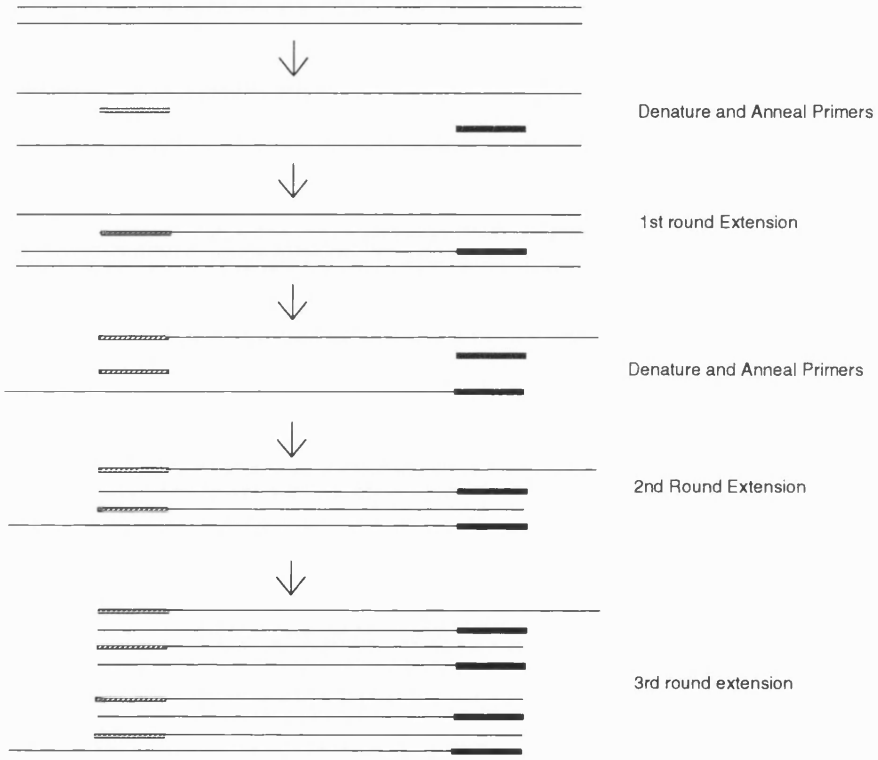

Figure 9. A diagram showing the theory behind the polymerase chain reaction. Double stranded DNA is heated to 95°C to denature and is then cooled, allowing the oligonucleotides to bind. During the first round TAQ polymerase extends the oligonucleotides producing long products. In the second round, the DNA is again melted and the primers anneal. Primers that have annealed to one of the long products of the first round will extend only as far as the first oligonucleotide producing short products. Because in subsequent rounds the oligonucleotides can bind to either long or short products to produce short products, the number of short products increases exponentially.

In this project we have used the polymerase chain reaction extensively to rapidly screen for the malarial glucose transporter. With the polymerase chain reaction

conditions can be varied and optimised until specific products are produced. Once produced the products can be cloned and sequenced rapidly. The polymerase chain reaction is fast, specific and sensitive.

A special adaptation of the polymerase chain reaction can be used to amplify the 3' ends of genes (Frohman and Martin, 1989; Frohman, 1990). This adaptation, known as RACE (Rapid Amplification of 3' cDNA Ends), uses cDNA that was synthesised using a special polyT primer containing a 3' stretch of 28 nucleotides (called R) of known sequence. In the PCR reaction one of the primers can be made to R so that the 3' end of the cDNA can be specifically amplified using a probe to the required gene. This method amplifies the gene but may also amplify unwanted gene products as well. The method can be made much more specific by repeating the method using 2 primers (one gene specific and one made to R) made to internal regions of the gene. This use of nested primers is a common way of removing unwanted contamination by non-specific contaminants in PCR reactions (figure 10).

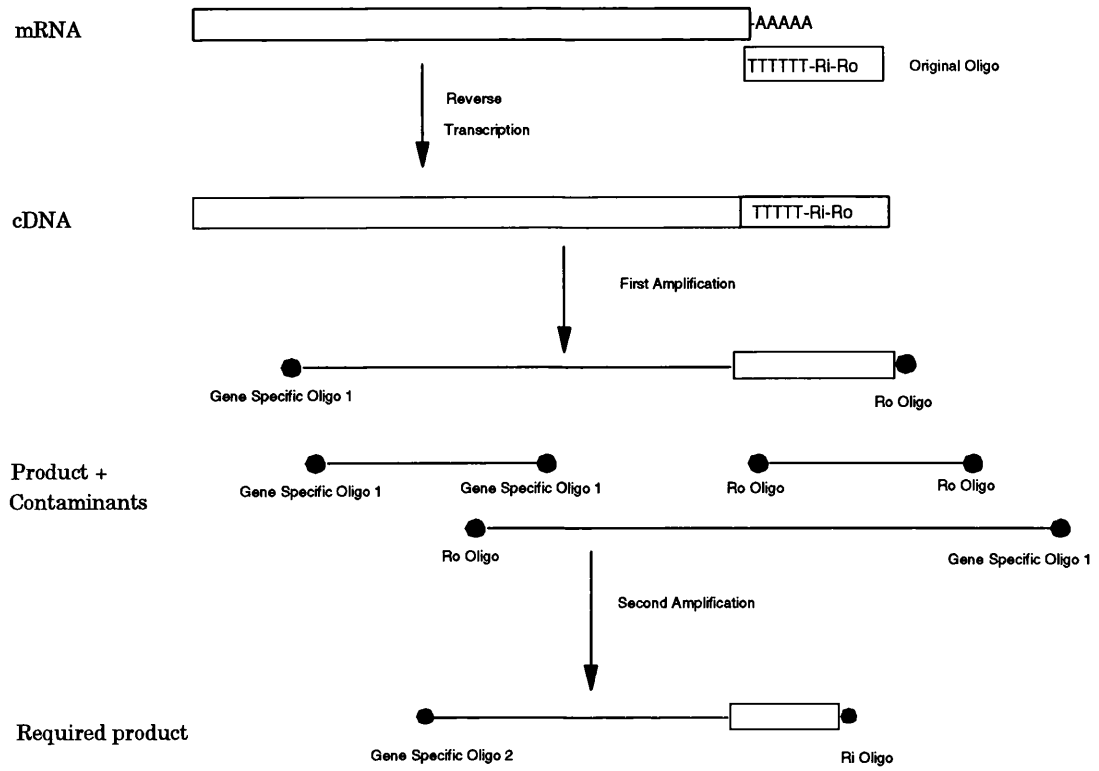

Figure 10. Rapid Amplification of 3' cDNA Ends (RACE) PCR (Frohman and Martin, 1989). Messenger RNA is purified and converted to cDNA using a polyT oligonucleotide that contains a 3' flanking region R 28 nucleotides long. The cDNA is mixed with an oligonucleotide made to the outer half of R (Ro) and a sense gene specific primer, and amplified using the polymerase chain reaction. Among the products will be the gene of interest. A second amplification using nested primers, i.e. to the inner half of R and a second sense gene specific oligonucleotide, will increase the specificity of the technique and hopefully yield a single product.

Although similar techniques can be used to clone genes from almost any organism, the methods often need to be adapted to suit the peculiarities of the particular organism under investigation. With malaria this is certainly the case. The malarial genome is extremely AT-rich (70% in coding regions and 92% in non-coding regions), contains very short introns and is haploid for the majority of its life cycle. The extreme AT-richness makes large pieces of DNA unstable in *E. coli*, which has made the preparation of malarial libraries extremely troublesome. These problems have only recently been circumvented using yeast artificial chromosomes (section 1.1.8.). The use of YACs however present their own problems: special equipment is required to electrophorese the samples and the DNA is propagated in comparatively slow growing

yeast rather than in *Escherichia coli*. The extreme AT bias of the malaria genome has also made the preparation of cDNA difficult. The first step in preparing cDNA is the purification of mRNA from total RNA (using a polyT oligonucleotide). This is difficult because contaminating DNA species with long polyA stretches and non-messenger RNA species containing polyA will copurify with the mRNA. The AT-richness lowers the hybridisation temperature of oligonucleotides during PCR, Southern and Northern blotting making the detection of non-specific products more likely. It has also been suggested that the low efficiency of expression of malarial genes is due to the AT-richness of the genome (Weber, 1987). For example, in *Plasmodium falciparum* the Asn codon AAT is strongly favoured over AAC (table 3); the reverse is true for highly expressed genes in both *E. coli* and yeast. Only in poorly expressed genes in these two species is AAT favoured (Saul and Battistutta, 1988). When mRNA is injected into *Xenopus oocytes* it is believed that supplying malarial tRNAs aids the efficient expression of malarial genes (Robson, 1991).

Many malarial genes have now been sequenced. It has been noted that many of these genes share common features that are unusual in other organisms. One feature is the presence of one of the two sequences `LFFLI` or `LFFII` in the signal sequences from many malarial genes (MacKay, 1987). These pentapeptide sequences are rare in other eukaryotic signal sequences (MacKay, 1987). Another feature is the highly repetitive nature of many regions of plasmodial genes (MacKay, 1987). For example the *P. falciparum* circumsporozoite protein (CSP), which covers the surface of the sporozoite, consists of a tandem repeat that occupies about a third of the molecule (Hyde, 1990). Introns are not common in malarial genes and when they do occur they are usually located towards the start of the gene and are usually only a few hundred bases long (MacKay, 1987; Saul and Battistutta, 1988).

As mentioned earlier, malaria has an extreme preference for A and T over G and C in its DNA. For amino acids where all 4 bases can be used in the last position, A and T are equally abundant (table 3). An A or a T in the third position is 3-5 times more likely than a G or a C in such cases (Saul and Battistutta, 1988). The high frequency

of A and T in the 3rd position accounts for the AT-richness of the malarial coding region.

It has also been reported that there is an overall bias for the A codon over the T codon in the mRNA sense strand (Weber, 1987). This is because the coding strand is purine-rich with the bulk of these being A (T:C:A:G is 26:14:43:17). The first base in the codon is a purine 70% of the time; the last base is only a purine 51% of the time (Hyde and Sims, 1987; Saul and Battistutta, 1988). The purine bias is therefore not accounted for by bias in the third position. Rather, the base composition is a reflection of the high abundance of asparagine, lysine, aspartate and glutamate (which use AA or GA in the first two positions) in malarial proteins (Hyde and Sims, 1987; Saul and Battistutta, 1988). Having a high purine/pyrimidine ratio in the sense strand may be advantageous to the malarial parasite in the production of mRNA because purines are readily available from the host and pyrimidines have to be synthesised *de novo* (Saul and Battistutta, 1988; Gero and O'Sullivan, 1990). Table 3 shows the codon usage in *Plasmodium falciparum* (Saul and Battistutta, 1988).

F	TTT	266.	S	TCT	149.	Y	TAT	362.	C	TGT	79.
F	TTC	91.	S	TCC	96.	Y	TAC	79.	C	TGC	18.
L	TTA	502.	S	TCA	198.	*	TAA	14.	*	TGA	2.
L	TTG	110.	S	TCG	23.	*	TAG	2.	W	TGG	32.
L	CTT	124	P	CCT	72.	H	CAT	154.	R	CGT	2.
L	CTC	19.	P	CCC	21.	H	CAC	65.	R	CGC	5.
L	CTA	42.	P	CCA	152.	Q	CAA	298.	R	CGA	26.
L	CTG	7.	P	CCG	9.	Q	CAG	35.	R	CGG	0.
I	ATT	243.	T	ACT	138.	N	AAT	1104.	S	AGT	180.
I	ATC	53.	T	ACC	53.	N	AAC	310.	S	AGC	65.
I	ATA	247.	T	ACA	228.	K	AAA	971.	R	AGA	159.
M	ATG	238.	T	ACG	21.	K	AAG	210.	R	AGG	28.
V	GTT	214.	A	GCT	144.	D	GAT	525.	G	GGT	175.
V	GTC	28.	A	GCC	54.	D	GAC	84.	G	GGC	11.
V	GTA	215.	A	GCA	131.	E	GAA	730.	G	GGA	219.
V	GTG	33.	A	GCG	9.	E	GAG	89.	G	GGG	19.

Table 3. The codon usage table for *Plasmodium falciparum*. The table shows the frequency that each codon is used per 10 000 codons for non-repetitive DNA in the first 17 *P. falciparum* proteins to be cloned. Reproduced from Saul & Battistutta, 1988.

Studies have been performed on superfamilies of genes where the malaria homologue has been sequenced. It has been found that, in general, genes cloned from *P. falciparum* appear to be most similar to higher eukaryote sequences than to lower eukaryotes or to prokaryotic sequences (Hyde *et al*, 1989). Recently the *Plasmodium falciparum* enolase gene has been isolated and found to have regions that are only found in higher plants (Read *et al*, 1993). It has been suggested that, where possible, oligonucleotide probes designed to clone malarial genes should be based on mammalian sequences in preference to trypanosome or yeast sequences (Hyde *et al*, 1989). It has also been suggested (Hyde and Sims, 1987) that dinucleotide frequencies in *Plasmodium falciparum* have characteristics indicative of vertebrates rather than lower eukaryotes (particularly the low frequency of CG and the high frequency of TG found in the coding strand). A phylogenetic tree based on the divergence of small subunit rRNA molecules confirms that *Plasmodium* is closer to *Saccharomyces* and mammalian sequences than to other lower eukaryotes like *Trypanosoma brucei* and *Euglena gracilis* (Hyde, 1990). The phylogenetic tree does suggest however that yeast sequences may be a good basis for the design of oligonucleotide probes.

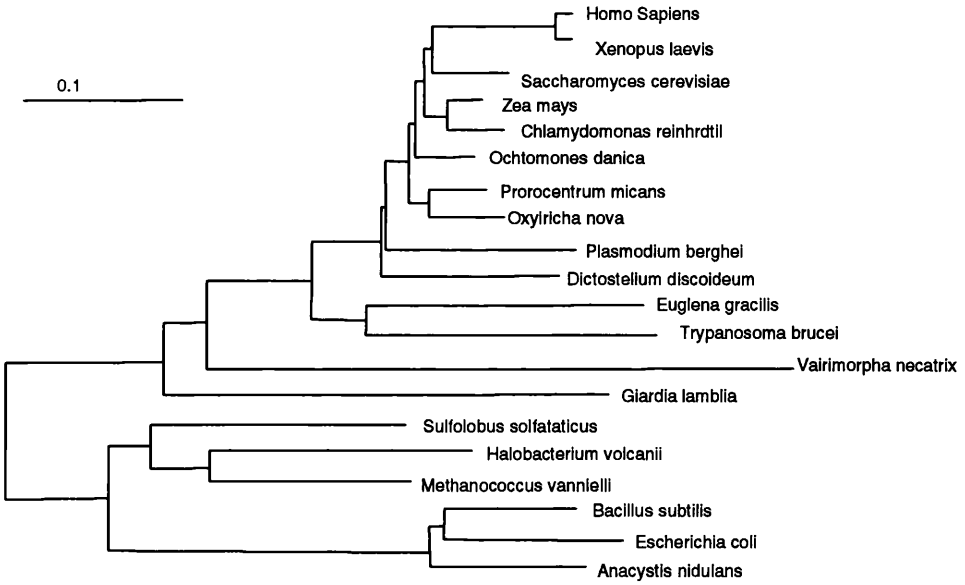

Figure 11. A phylogenetic tree showing the convergence of small subunit rRNAs from a wide range of organisms. Note that *Plasmodium* is closer to yeast and mammalian sequences than to trypanosome and *Euglena* sequences. Redrawn from Hyde (1990).

There is no sequence information for the malarial glucose transporter and such data would be very hard to obtain. For this reason, an accurate alignment of the sugar transporters from other species is necessary so that conserved regions can be identified and oligonucleotides can be designed to them. The alignment used during this project is shown in Appendix 1. Since this alignment was produced further members of this superfamily have been isolated (section 1.2.3.). Visual alignment of these new sequences confirms that the regions chosen are indeed conserved. Figure 12 shows these conserved regions in a two dimensional model of the glucose transporter.

A comparison of the alignment with alignments from other superfamilies suggests that the facilitative sugar superfamily may not be highly conserved. For instance the *Leishmania* transporter appears to be lacking both the PESPRFL and VPETKG regions completely. Both GABA receptors and insect opsins have a much tighter conservation.

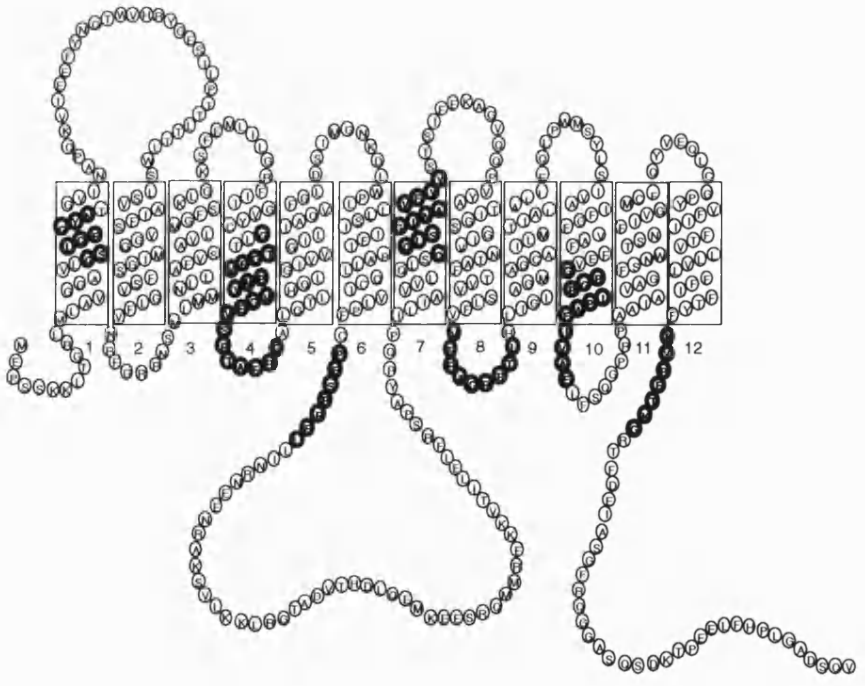

Figure 12. Human GLUT1 glucose transporter showing regions that are conserved with other members of the superfamily.

3.2. Methods

3.2.1. PREPARATION OF cDNA FROM *PLASMODIUM YOELII*

Plasmodium yoelii parasites were cultured in albino CDC mice (section 2.2.10.) until the parasites occupied more than 90% of the mouse erythrocytes. The blood from ten mice was pooled and passed through a cellulose column to remove contaminating white blood cells (section 2.2.12.). The red blood cells were then lysed with saponin (section 2.2.6.) to yield free parasites. The haemoglobin and red blood cell debris were washed away, by two washes with PBS, and then RNA was extracted from the parasites using guanidinium isothiocyanate (section 2.4.31.). Messenger RNA was purified from the total RNA, using DYNA™ beads (section 2.4.32.) and used immediately to make cDNA (section 2.4.33.). The cDNA was synthesised using polyT oligonucleotide containing a 28bp trailer sequence so the cDNA was suitable for RACE PCR (3.2.4.).

3.2.2. PREPARATION OF GENOMIC DNA FROM *PLASMODIUM FALCIPARUM*

Plasmodium falciparum parasites were grown in culture (section 2.2.), harvested and the red blood cells lysed using saponin (section 2.2.6.). Genomic DNA was then prepared from the cells using Proteinase K and phenol (section 2.4.29.).

3.2.3. AMPLIFICATION USING TWO GENE SPECIFIC PRIMERS

The genomic DNA or cDNA was used in the polymerase chain reaction (section 2.4.34.) using two internal primers. The products were electrophoresed on a 1% agarose gel (section 2.4.2.) and any bands of the anticipated molecular weight were excised from the gel using a new razor blade. The DNA was extracted from the agarose by the freeze squeeze method (section 2.4.3.) and 1/1000th of it reamplified using the same primers. The single products were again electrophoresed on a 1% agarose gel (section 2.4.2.). The band of DNA was excised and purified from the agarose using the GeneClean™ method (section 2.4.4.), blunt ended with T4 DNA Polymerase I (section 2.4.27.), and ligated (section 2.4.19.) overnight with SmaI digested (section 2.4.6.), phosphatased (section 2.4.18.) pUC19 vector (Promega). Competent *Escherichia coli* cells (section 2.4.22.) were transformed with the ligated

vector (section 2.4.19.) and screened for inserts using the alkaline lysis miniprep method (section 2.4.23.). Vector from cultures containing the correct size inserts were purified using MagicMiniprep™ columns (section 2.4.24.) and sequenced (section 2.4.26.).

3.2.4. Amplification Using the RACE Method

The cDNA (20ng) was amplified (section 2.4.34.) with a primer to the outer half of the trailing sequence (R_o) and an internal sense oligonucleotide. A sample ($1/1000^{th}$) of the above reaction was subjected to a second round of amplification using an oligonucleotide to the inner region of the trailing sequence (R_i) and a second gene specific primer downstream of the first. For the control, the sample was electrophoresed (section 2.4.2.), transferred to hybridisation membrane (section 2.4.28.1.) and blotted (section 2.4.28.2.) with a third gene specific oligonucleotide. The samples were cloned by reamplifying (section 2.4.34.) with a gene specific oligonucleotide containing a 5' restriction site and R_i (which also contains a restriction site), and ligating (section 2.4.19.) with pUC19 vector which has been digested with the same restriction enzymes. Competent *Escherichia coli* cells (section 2.4.22.) were transformed with the ligated vector (section 2.4.19.) and screened for inserts of the correct size (section 2.4.23.). Vector from cultures containing the correct size inserts was purified on a large scale (section 2.4.25.) and sequenced (section 2.4.26.).

3.2.5. Checking the Quality of the cDNA

During the cDNA synthesis a fraction of the sample was labelled with ^{32}P and then electrophoresed on an alkaline gel (section 2.4.33.). Once the synthesis was complete, a sample of the cDNA was used to amplify the *Plasmodium yoelii* actin gene. For this the RACE method was used. Two rounds of amplification were performed and the products electrophoresed on an agarose gel (section 2.4.2.). The PCR products were transferred to nitrocellulose and blotted with a third actin specific oligonucleotide (section 2.4.28.). Only cDNA that successfully amplified actin was used.

3.2.6. Checking the Quality of the Genomic DNA

Only genomic DNA that had a molecular weight larger than λ and that could be digested with EcoRI was used for PCR amplification. As an internal control a portion of the *P. falciparum* TATA-binding protein (TBP) was amplified. The oligonucleotides for this reaction were supplied by Dr J. E. Hyde. Whenever PCR on the genomic DNA was performed a tube was prepared containing the genomic DNA and the two TBP oligonucleotides. After electrophoresis (section 2.4.2.) it was always found that these oligonucleotides produced a single intense product that was 700bp long.

3.2.7. Analysis of the Clones using a Computer

Sequences were read from the autoradiograph and translated to protein using the Staden suite of programs and an IBM compatible personal computer. The sequences were then transferred to a Silicon Graphics computer and further analysed using the Wisconsin GCG computer package. Codon usage and a search for similarity with sequences held in the EMBL database were performed on each sequence using the GCG programs COMPOSITION and FASTA respectively.

3.3. Results

3.3.1. Testing the Integrity of *P. yoelii* cDNA

The integrity of the cDNA prepared from *P. yoelii* was confirmed by two methods (figure 14). Firstly, during synthesis a fraction of the cDNA was radiolabelled and then electrophoresed on an alkaline gel. As can be seen in figure 14a molecules between 200 and 4000 nucleotides were labelled during the synthesis. This suggests that a large proportion of the mRNA was intact during the synthesis producing full length cDNA transcripts. Secondly, actin was amplified from the cDNA pool using the RACE PCR method and two rounds of amplification (Goodyer *et al*, 1991). To do this, three actin specific oligonucleotides were synthesised based on the published *P. falciparum* PFActinI gene (Wesseling *et al*, 1988). The method of amplification and the sequence of the actin specific oligonucleotides is shown in figure 13. The results (figure 14b) show that actin is specifically amplified in both rounds of the PCR reaction and that the cDNA is intact.

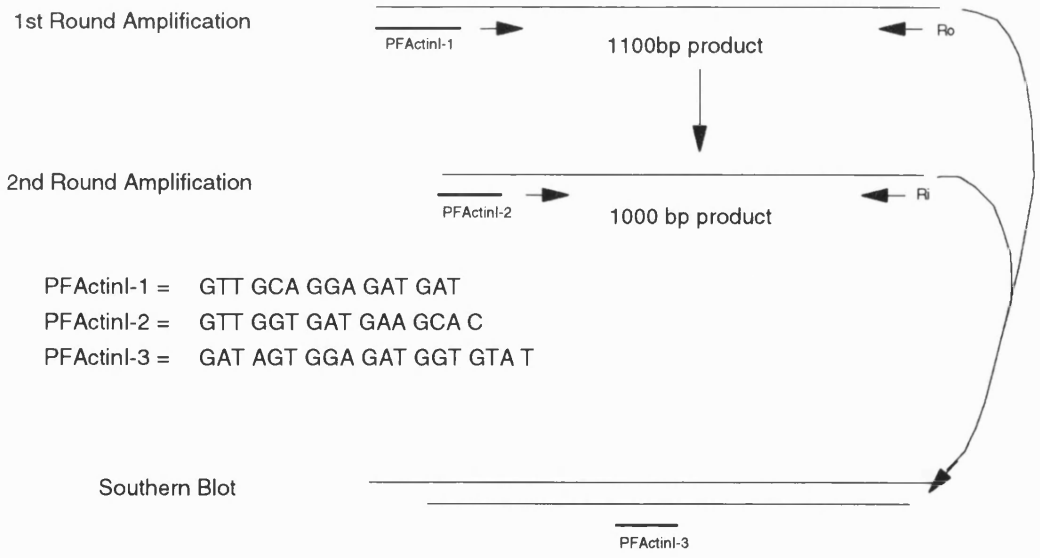

Figure 13. The strategy used to amplify *Plasmodium yoelii* actin. In the first PCR reaction *P. yoelii* cDNA is amplified with Ro and PFActinI-1 oligonucleotides producing a 1100 nucleotide long product. In the second round $1/1000^{th}$ of the products formed in the first reaction were reamplified with Ri and PFActinI-2 oligonucleotides producing a 1000 nucleotide product. An aliquot ($1/10^{th}$) of both the first and second round reactions are then blotted with a third actin specific oligonucleotide PFActinI-3.

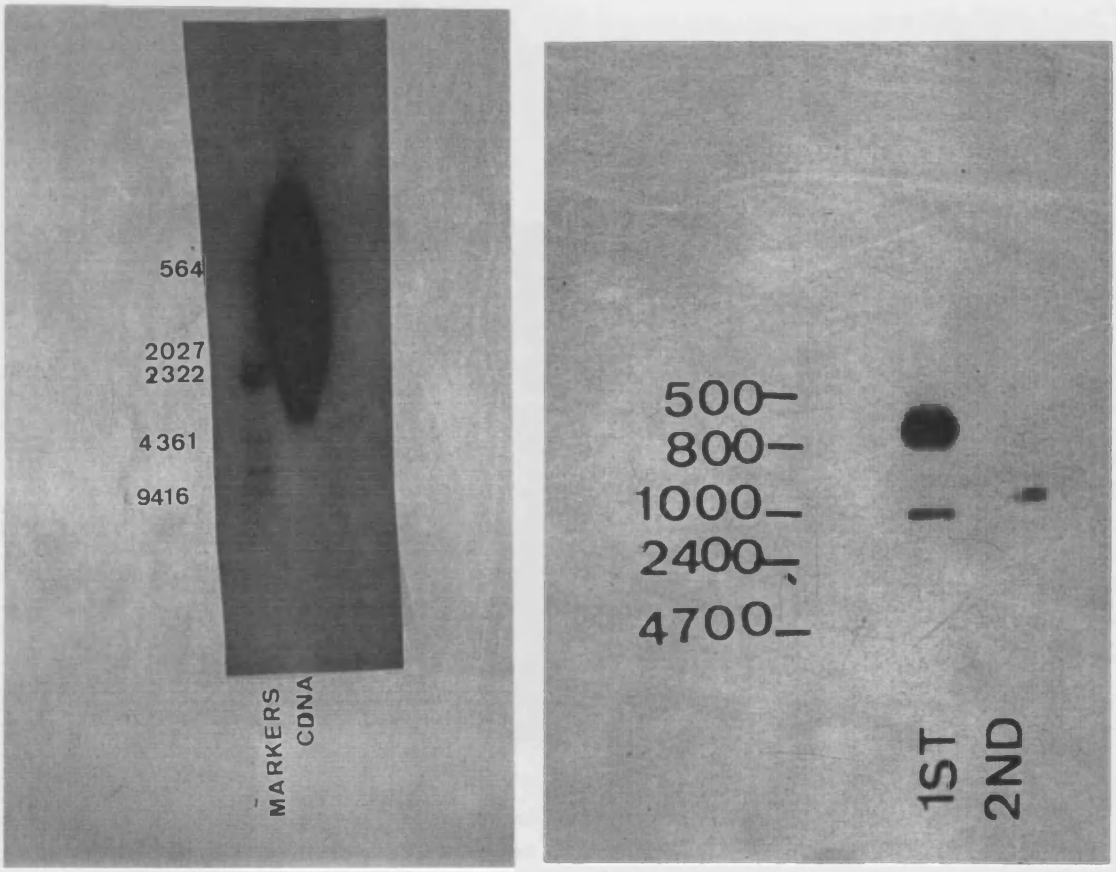

Figure 14. Testing the integrity of *P. yoelii* cDNA. a) The synthesised cDNA has a molecular weight ranging between 200 and 4000 nucleotides b) Southern blot showing amplification of actin from *P. yoelii* cDNA by two rounds of RACE PCR.

3.3.2. TESTING THE INTEGRITY OF *P. FALCIPARUM* GENOMIC DNA

The *Plasmodium falciparum* genomic DNA appeared to be of a high quality. Figure 15a shows that the malarial DNA is larger than bacteriophage λ and shows virtually no shearing. Figure 15b shows that the genomic DNA can be digested by the common restriction endonucleases EcoRI and HindIII. The digestion is important as it shows that the DNA is purified from histones and other DNA associated proteins and suggests that it will also be accessible to TAQ polymerase during the polymerase chain reaction.

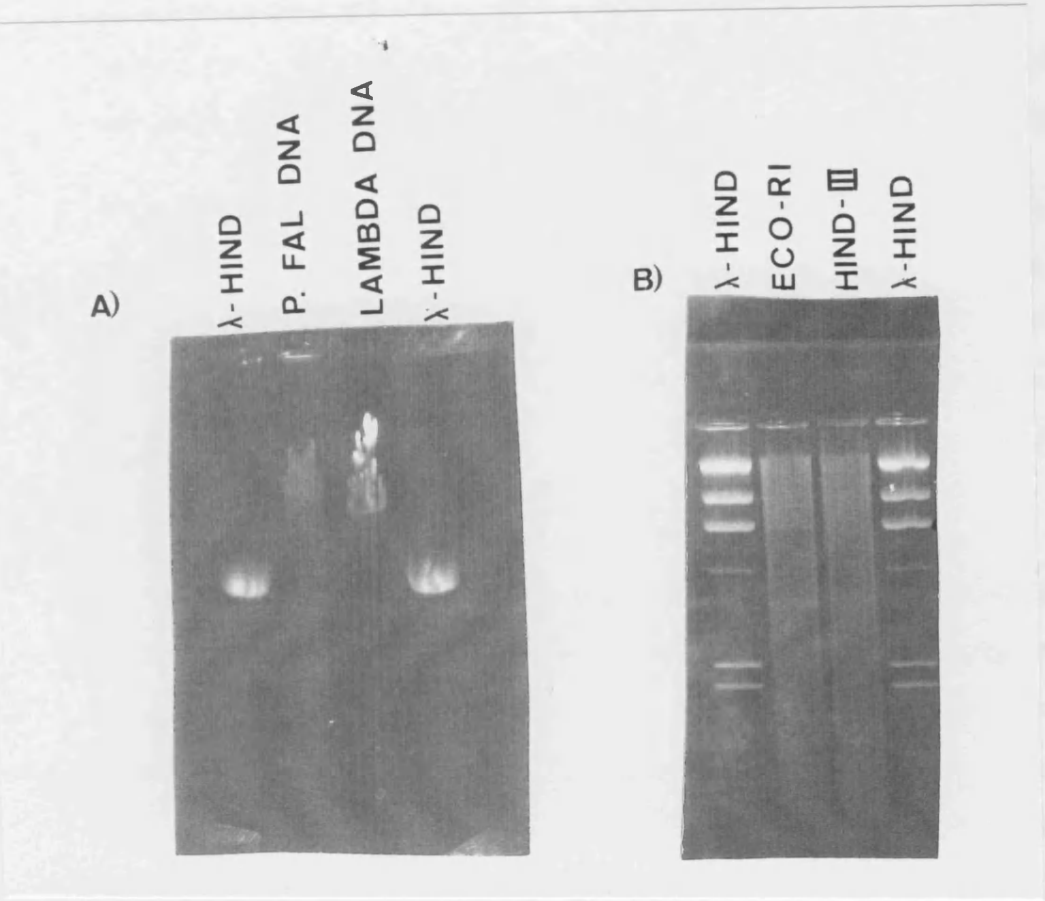

Figure 15. *Plasmodium falciparum* genomic DNA used during this project. a) The malarial genomic DNA is larger than λ and shows little shearing, b) The DNA can be digested by either HindIII or EcoRI endonuclease.

3.3.3. PRIMARY SCREEN - CLONING OF THE *P. YOELII* GLUCOSE TRANSPORTER

3.3.3.1. Design of Oligonucleotides

In the early attempts to clone the malarial glucose transporter oligonucleotides were designed using the rules accepted at the time (Hyde *et al*, 1989; Hicks *et al*, 1991). These included:

i) the malarial sequence will be most similar to previously isolated vertebrate sequences (Hyde *et al*, 1989).

ii) the redundancy of the oligonucleotides should be kept low so that the matching oligonucleotide is not diluted by probes that do not match. The degeneracy can be kept low when cloning malaria genes by utilising the unusual codon usage found in *Plasmodium* (table 3). For example, the proline residue is coded for by the triplet codons CCA, CCC, CCG and CCT, but in malaria

approximately 90% of prolines are encoded for by CCA or CCT. For prolines we therefore chose the less degenerate CC$^{A}/_{T}$ combination (Hyde *et al*, 1989).

iii) the oligonucleotides can be slightly degenerate at the 3' end. It is believed that TAQ polymerase requires the 3' end bases to be an exact match for efficient translation. Mismatches are less serious at the 5' end of the oligonucleotide. For this reason, redundancy was incorporated into the 3' end of the probe to help ensure an exact match in at least a fraction of the oligonucleotides (Hicks *et al*, 1991).

iv) the probes should be quite long (~35-40 nucleotides). This increases the melting temperature of the oligonucleotide and thus increases the probability of efficient priming (Hyde *et al*, 1989). It was not possible to follow this strictly because the conservation found in the glucose transporter superfamily is quite low and the conserved regions that exist are often short. In practice primers were synthesised that were as long as possible.

Five oligonucleotides were synthesised to four conserved regions in the facilitative sugar transporter superfamily. All these regions were highly conserved in the mammalian sequences and showed extensive conservation throughout the superfamily. The sequence of these oligonucleotides are shown in table 4.

Protein Sequence	Position	Sense or antisense	Length	Nucleotide sequence
PESPR$^{F}/_{Y}$L	386-392	Sense	20mer	CC$^{A}/_{T}$ GAA $^{A}/_{T}{}^{C}/_{G}$T CC$^{A}/_{T}$ AG$^{A}/_{T}$ T$^{A}/_{T}$T TT
QQLSGIN$^{A}/_{G}/_{V}$ VFYY	468-479	Sense	36mer	CAA CAA TTA $^{T}/_{A}{}^{C}/_{G}{}^{T}/_{A}$ GG$^{A}/_{T}$ AT$^{A}/_{T}$ AAT G$^{T}/_{G}{}^{A}/_{T}$ $^{A}/_{G}$T$^{A}/_{T}$ TTT TAT TAT
VERAGRRTL	518-526	Sense	26mer	GT$^{A}/_{T}$ GAA AGA GC$^{A}/_{T}$ $^{C}/_{G}$G$^{A}/_{T}$ AGA AGA ACA TT
VERAGRRTL	526-518	Antisense	27mer	TAA TGT TCT TCT $^{A}/_{T}$C$^{C}/_{G}$ $^{A}/_{T}$GC TCT TTC $^{T}/_{A}$AC
KVPETKG	670-663	Antisense	17mer	CC TTT AGT TTC $^{T}/_{A}$GG $^{T}/_{A}$AC

Table 4. Oligonucleotides synthesised to the facilitative glucose transporter superfamily. The numbers in the position column refer to the place the protein sequence occurs in the alignment in Appendix 1.

3.3.3.2. PCR conditions

Using these oligonucleotides in combination there are six possible pairings. Every combination was tried using a variety of PCR conditions. The target was in each case the cDNA prepared from *P. yoelii* that was found to be intact by the amplification of actin. It has been reported that the concentration of magnesium in the reaction mixture is critical (Innis and Gelfand, 1990). The magnesium concentration affects primer annealing, the formation of primer-dimer artefacts and enzyme activity. We found that the concentration of magnesium in the 10X TAQ buffer (1.5mM) was sufficient and extra magnesium did not increase either the number or yield of products. Initially the annealing temperature was set high (55°C) in an attempt to reduce non-specific binding. At 55°C however, no products were obtained. The amplification was repeated lowering the temperature until discreet products were observed. The optimal temperature was found to be 42°C. At this temperature the following conditions were used.

Denaturation - 95°C for 5 minutes before the enzyme was added followed by 40 cycles of

94°C - 1 minute, 42°C - 1 minute 30 seconds, 72°C - 3 minutes.

3.3.3.3. Products cloned and sequenced

Four bands were evident following the PCR reaction: one band (550 nucleotides) was produced using the PESPRFL sense and the VERAGRRTL antisense oligonucleotides; two bands (400bp and 700bp) were produced using VERAGRRTL sense and KVPETKG antisense; and one band (350bp) was produced using QQLSGINAVFYY sense and KVPETKG antisense. Each band was excised from an agarose gel, reamplified and cloned into pUC19 vector. After minipreps and sequencing it was found that 8 different clones were produced. A summary of these products is shown in table 5 and the sequences of these products are found in appendix 2.

Clone	Sense primer	Antisense primer	%AT	Size in Human Glut1	Actual Size
A	PESPRFL	VERAGRRTL	FORWARD = 59% REVERSE = 60%	386	550
B	VERAGRRTL	KVPETKG	55%	390	342
C	VERAGRRTL	KVPETKG	FORWARD = 69% REVERSE = 54%	390	700
D	VERAGRRTL	KVPETKG	FORWARD =47% REVERSE = 56%	390	700
E	VERAGRRTL	KVPETKG	49%	386	450
H	QQLSGINAVFYY	KVPETKG	FORWARD = 37% REVERSE = 46%	530	550
I	QQLSGINAVFYY	KVPETKG	47%	530	350
J	QQLSGINAVFYY	KVPETKG	FORWARD = 50% REVERSE = 47%	500	350

Table 5. The size and base composition of cloned PCR products using *P. yoelii* cDNA. The AT composition was determined by sequencing the cloned product with either forward or reverse primer.

Product A, which was produced with the sense PESPR$^{F}/_{Y}$L and the antisense VERAGRRTL oligonucleotides, is slightly larger than the size predicted for human GLUT1. It is AT-rich, and a homology screen revealed that this sequence has not been reported before and has no homology to previously reported sequences. Both the sense and antisense oligonucleotides have been identified in the clone but there is an unambiguous stop sequence in frame with the sense oligonucleotide. This suggests that the sense oligonucleotide in not in the coding strand. Another reading frame of the forward sequence is open and the antisense primer is also in an open reading frame. This product shows no homology to the facilitative glucose transporter superfamily.

Product B was sequenced in full and found to be slightly AT-rich (55%). Only one oligonucleotide (VERARRRTL) was found in the clone. The product was about the anticipated size and had stretches of hydrophobic residues that suggested that the sequence may encode a membrane protein. A homology search revealed that this sequence had not previously been described. Despite extensive searching this sequence did not show any homology to the facilitative transporters.

Products C and D were larger than predicted by homology to the other members of the glucose transporter superfamily. As malarial gene products are frequently larger than their vertebrate homologues it was decided to clone and sequence these products.

Product C was very AT-rich (about 60%), product D is only slightly AT-rich. Both contained the sense oligonucleotide VERARRRTL but in both the antisense oligonucleotide was missing. Neither sequence had been reported before, but the forward sequence of D was 70% homologous to the three long terminal repeats H11, H29 and H16 from rat (Furter *et al*, 1992). In the reverse direction sequence D appears to have approximately 70% homology to many rat and mouse clones suggesting, along with the only slight AT bias, that product D may be a contaminant from the murine host.

Product E is not AT-rich, lacks an oligonucleotide at either end, is not found in the EMBL database, is smaller than expected and does not show any homology to facilitative glucose transporters. It is most likely that this clone is a contaminant.

Product H is not AT-rich and does not have the sense oligonucleotide present. Initially these findings led us to discard this sequence. However, on performing a homology search we found that this was a cDNA clone of the human Glut4 sequence: a clone that had been used in the laboratory. A very small amount of this must have contaminated the PCR reaction because the band that was cloned was not particularly strong and contained other bands that were also sequenced (clones I and J). This demonstrates clearly that because an oligonucleotide is not found at one end of a clone it should not be dismissed without thorough investigation.

Clones I and J were both obtained from the same band on the gel; both are novel sequences with very little similarity to any other sequences reported to date. Both lack oligonucleotides at either end of the clone, and neither are AT-rich or show any homology to facilitative transporters.

RACE PCR was also performed on the *P. yoelii* cDNA using the three sense oligonucleotides shown in table 4 and the oligonucleotide Ro. It was found that 30 cycles with an annealing temperature of 48°C gave the best results. This method produced a band with the VERA$^{G}/_{R}$RRTL sense oligonucleotide. This product was 1500 nucleotides long, which was the expected size for the product, considering the inclusion of the 3' untranslated region. The product was purified and reamplified with

Ri and an oligonucleotide to the VERA$^{G}/_{R}$RRTL region that was synthesised with a GC-rich 5' anchor and a PstI site to aid directional cloning. The sequence of the RACE specific oligonucleotides are shown in table 6.

Oligonucleotide	Sequence
Ro	GACTACGTTAGCATTA
Ri	CATCTAGAATTCTCGAG
CG-pst-VERAGRRT	GCGCGGCG-CTGCA-GT$^{A}/_{T}$ GAA AGA GC$^{A}/_{T}$ CG$^{A}/_{T}$ AG

Table 6. Oligonucleotides synthesised for RACE PCR. Both Ro and Ri have a series of restriction sites. The gene specific oligonucleotide has a 5' GC-rich region that allows the restriction enzyme to anchor efficiently, a PstI restriction site and the gene specific sequence.

In the Ri oligonucleotide is an XbaI site. The PCR product was purified (section 2.4.24.) and digested with XbaI and PstI. The PCR product had an internal XbaI site 460 nucleotides from the terminal PstI. This 460 bp fragment was directionally cloned into pUC19 vector and sequenced. The product was found to be a novel sequence that was 51% AT and showed no significant homology to facilitative transporters. In frame with the oligonucleotide is a stop codon. It is therefore extremely unlikely that this clone is part of the malarial glucose transporter.

During my project the successful cloning of genes using PCR with highly redundant oligonucleotides (over 100 000 degeneracy) was reported (Criado, 1992). It was important to ensure that the redundancy was low at the 3' end of the oligonucleotide, preferably with an exact match, whereas the 5' end could be highly redundant (Compton, 1990).

The advantage of highly degenerate probes is that it is possible to make longer oligonucleotides based on fairly poor amino acid conservation. Two conserved regions with an intervening variable region can be used to make a probe by allowing high redundancy in the variable region. High degeneracy allows the covering of many amino acids in one particular position. For example if a residue is hydrophobic (phenylalanine, leucine, isoleucine or valine) all these can be covered adequately in malaria genes by NT$^{A}/_{T}$.

The certainty required for the 3' end of the oligonucleotide requires a careful design. Residues that are encoded by one triplet (i.e. methionine and tryptophan) are most favourable because if this residue is present redundancy in not required. In an organism with a pronounced AT bias, residues encoded by two triplets, one ending in an A or a T and the other ending in a C or a G are almost as favourable, and can usually be predicted with certainty. Hyde *et al* (1989) ranked the amino acids in order of preference for the designing of oligonucleotides in cloning malaria genes. He produced a numeric measure of the favourability of each residue that he called the match index. This was used in the design of oligonucleotides with low degeneracy at the 3' end.

Initially two new oligonucleotides were synthesised which were multiply degenerate. The sequences of these probes are shown in table 7.

Protein Sequence	Position	Sense or antisense	Length	Nucleotide sequence
GEVSPTAFR G	312-321	Sense	28mer	$^{G}/_{C}{}^{A}/_{T}$ GAA $N^{T}/_{C}{}^{G}/_{A/T}$ $^{T}/_{A/G}{}^{G}/_{C}{}^{A}/_{T}$ $CC^{A}/_{T}$ $^{A}/_{G/T}{}^{A}/_{C/T}$T $N^{A}/_{C/G}{}^{T}/_{A}$ $N^{T}/_{A}{}^{T}/_{A}$ AGA GG
VERAGRRTL	526-518	Antisense	27mer	$^{C}/_{A/T}{}^{G}/_{C/T}{}^{C}/_{T/G}$ $^{T}/_{A}{}^{T}/_{A/C}{}^{T}/_{A/C}$ $T^{C}/_{T}$T TCT $^{T}/_{A}$CC $^{C}/_{T/A}{}^{C}/_{A/G}{}^{A}/_{C}$ $T^{C}/_{T}$T $^{T}/_{A}$TC $^{T}/_{A}$AC

Table 7. Multi-redundant oligonucleotides raised against conserved regions in the mammalian sugar transporter superfamily. Note the comparatively low redundancy at the 3' end of the probes. The position column refers to the place the amino acid sequence occurs in the alignment in appendix 1.

These two oligonucleotides were used in combination with the ones synthesised previously using the same conditions. This produced one more product that was 800 nucleotides in length, using the sense QQLSGINAVFYY and the antisense VERAGRRTL multiply redundant oligonucleotides. The expected size for this product is approximately 190 nucleotides so it is much larger than expected. The antisense VERAGRRTL probe was found; but the sense oligonucleotide could not be located. A homology search revealed that this sequence was novel but does not have homology to facilitative transporters. The sequence is 58% AT and hence probably of malarial origin.

3.3.4. SECOND SCREEN - CLONING OF THE *P. FALCIPARUM* GLUCOSE TRANSPORTER

3.3.4.1. Design of Oligonucleotides

More oligonucleotides were synthesised using the highly redundant strategy. The sequences of these oligonucleotides are shown in table 8. These sequences, especially the sense ones, are based more on the non-mammalian sequences. Some are based generally on the non-mammalian (yeast and bacterial sequences) and some are biased towards the trypanosome sequences. It was decided to try these non-mammalian sequences because it was thought that the mammalian sequences had been exhausted.

Protein Sequence	Position	Sense or antisense	Length	Nucleotide sequence
GG-LFGYD (invertebrate probe)	118-110	Sense	23mer	GGN GGN NTN $N^{T}/_{A}N\ ^{C/A}/_{T}\ ^{A}/_{T}N$ GGN $TA^{T}/_{C}$ GA
GG-LFGYV (Trypanosome specific probe)	120-128	Sense	23mer	GGN GGN NTN $N^{T}/_{A}N\ ^{C/A}/_{T}\ ^{A}/_{T}N$ GGN $TA^{T}/_{C}$ GT
$G^{F}/_{L}$VPMY$^{V}/_{I}$ GE	303-313	Sense	26mer	$GG^{A}/_{T}\ ^{T}/_{C}$TN $G^{T}/_{G}\ ^{T}/_{A}$ $CC^{T}/_{A}$ ATG $TA^{T}/_{C}$ $^{G}/_{A}T^{T}/_{A}$ $GG^{A}/_{T}$ GA
PESPR$^{F}/_{Y}$L	392-386	Antisense	21mer	$NA^{A/T}/_{G}\ ^{T/A}/_{G}\ ^{T/A}/_{G}$T $N^{T}/_{C}\ ^{G}/_{T}$ NGG $N^{C}/_{G}\ ^{T}/_{A}\ ^{T}/_{C}$TC NGG
QLSGIN^{A}/G/$_{V}$ VFYY (General Probe)	479-468	Antisense	23mer	$^{A}/_{G}$C $^{A}/_{G}$TT $N^{A}/_{T}\ ^{T/C}/_{G}$ NCC $N^{C}/_{G}\ ^{A}/_{T}$ $NA^{A}/_{G}\ ^{T}/_{C}$TG $^{T}/_{C}$TG
QLSGIN^{A}/G/$_{V}$ VFYY (Trypanosome probe)	479-468	Antisense	20mer	$^{A}/_{G}$C $^{A}/_{G}$TT $N^{A}/_{T}\ ^{T/C}/_{G}$ NCC $N^{C}/_{G}\ ^{A}/_{T}$ $NA^{A}/_{G}\ ^{T}/_{C}$TG
GPGPIPWF$^{I}/_{M}$ VAE	593-582	Antisense	36mer	$^{C}/_{T}$TC $^{A/T}/_{G}$GC $^{T}/_{A}$AC NAT $^{A}/_{G}$AA CCA $^{A}/_{T}$GG $^{A}/_{T}$AT $^{A}/_{T}$GG $^{A}/_{T}$CC $^{A}/_{T}$GG $^{A}/_{T}$CC

Table 8. Sequences of oligonucleotides synthesised after alignment of facilitative glucose transporters from various species. The position column describes the position the residues appear in the alignment in appendix 1. Note the high redundancy at the 5' end and the lower redundancy at the 3' end of the oligonucleotides.

3.3.4.2. PCR conditions

Using all the sense and antisense oligonucleotides in combination gives 52 pairings that could be tried. Every oligonucleotide combination was prepared with *P. falciparum* genomic DNA. The samples were denatured at 95°C for 5 minutes before the enzyme

was added and then subjected to 40 cycles in the PCR machine. Each cycle consisted of:

95°C for 1 minute to denature,

42°C for 1 minute 30 seconds to anneal,

72°C for 3 minutes to extend.

After the first round of amplification an aliquot of each sample was electrophoresed (section 2.4.2.). None of the reactions gave bands on the gel (except for the positive control that was a fragment of the malarial TATA-binding protein). There have been reports (Criado, 1992) that reamplification in these circumstances produces the desired gene. For this reason $1/20^{th}$ of the PCR products were reamplified with the same probes used in the first round. This time, after electrophoresis, products were evident in 5 lanes of the gel produced by 5 different combinations of oligonucleotides. These bands, which were named P, Q, R, S and T were purified and subsequently cloned into pUC19 vector. Each band (except Q) contained more than one product. A summary of the products that were produced are shown in table 9 and the partial sequences of the products are found in appendix 2.

Clone	Sense primer	Antisense primer	%AT	Size in Human Glut1	Actual Size
P2	GGLFGYD (norm)	VERAGRRTL	FORWARD = 60% REVERSE = 67%	950	800
P4	GGLFGYD (norm)	VERAGRRTL	FORWARD = 56% REVERSE = 61%	950	850
Q	GEVSPT.....RG	QQLSGIN (norm)	FORWARD = 64% REVERSE = 67%	450	560
R2	GFVPMYVGE	QQLSGIN (norm)	FORWARD = 59%	450	500
R6	GFVPMYVGE	QQLSGIN (norm)	FORWARD = 48%	450	500
R7	GFVPMYVGE	QQLSGIN (norm)	71%	450	400
R10	GFVPMYVGE	QQLSGIN (norm)	FORWARD = 49% REVERSE = 51%	450	500
R14	GFVPMYVGE	QQLSGIN (norm)	FORWARD = 70% REVERSE = 61%	450	520
S1	GFVPMYVGE	QQLSIN (Trp)	70%	450	480
S3	GFVPMYVGE	QQLSIN (Trp)	FORWARD = 65% REVERSE = 65%	450	450
S6	GFVPMYVGE	QQLSIN (Trp)	FORWARD = 67% REVERSE = 67%	450	580
S8	GFVPMYVGE	QQLSIN (Trp)	FORWARD = 77% REVERSE = 56%	450	480
S11	GFVPMYVGE	QQLSIN (Trp)	FORWARD = 59% REVERSE = 59%	450	450
T3	GFVPMYVGE	VERARRRTL	FORWARD = 75%	597	520
T8	GFVPMYVGE	VERARRRTL	FORWARD = 57% REVERSE = 58%	597	582

Table 9. PCR products cloned and sequenced using *P. falciparum* genomic DNA as a target. The forward and reverse primers are shown along with the AT-richness and the actual and predicted sizes of the products.

Clone P2 does not have significant homology to any sequence in the EMBL database. The antisense oligonucleotide is located at the end of this AT-rich sequence. The sequence is probably malarial but is unlikely to be the malarial glucose transporter because there does not appear to be any homology to the facilitative sugar transporter superfamily.

Like P2 the sequence of P4 is AT-rich and is not similar to any sequence found in the EMBL database. The clone also does not show significant homology to the glucose transporter superfamily.

Clone Q is AT-rich and typically malarial. The sequence has not been reported before and the clone does not show any homology to the glucose transporters.

Clone R2 has 96% identity with human α-satellite DNA indicating that it is a contaminant from the host. The antisense oligonucleotide (QQLSGINAVFYY) is found and has homology to the repetitive DNA sequence.

Clone R6 is not AT-rich, does not have significant homology to any known sequence and is not similar to glucose transporters.

Clone R7 is extremely AT-rich and has high homology to the enolase family of proteins. The clone is most similar to mammalian and yeast sequences and is a fragment of the *P. falciparum* enolase gene. The complete sequence of the *Plasmodium falciparum* enolase gene has recently been reported (Read *et al*, 1993). In appendix 2 the enolase amino acid sequence is shown in bold.

Clone R10 is a contaminant. The sequence is identical with the rat glut2 sequence (commonly known as RATGTL). This clone has been used for probing libraries by other workers in our laboratory.

Clone R14 is extremely AT-rich but neither oligonucleotide could be observed. A homology search revealed no significant identity with any sequence in the EMBL database.

Clone S1 is extremely AT-rich, but has the same oligonucleotide at each end of the clone. This sequence was also cloned from band R that used the GFVPMYVGE sense oligonucleotide. A comparison with sequences previously deposited in the EMBL database revealed that this sequence had 70% identity to human line-1 repetitive DNA and was probably a contaminant from the host.

Both clones S3 and S6 are highly AT-rich (65%) and have no homology with any sequence in the EMBL database. After the sense oligonucleotide, however, both sequences contain an in frame, unambiguous stop codon.

Clone S8 is AT-rich and has 60% identity to a number of *Plasmodium falciparum* genes (including calmodulin and tubulin) suggesting that this DNA has a malarial origin. This sequence however has the same oligonucleotide (GFVPMYVGE) on both ends of the clone.

Clone S11 is AT-rich, contains the sense oligonucleotide and does not have any homology to previously reported sequences. The clone also does not have homology to the facilitative glucose transporter superfamily.

Clone T3 leaves the oligonucleotide and enters a polyAT region. The sequence has identity as the nucleotide level with a number of malarial proteins including 69% identity with the *P. falciparum* knob protein. It does not have homology with facilitative glucose transporters.

Clone T8 is AT-rich and has significant homology to glucose transporters. It has, however, over 90% identity with the human glucose transporter pseudogene (GLUT6). The clone changes reading frame and contains stops at the same positions as the pseudogene and so is almost certainly a variant of it.

3.4. Discussion

The size and the specific amplification of malarial actin suggests that the cDNA used during the experiments was of a high quality. Similarly the size and digestion by restriction enzymes suggests that the *P. falciparum* genomic DNA was also of a suitable standard for cloning using the polymerase chain reaction.

The amplification of actin from *P. yoelii* cDNA was the first corroboration of the RACE method (Frohman and Martin, 1989; Frohman, 1990) and the first time it was used in an organism as AT enriched as *Plasmodium* (Goodyer *et al*, 1991).

The polymerase chain reaction is a very sensitive and specific technique. There are a large number of variables that can be altered during PCR and each one will affect the products formed both quantitatively and qualitatively. Among the variables are different oligonucleotide pairings, differing magnesium concentrations, differing annealing temperatures, different number of cycles, different ramp times (the time taken to move between temperatures) and different extension protocols after the reaction has finished. Ideally all possible combinations will be tried, but with over 50 reactions using all the oligonucleotide combinations only a few specific conditions can be tested in full. This means that finding the correct conditions and hence obtaining the desired product is often a matter of chance.

Apart from PCR there are a number of other methods that have successfully been used to isolate genes. Complementation in *E. coli* is unlikely to be successful in the isolation of the malarial glucose transporter because, although a very powerful method for cytostolic enzymes, it has never been successfully used to isolate a membrane protein. It is uncertain whether the malarial glucose transporter would fold and insert correctly in a prokaryotic membrane and obtaining a Δ*glucose transport* mutant of *E. coli* would be very hard.

Cloning by functional expression in a eukaryotic host, however, may be a possible route for isolating the gene. *Xenopus laevis* oocytes appear to be an ideal host for

such studies. Basal *Xenopus* oocyte glucose transport levels are very low and glucose transporters from mammals have been successfully expressed in this host (Gould and Lienhard, 1989; Gould *et al*, 1991). These expression studies involved the injection of mRNA. Using *in vitro* transcription of a cDNA library large amounts of malarial mRNA could be obtained. It is unclear how much mRNA would have to be injected, but it is possible that the capacity of the egg may be a limiting factor. The unusual codon usage of malarial genes may also pose a problem. It has been noticed that malarial genes have a poor rate of expression in hosts such as *Xenopus laevis* oocytes unless *Plasmodium* derived tRNAs are also injected (Robson, 1991).

Unlike PCR, where oligonucleotides can be synthesised to only poorly conserved regions by incorporating large amounts of degeneracy, screening libraries with highly degenerate probes will produce a very high background of unwanted non-specific binding. For this reason, combined with the difficulty of producing an intact and stable *Plasmodium* library it was decided not to try and clone the malarial glucose transporter by library screening.

Although cloning genes by 'genomic blotting' suffers similar limitations to screening a library an attempt was made to clone the malarial glucose transporter using this method. Each oligonucleotide shown in table 4 (and a cDNA rat GLUT2 probe) was used as a probe on a Southern blot of EcoRI or HindIII digested *P. falciparum* genomic DNA. Unfortunately it was not very successful. Only one oligonucleotide (VERA$^{G}/_{R}$RRTL) annealed. The blots had to be exposed to film for approximately 3 months, indicating that the hybridisation was very weak. The long exposure times made repeating the experiment more than twice impractical. The band also had to be cloned. *Plasmodium falciparum* DNA that has been digested with EcoRI or HindIII does not show a typical banding pattern as seen with other organisms. In humans the large amount of repetitive DNA produces bands after digestion. Also with organisms with a smaller genome than malaria (e.g. most archaebacteria) banding is seen after restriction digestion of genomic DNA due to the non-uniform size distribution of the DNA fragments. The absence of banding in digested malarial DNA made the exact location of the hybridising band harder to achieve.

The cloning of genes is much easier if information about the protein sequence of the gene is known. Designing oligonucleotides to conserved regions in an alignment has its disadvantages. Firstly the amino acid sequence has to be guessed and then the codons encoding the sequence must be guessed. This 'double guessing' makes oligonucleotides synthesised in this way less reliable than ones designed against a known protein sequence. Unfortunately, obtaining a partial protein sequence for the malarial glucose transporter was not possible. Purification of membrane proteins is notoriously difficult. For standard purification techniques the protein must be extracted from the lipid. The loss of function associated with the disruption of the lipid bilayer makes monitoring the purification difficult. Another problem is that a large amount of material is needed. Huge quantities of erythrocytes are readily available and the glucose transporter protein is at an unusually high concentration (5% of total membrane protein) in erythrocytes. It is no coincidence that the only glucose transporter that has been successfully purified is GLUT1 from human erythrocytes (Baldwin *et al*, 1982; Baldwin and Lienhard, 1989).

The large number of PCR products that have been cloned and sequenced during this project demonstrates that the PCR, 'magic' minipreps and sequencing form an efficient method of screening for genes.

None of the clones that were obtained were the malarial glucose transporter, however important information can be obtained from their analysis. Looking at the contaminating glucose transporter-like clones, whose sequences are already known, it is possible to gain an insight into how close the oligonucleotides need to be to the target sequence for them to be successful using PCR. Figure 16 shows the published sequences of the rat GLUT1, human GLUT4 and human GLUT6 sequences where the oligonucleotides bound. Also shown in figure 16 is the sequence of the closest oligonucleotide in the redundant probe. This confirms that the oligonucleotides must have a high match to the target sequence especially at the 3' end (Compton, 1990). Each oligonucleotide has at least 6 bases of exact match at the 3' end. Although most have a high match throughout the sequence (100% for the sense rat GLUT2 primer),

some (e.g. human GLUT6 antisense) have only a poor match at the 5' end. This indicates that the match at the 5' end is less important than that at the 3' end. Clone R2, which has been found to be human α-satellite DNA, has the antisense oligonucleotide in the clone. This oligonucleotide has, by chance, an exact match with the first seven 3' end bases in the human repetitive sequence.

```
Sense oligonucleotide            GGACTGGTTCCAATGTACATTGGTGA
                                 ||||||||||||||||||||||||||
Rat GLUT2 sequence               GGACTGGTTCCAATGTACATTGGCGA

Antisense Oligonucleotide        ACATTGATTCCACAGAACTGCTG
                                  |||||||||||||||||||||
Rat GLUT2 rev comp               CCATTGATTCCACAGAACTGCTG

Sense oligonucleotide            CAACAATTATCTGGAATAAATGTTGTTTTTTATTAT
                                 || ||  | ||||| || |||| |||||| ||||||
Human Glut4 sequence             CAGCAGCTCTCTGGCATCAATGCTGTTTTCTATTAT

Antisense oligonucleotide        CCTTTAGTTTCAGGTAC
                                 |||  ||||| ||||||
Human Glut4 sequence rev comp    CCTCGAGTTTGAGGTAC

Sense Oligonucleotide            GGTTTTGTTCCAATGTACATTGGAGA
                                  ||||||| || |||||||||||||
Human GLUT6 SEQUENCE             AGTTTTGTGCCCATGTACATTGGAGA

Antisense Oligonucleotide        TAATGTTCTTCTTCGTGCTCTTTCAAC
                                    |    | |||| ||| ||||||||
Human Glut6 sequence rev comp    ATGTAAAGTCCTTCCTGCCCTTTCAAC
```

Figure 16. Sequences of the oligonucleotides and targets that hybridised to produce glucose transporter like sequences using PCR.

It is also interesting to consider how these clones came to contaminate the malarial nucleic acid. With rat GLUT2 and human GLUT4 it is fairly obvious that a small fraction of the cDNA clones that have been used previously in our laboratory contaminated the PCR reaction. The human GLUT4 sequence obtained is from a cDNA clone because in genomic DNA the sequence would have been interrupted by an intron (Fukumoto *et al*, 1989). The human GLUT6 sequence must have been a contamination from the very small number of white blood cells that contaminated the *P. falciparum* genomic DNA preparation. During *in vitro* culturing of the malarial parasites packed blood cells are obtained from the transfusion service. These cells are washed extensively and each time a generous top layer of blood cells is removed to remove the majority of white blood cells in the Buffy coat. During microscopic

examination of thin smears of Giemsa stained cultures it is extremely rare to find a white blood cell. Using these procedures however, very small amounts of nucleic acid are required. One molecule of human genomic DNA can produce 2^{40} ($1x10^{12}$) molecules or 1.7pmoles of GLUT6 during the first round. Although this small amount of DNA could not be detected by electrophoresis, reamplification using $1/20^{th}$ of this sample could produce in theory produce up to (($10^{12}x2^{40}$)/20) molecules or 83mmoles of target. As 83mmoles of a 400bp fragment of double stranded DNA would weigh approximately 21 kilograms, it is obvious that less DNA than the theoretical maximum will be produced, and that easily enough DNA to clone will be present even if only one copy of the human genome is present.

Being a non-functional protein mutations in this region are unlikely to be fatal, and so the mutation rate in this gene would be expected to be high. This may explain why the sequence of the published human pseudogene differs (by about 5%) from the sequence found in this project. It is also possible that our clone differs from the published one due to TAQ polymerase introduced changes in our sequence.

Human white blood cells contain about as much DNA as 500 malarial parasites (Hyde, 1990b) and a major proportion of human DNA is repetitive. It is therefore not too surprising that some of the clones appear to be human repetitive DNA.

Analysis of clone R7 revealed that this clone encodes part of the *P. falciparum* enolase gene. An alignment of this clone with the known enolase genes is shown in figure 17. Analysis of the clone revealed the binding sites for the oligonucleotides. The clone had an exact match to the last 10 3' bases of the sense $G^{F}/_{L}VPMY^{V}/_{I}GE$ oligonucleotide. The antisense primer $QLSGIN^{A/G/}{}_{V}VFYY$ was not a such good match with only 9 of the 13 3' most bases matching (table 10).

Sense oligo $G^{F}/_{L}VPMY^{V}/_{I}GE$	GGTTTTGTTCCTATGTATGTAGGTGA
	||| ||| | | | ||||||||
Enolase Sequence	GGTATTGATGCAACCAATGTAGGTGA
Antisense Oligo $QLSGIN^{A}/G/_{V}VFYY$	CAACAGTTTTGGGGAAATAATGC
	||| | |||| |||| ||||
Enolase Sequence	CAAGATGATTGGGAAAATTATGC

Table 10. Alignment of two oligonucleotides used during this project (raised against conserved regions of the glucose transporter superfamily) to the recently published *P. falciparum* sequence (Read *et al*, 1993). The sense oligonucleotide is displayed as 5'→ 3', the antisense oligonucleotide has been reversed and complemented and is displayed as 3'→5'. The 3' ends of each oligonucleotide show a significant match to the reported enolase sequence.

The *P. falciparum* enolase sequence reported here differs slightly from the previously reported sequence (Read *et al*, 1993). This is almost certainly due to inaccurate sequencing during this project. The differences are only in the middle of my clone with the two ends being identical to the sequence of Read *et al* (1993). Using the rapid screening procedure employed during this project the clones are only sequenced once and only with oligonucleotides complimentary to pUC. This means that with clones longer than 300bp the middle part of the sequence is inaccurate because it is located towards the top of the sequencing gel and is difficult to read accurately. It is interesting to notice that at either end of the clone the sequence in this project is identical to the reported sequence. My clone is almost certainly part of the same gene cloned by Read *et al*, 1993. Most of the other clones obtained during this project were highly AT-rich and probably of malarial origin.

```
HUMAN_ALPHA     HNLKNVIKEKYGKDATNVGDEGGFAPNILENKEGLELLKTAIGKAGYT--
HUMAN_BETA      HHLKGVIKAKYGKDATNVGDEGGFAPNILENNEALELLKTAIQAAGYP--
HUMAN_GAMMA     HTLKGVIKDKYGKDATNVGDEGGFAPNILENSEALELVKEAIDKAGYT--
MALARIA         ----------------VGDEGGFAPNILNANEALDLLVTAIKSAGYE--
YEAST_1         HNLKSLTKKRYGASAGNVGDEGGVAPNIQTAEEALDLIVDAIKAAGHD--
YEAST_2         HNLKSLTKKRYGASAGNVGDEGGVAPNIQTAEEALDLIVDAIKAAGHD--
DROSOPHILA      HHLKNVIKAKFGLDATAVGDEGGFAPNIQSNKEALNLISDAIAKAGYT--
XENOPUS         HNLKNVIKEKYGKDATNVGDEGGFAPNILENKEALELLKTAINKAGYP--
TOMATO          HHLKAVIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKTAIEKAGYT--
MAIZE           HNLKSIIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKAAIEKAGYT--
ARABIDOPSIS     HHLKSVIKKKYGQDATNVGDEGGFAPNIQENKEGLELLKTAIEKAGYT--
CLYDOMONAS      HALKGLIKAKYGQDACNVGDEGGFAPNIGSNDEGLNLVNEAIEKAGYT--
E_COLI          HNLKSLTKKKYGQSAGNVGDEGGVAPDIKTPKEALDLIMDAIDKAGYK--
ZYMOMONAS       HTLKKELSAK--GMNTNVGDEGGFAPSLDSASSALDFIVDSISKAGYKPG
                                ****** ** .     .*...  .*  **

HUMAN_ALPHA     DKVVIGMDVAASEFF--RSGKYDLDFKSP-DDPSRYISPDQLADLYKSFI
HUMAN_BETA      DKVVIGMDVAASEFY--RNGKYDLDFKSP-DDPARHITGEKLGELYKSFI
HUMAN_GAMMA     EKMVIGMDVAASEFY--RDGKYDLDFKSP-TDPSRYITGDQLGALYQDFV
MALARIA         GKVKIAMDVASSEFR-QVNKTYDLDFKTPNNDKSLVKTGAQLVDLYIDLV
YEAST_1         GKVKIGLDCASSEFF--KDGKYDLDFKNPNSDKSKWLTGPQLADLYHSLM
YEAST_2         GKVKIGLDCASSEFF--KDGKYDLDFKNPESDKSKWLTGVELADMYHSLM
DROSOPHILA      GKIEIGMDVAASEFY--KDGQYDLDFKNEKSDKSQWLPADKLANLYKEFI
XENOPUS         DKIVIGMDVAASEFY--RDGKYDLDFKSP-DDPSRYISPDKLAELYMSFV
TOMATO          GKVVIGMDVAASEFYG-KDKSYDLNFKEESNDGSQKISGDQLKDLYKSFV
MAIZE           GKVVIGMDVAASEFFGEKDKTYDLNFKEENNDGSNKISGDSLKDLYKSFV
ARABIDOPSIS     GKVVIGMDVAASEFYS-EDKTYDLNFKEENNNGSQKISGDALKDLYKSFV
CLYDOMONAS      GKVKIGMDVASSEFYTEDGM-YDLDFKNQPNDGSQKKTKEQMLELYNEFC
E_COLI          GKVGIAMDVASSEFY--KDGKYDLDFKNPESDPSKWLSGPQLADLYEQLI
ZYMOMONAS       EDVFIALDAASSEFYNKDQNIYDLK------GEGRKLTSAQLVDYYVELC
                  . *..* *.***       ***          .       .   *  .

HUMAN_ALPHA     KDYPVVSIEDPFDQDDWGAWQKFTASAG--IQVVGDDLTVTNPKRIAKAV
HUMAN_BETA      KNYPVVSIEDPFDQDDWATWTSFLSGVN--IQIVGDDLTVTNPKRIAQAV
HUMAN_GAMMA     RDYPVVSIEDPFDQDDWAAWSKFTANVG--IQIVGDDLTVTNPKRIERAV
MALARIA         KKYPIVSI----------------------------------------
YEAST_1         KRYPIVSIEDPFAEDDWEAWSHFFKTAG--IQIVADDLTVTNPKRIATAI
YEAST_2         KRYPIVSIEDPFAEDDWEAWSHFFKTAG--IQIVADDLTVTNPARIATAI
DROSOPHILA      KDFPIVSIEDPFDQDHWEAWSNLTGCTD--IQIVGDDLTVTNPKRIATAV
XENOPUS         KNYPVVSIEDPFDQDHWEAWTKFTAASG--IQVVGDDLTVTNPKRIAKAV
TOMATO          SEYPIVSIEDPFDQDDWETYAKLTAEIGEQVQIVGDDLLVTNPKRVAKAI
MAIZE           SEYPIESIEDPFDQDDWSTYAKLTDEIGQKVQIVGDDLLVTNPTRVAKAI
ARABIDOPSIS     AEYPIVSIEDPFDQDDWEHYAKMTTECGTEVQIVGDDLLVTNPKRVAKAI
CLYDOMONAS      KKYPVISIEDPFEQDDWEPCAKLTTE--NICQVVGDDILVTNPVRVKKAI
E_COLI          SEYPIVSIEDPFAEDDWDAWVHFFERVGDKIQIVGDDLTVTNPTRIKTAI
ZYMOMONAS       GKYPIYSIED-LAEDDFEFWKILTEKLGDKVQLVGDDLFVTNVKRLSDGI
                  .*. **
```

Figure 17. An alignment of the putative malarial enolase gene with other enolase genes.

There are many reasons why the cloning of the malarial glucose transporter may have been unsuccessful. The sensitivity of the polymerase chain reaction led to misleading contaminants. Also the infinite variation with the polymerase chain reaction is a problem. Although a lot of time was spent altering the PCR conditions, with so many variables (e.g. different oligonucleotide variations, different ramp times, varying magnesium concentrations, different numbers of cycles and annealing temperature) optimising the conditions is very hard. The project was harder due to the peculiarities of the malarial genome. The oligonucleotides were AT-rich and therefore had a lower annealing temperature. The gene may have a large intervening coding sequence as found in many other malarial proteins. This would make it less likely to be amplified during PCR, make it harder to clone and, as the sequence would be larger than predicted by homology with other glucose transporter sequences, mean it may not be selected for further analysis.

It is also possible that either *Plasmodium falciparum* does not possess a glucose transporter (see discussion in section 4.) or that the glucose transporter is not homologous to the facilitative sugar superfamily.

Assuming malaria does possess a functional glucose transporter probably the best way to clone the gene is by expression cloning in *Xenopus* oocytes. It may also be possible that the glucose transporter gene is linked to either glycolytic or pentose phosphate enzymes that have already been isolated. By using YACs and chromosome walking the transporter sequence may be obtained. This method was used to determine the *Zymomonas* glucose transporter sequence (section 1.2.3.3.5.).

4. Characterisation of Glucose Transport in Human Red Blood Cells Infected with *Plasmodium falciparum* Trophozoites

4.1. Introduction

The malarial parasite requires a large amount of glucose for growth. A mature *Plasmodium falciparum* schizont inside a human red blood consumes about 100X as much glucose per cell as a normal, mature, uninfected red blood cell (Sherman and Tanigoshi, 1974; Sherman, 1979). The increased demand for glucose by infected erythrocytes has been known for some time (Sherman and Tanigoshi, 1974), but the mechanism by which it enters the cell has remained unclear. The intraerythrocytic life style of the asexual parasite has many advantages including protection from the host's immune system. One of the disadvantages, however, is that the substrates that are required for growth are harder to obtain.

4.1.1. Malaria Induced Transport Systems

Four routes have been proposed that may help the intraerythrocytic parasite obtain the large quantity of nutrients they require and to excrete the large amount of waste products they produce. These four methods (a malaria induced anionic pore, direct diffusion across the membrane, through a parasitophorous duct, and by modification of the host's native transporter) are discussed below.

4.1.1.1. The Anionic Pore

The first parasite induced transport pathway to be described was the pore. Early experiments used the specific band 3 modifier H_2-DIDS (figure 18). In erythrocytes infected with ring stage parasites only band 3 was labelled. Using erythrocytes infected with *Plasmodium falciparum* trophozoites and schizonts it was found that H_2-DIDS entered the red blood cell and labelled haemoglobin as well as band 3 (Cabantchik *et al*, 1982; Kutner *et al*, 1982). This suggested that malaria induced another transport pathway in infected red blood cells that facilitated the uptake of the normally impermeant H_2-DIDS.

Sorbitol has also been used to study transport processes in malaria-infected red blood cells. Sorbitol enters normal red cells through the host's glucose transporter (GLUT1 - see section 1.2.3.1.1.), but enters trophozoite and schizont-infected cells much faster. The uptake into infected cells appears to be unsaturable (Ginsburg *et al*, 1983). The entry of sorbitol causes a large osmotic stress that lyses infected erythrocytes (Lambros and Vanderberg, 1979). The release of haemoglobin has been used as a measure of transport (Ginsburg *et al*, 1983). A potent inhibitor of the host's glucose transport system, cytochalasin B (figure 18), does not protect the cells from lysis indicating that sorbitol enters through a novel transport pathway (Ginsburg *et al*, 1983). Phloretin, phlorizin (figure 18) and maltose block the sorbitol induced lysis of infected cells, suggesting that these compounds specifically inhibit the induced pathway (Ginsburg *et al*, 1983).

It was later found that the rate of transport of glutamine, β-alanine, H_2-DIDS, alanine, glycine, sorbitol, myoinositol, glutamine, isoleucine, fructose and sorbitol (Kutner *et al*, 1985; Ginsburg *et al*, 1986), and the export of NBD-taurine (Cabantchik *et al*, 1982), all increase with parasite age. These compounds have widely differing charges and sizes suggesting that the induced pathway is non-specific. In the presence of cyclohexamide, a specific inhibitor of protein synthesis, the new transport pathway does not develop (Kutner *et al*, 1985). It has been suggested, based on these results, that a protein forms a pore that transports all of these compounds and the number of pores increases with parasite age (Ginsburg *et al*, 1986). It has also been suggested (Ginsburg *et al*, 1983, 1986) that the pore is an anionic transporter. It has been found that negatively charged molecules, like lactate, are transported by the pore more efficiently than neutral molecules, and that cations are poorly transported by this pathway (Ginsburg *et al*, 1986).

The induced pathway is impermeant to disaccharides, but permeant to monosaccharides (Homewood and Neame, 1974; Ginsburg *et al*, 1986) suggesting that the pore has a diameter of about 7Å (Ginsburg *et al*, 1986). Using the rate of uptake, the area of the cell, the width of the membrane and the concentration of the solute, it

has been estimated that there are 8-16 pores/cell on a trophozoite-infected cell (Ginsburg *et al*, 1986).

There has recently been provided very strong evidence that a pore also exists in the parasitophorous vacuolar membrane (PVM) of *Plasmodium falciparum*-infected red blood cells (Desai *et al*, 1993). Using patch-clamp studies on malaria cells, which have been removed from the erythrocyte, the authors found channels in the PVM. These channels were large enough to allow the entry of fairly large molecules ($lysine^+$ and $Tris^+$) into the cell. The authors suggested that these pores had a diameter of about 11Å and were open about 98% of the time. These pores had some similarities to those reported in the erythrocyte membrane, i.e. a preference for anions over cations, and the capacity to transport amino acids and monosaccharides. The authors did not investigate the pores found in the erythrocyte membrane to find out if they had identical characteristics.

4.1.1.1.1. Inhibitors of the Malaria Induced Pore

The anti-malarial action of anion transport inhibitors (figure 18) has been investigated (Cabantchik *et al*, 1982). Two classes of inhibitors were tested: i) hydrophilic compounds that are impermeant to uninfected cells (e.g. DIDS, DNDS, and phlorizin) and ii) relatively lipophilic compounds that can permeate through membranes (e.g. niflumic acid and furosemide). Although relatively poor antimalarials, niflumic acid and furosemide, which are considered inhibitors of chloride channels, have more recently been shown to specifically inhibit this malaria induced pathway (Kirk *et al*, 1992b). Phlorizin was worst at inhibiting anion transport but was the most effective antimalarial (Cabantchik *et al*, 1982).

The effect of phlorizin (figure 18) on malaria-infected red blood cells has been investigated in detail (Cabantchik *et al*, 1982; Tripatara and Yuthathong, 1986; Kutner *et al*, 1987). It has been reported that phlorizin inhibits sorbitol uptake into *P. falciparum* trophozoite-infected red blood cells with a Ki of 17μM and arrests parasite growth with an IC_{50} of 16μM (Kutner *et al*, 1987). Uninfected red blood cells are impermeable to phlorizin, whereas the inhibitor entered infected cells through the

malaria induced pore (Kutner *et al*, 1987). The uptake of phlorizin was measured directly using radiolabelled inhibitor. Phlorizin, at 100μM, reached equilibrium after about 1 hour in trophozoite-infected cells, but was hardly transported at all in ring-infected cells. Phlorizin blocked the uptake of sorbitol, whether present during the experiment or after pre-incubation and extensive washing of the cells in phlorizin free buffer. This is because the phlorizin that entered the cell was effective at inhibiting sorbitol uptake into infected cells. Phloretin inhibited to a similar extent when present during the sorbitol lysis but did not have any effect with the pre-incubation experiment (Kutner *et al*, 1987). This indicates that the probable site of action of phlorizin is on the cytoplasmic side of the membrane. Phlorizin did not inhibit uptake into ring stage parasites because phlorizin is impermeant to erythrocytes infected with ring stage parasites. An estimate of 6000 phlorizin binding sites per cell was obtained (Kutner *et al*, 1987).

Because phlorizin is a potent inhibitor of the Na^+/glucose symport system in mammalian small intestine and kidney (Koepsell and Madrala, 1987), it cannot be used as an antimalarial drug *in vivo* (Silfen *et al*, 1988). Various analogues of phlorizin have been tested to determine whether a drug, which inhibits malarial transport but which is ineffective as a Na^+/glucose symport inhibitor, could be found. The compound 3-isothiocyano-phlorizin (figure 18) was found to bind irreversibly (unlike phlorizin that was reversible) to the malarial pore with a high affinity and was ineffective against the mammalian Na^+/glucose symporter (Silfen *et al*, 1988). The transport of small molecules into malaria-infected red blood cells was totally blocked by incubating with the phlorizin analogue at 50μM for 5 minutes (Silfen *et al*, 1988). As phlorizin is selectively permeant to infected cells, an experiment has been performed to determine whether phlorizin interacted with the parasite as well as being a blocker of the malaria induced red blood cell transporter (Silfen *et al*, 1988). Using Sendai virus treated cells (that can freely transport anything up to a M.W. of 10 000 across the red blood cell membrane) it appeared that phlorizin inhibits the *in vitro* growth of *P. falciparum* in a concentration dependent fashion (Silfen *et al*, 1988). This showed

that phlorizin not only inhibits the transport into the red blood cells but also has some effect directly on the parasite.

At pH9.0, H_2-DIDS blocks uptake through the pore possibly by chemically modifying the pore protein (Breuer *et al*, 1987). Using this method, three proteins of parasite origin have been identified in the red blood cell membrane that may be constituents of the pore (Breuer *et al*, 1987).

It has been noticed that phloretin (figure 18) partially blocked the labelling of haemoglobin by H_2-DIDS in trophozoite-infected cells suggesting that phloretin may be an inhibitor of the induced pathway (Kutner *et al*, 1983). As well as being a powerful inhibitor of sugar transport, there have been reports of phloretin acting as a non-specific inhibitor of transport by disrupting the plasma membrane lipid structure (Melnik *et al*, 1977).

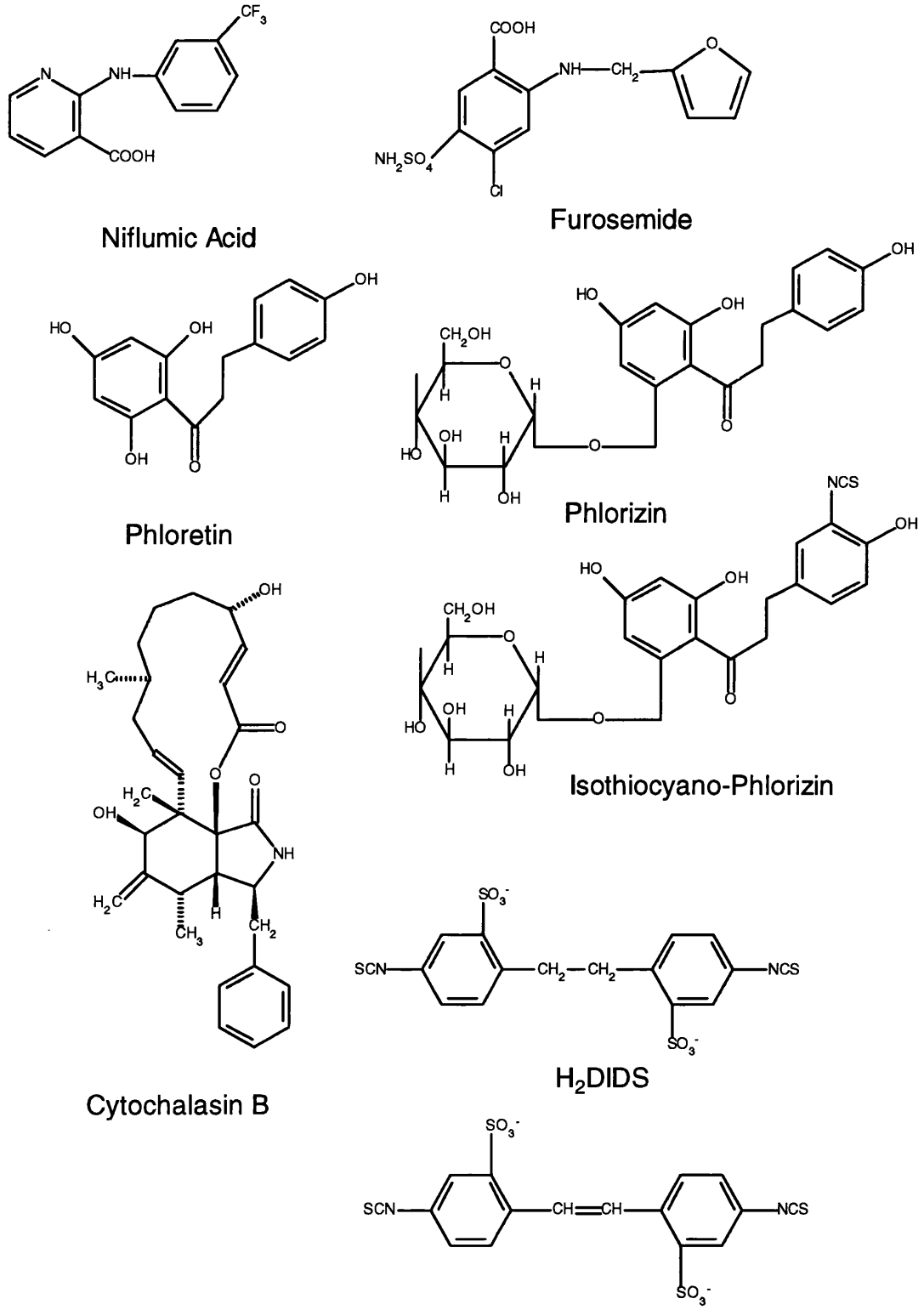

Figure 18. The inhibitors used in malaria transport experiments.

4.1.1.2. Non-Stokesian Diffusion Across the Membrane

Although the pore model explains many of the transport properties observed in malaria-infected cells, it is now generally accepted that it cannot explain the permeability of many neutral molecules (Cabantchik, 1989a, 1990). The transport of glycerol, thiourea and cysteine, although markedly increased during parasite development, is not inhibited by phlorizin (Cabantchik, 1989a). It is thought that non-electrolytes cross an artificial lipid bilayer by a process termed non-Stokesian diffusion (Lieb and Stein, 1986). This model suggests that the lipid chains form a soft polymer-like network, and that the diffusing molecules move across the membrane by occupying temporary holes formed in this network. Non-Stokesian diffusion predicts that small molecules move faster than large molecules (as opposed to Stokesian diffusion where diffusion rate is independent of size). In biological membranes the lateral movement of the hydrocarbon chains is constrained to a large extent by cholesterol and proteins (some of which are bound to the cytoskeleton) resulting in limited transport of large volume non-electrolytes (Lieb and Stein, 1986). It has been proposed that during malaria development these constraints in the erythrocyte membrane are relaxed allowing these molecules to diffuse across the membrane (Ginsburg and Stein, 1987).

4.1.1.3. The Duct

Large molecules appear to have direct access to the aqueous space surrounding the parasite (Pouvelle *et al*, 1991). It has been shown that fluorescently labelled dextrans and antibodies label the parasite membrane under conditions that do not allow endocytosis (i.e. without ATP and at 4°C). These molecules are too large to enter the erythrocyte through the pore. It has been postulated that these molecules have access to the parasite membrane through a 'parasitophorous duct' (Pouvelle *et al*, 1991). This malaria induced structure is continuous with the host plasma membrane and the parasitophorous plasma membrane. It is also believed that small molecules have direct access to the parasite membrane through the duct.

Since the proposal of the parasitophorous duct, its presence has been a matter of some debate (Sherman and Zidovetzki, 1992). An early criticism was that the fluorescently labelled dextrans could break-down to dextran and the fluorescent tag, and only the fluorescent tag gain access to the parasite (through the pore). This was conclusively refuted (Schneider *et al*, 1992; Taraschi *et al*, 1992). Experiments indicated that the free dye had not dissociated and work with free ruthenium red dye showed that this electron dense dye only entered the parasite under conditions that allow endocytosis. Under no circumstances was the cytosol of the erythrocyte labelled (Schneider *et al*, 1992) suggesting that the parasite had direct access to the dye and that it could not enter the erythrocyte through the pore. At 4°C and in the absence of ATP, it has been shown that ruthenium red labels the host and parasite plasma membranes, (showing that the parasite has direct access to the dye) but it does not enter the parasite (Schneider *et al*, 1992; Taraschi and Pouvelle, 1993). If this labelling is genuine, even if the dye had dissociated from the dextran the result would have been the same, as the dye labels the parasite membrane by the same mechanism as the labelled dextran (Schneider *et al*, 1992). Recently it has been reported that ruthenium red is excluded from malaria-infected human erythrocytes at 4°C (Ferguson and Elford, 1993) but these experiments may have been incorrectly performed (Taraschi and Pouvelle, 1993).

Another criticism of the duct model is that the confocal fluorescent electron micrographs that claimed to visualise the duct (Pouvelle *et al*, 1991) are in fact pictures of the tubovesicular system that had been known for many years (Elford *et al*, 1985; Elford and Ferguson, 1993). However, even if the micrographs are not of the duct this does not preclude its existence. There are many reasons why the duct may be hard to visualise. Firstly the electron microscopy procedure could easily destroy a fragile structure like the parasitophorous duct. Secondly, it has been noted that the parasite does not occupy the centre of the red blood cell; rather the parasite is found close to an edge of the erythrocyte. This means that the duct may be very short or even that the host's plasma membrane and the parasitophorous vacuolar membrane may come into contact.

Evidence for a duct is quite strong. It was suggested in 1985 that a 'metabolic window' connecting the parasitophorous vacuolar membrane and the host's plasma membrane was the most reasonable explanation for results obtained when studying L-glutamine uptake (Elford *et al*, 1985). The authors found that L-glutamine was transported into infected cells reaching equilibrium after about an hour in trophozoite-infected cultures, but that the parasite used the L-glutamine immediately (i.e. the rate of protein synthesis was constant throughout the experiment). The authors suggested that the best explanation for this was that 'the parasite was effectively in direct communication with the extracellular medium, which was at almost constant specific activity during the influx period' (Elford *et al*, 1985).

Further evidence for the parasitophorous duct has been provided by work using iron chelators. Desferrioxamine (DFO) and N-methylanthranilic-desferrioxamine (MA-DFO) are both effective antimalarials. They have IC_{50} values of $3\pm1\mu M$ when placed in culture with *Plasmodium falciparum*-infected red blood cells over a 24 hour period (Loyevsky *et al*, 1993; Lytton *et al*, 1993). These compounds are too large to enter the infected cells through the pore, and so for a while their method of entry into the parasite was not known. In an elegant experiment, red blood cells were lysed in the presence of millimolar concentrations of DFO or MA-DFO, and resealed (Loyevsky *et al*, 1993). The resealed red blood cells encapsulated the iron chelator at concentrations far in excess of the amount required for antiplasmodial activity. Despite this, when these resealed erythrocytes were added to a malaria culture it was found that *P. falciparum* merozoites invaded these cells and developed into mature parasites normally. This experiment conclusively showed that the iron-chelators bypass the red blood cell and have direct access to the parasite (Loyevsky *et al*, 1993) presumably via the parasitophorous duct.

4.1.1.4. Modification of the Host's Transporters

Along with the pore, the non-Stokesian route, and the duct, a fourth method that increases substrate flux across the plasma membrane of a malaria-infected erythrocyte has been proposed. Many of the compounds that have increased rates of transport in malaria-infected cells are transported by uninfected cells using its constitutive

transporters (e.g. glucose, adenosine, lactate, tryptophan and choline). It has been suggested that malaria modifies these constitutive transporters thereby increasing their capacity (Ginsburg and Krugliak, 1983; Ancelin *et al*, 1985; Tripatara and Yuthathong, 1986; Gero *et al*, 1988; Cabantchik, 1990; Kanaani and Ginsburg, 1991). Figure 19 shows all the routes that are currently believed to transport small molecules in an erythrocyte infected with a *Plasmodium falciparum* trophozoite or schizont.

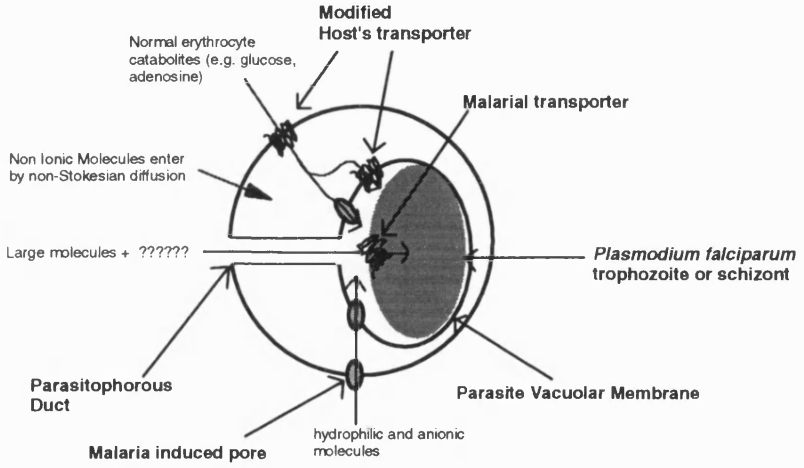

Figure 19. Small molecules can enter the parasite-infected red blood cell by one of four methods: i) through the host's constitutive transporter (there have been suggestions that the properties of the constitutive transporters are altered by the parasite), ii) through the malaria induced pore, iii) through the parasitophorous duct and a transporter in the malarial parasite, and iv) by non-Stokesian diffusion through the lipid bilayer.

4.1.2. Transport of Small Molecules into Malaria-Infected Erythrocytes

4.1.2.1. Nucleoside Transport

The transport of nucleosides and their analogues (figure 20) into malaria-infected and uninfected red blood cells has been extensively investigated (Gati *et al*, 1987; Gero *et al*, 1988). In uninfected red blood cells transport of nucleosides (e.g. adenosine) and their analogues, such as tubercidin, occurs through a facilitative nucleoside transporter (Gero *et al*, 1988). This transporter is inhibited by nitrobenzylthioinosine (NBMPR),

dipyridamole and dilazep (figure 20). These inhibitors are known to bind reversibly to the nucleoside binding site of the transporter (Gero *et al*, 1988; Cabantchik, 1989b). *Plasmodium falciparum* is incapable of *de novo* purine biosynthesis being totally reliant on its host for their supply (Gero and O'Sullivan, 1990). Human erythrocytes infected with *P. falciparum* show a marked decrease in sensitivity to NBMPR, nitrobenzylthioguanosine (NBTGR), dipyridamole and dilazep (Gati *et al*, 1987; Gero *et al*, 1988). After thirty minutes incubation with 50μM adenosine or 50μM tubercidin all the available nucleoside is utilised (converted into higher molecular weight molecules that cannot leave on the nucleoside transporter) in infected and uninfected cells when no inhibitors are present (Gero *et al*, 1988). In the presence of an inhibitor, the uptake into uninfected red blood cells is reduced to about 80% of the total amount. *Plasmodium falciparum*-infected red blood cells use all the radiolabel available to them (Gero *et al*, 1988). It has been suggested that the inhibitors are ineffective in malaria-infected cells due to a decreased sensitivity of the host's transporter and the presence of an inhibitor insensitive transport system (Gero *et al*, 1988).

Many of the experiments on nucleoside transport use adenosine itself as a substrate (Gero, 1989). In these experiments, uptake rather than transport is measured. Uptake is the amount of compound that has been transported. Uptake will be higher if the compound is metabolised and retained within the cell. In contrast, transport rate is the rate at which the molecule crosses the membrane. Transport is independent of metabolism but can only be effectively measured using an analogue that remains unaltered (i.e. is not metabolised). Labelled adenosine is transported into the parasite and the erythrocyte at comparable rates. The cells reach equilibrium after 3 seconds, but then the adenosine begins to accumulate as it is converted into metabolites that cannot leave the cell. NBMPR completely blocks the uptake into normal cells and cuts the accumulation by over half for the *P. falciparum*-infected cells. Similar results are obtained using tubercidin instead of adenosine (Gero *et al*, 1988).

Tubercidin transport into trophozoite-infected cells is rate limiting for its subsequent metabolism. The majority is not inhibited by NBMPR in infected cells, whereas in uninfected cells the uptake is linear and almost totally abolished by the nucleoside

transport inhibitor, indicating that the rate limiting step has changed in malaria-infected cells (Gero *et al*, 1988). Tubercidin is toxic to cells when it is incorporated into nucleic acids. In mice its toxic effect can be resisted by administering a nucleoside inhibitor at the same time (Gati *et al*, 1987; Gero *et al*, 1989; Gero and Upston, 1992). This is because the nucleoside protects the host cells from the cytotoxic effect of tubercidin but does not protect malaria infected cells. In mice with malaria, tubercidin and NBMPR effectively cleared malaria from the system without being cytotoxic to the host (Gero *et al*, 1989). This has potential as a therapeutic approach to treat malaria infected patients.

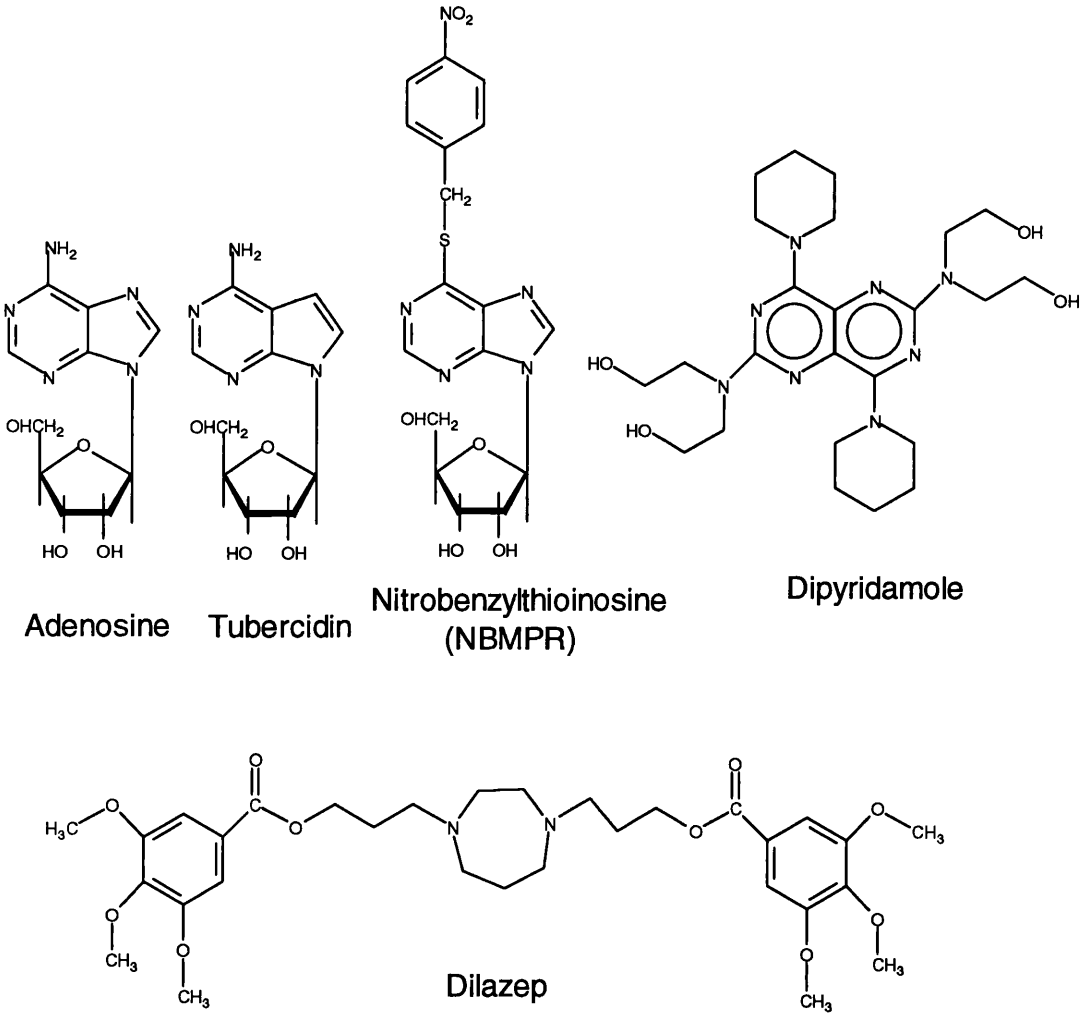

Figure 20. Nucleosides, nucleoside analogues and inhibitors of mammalian nucleoside transport.

4.1.2.2. Glucose Transport

An increased rate of L-glucose uptake has been found in *P. berghei*-infected mouse erythrocytes (Homewood and Neame, 1974; Tripatara and Yuthathong, 1986). It has been reported that this uptake is insensitive to the classic sugar transport inhibitors cytochalasin B and phloretin.

Using *Plasmodium lophurae*-infected duck erythrocytes, an increased rate of uptake of D-glucose was observed compared to uninfected cells (Sherman and Tanigoshi, 1974). The rate of transport of 3-O-methyl-D-glucose was also found to be increased in these malaria-infected duck cells. The transport into these cells was, however, very slow. Uninfected duck erythrocytes took 8 hours to equilibrate the sugar analogue at 2.5mM (Sherman and Tanigoshi, 1974). This is in contrast to human erythrocytes that reach equilibrium within seconds.

Some experiments on D-glucose uptake into malaria-infected erythrocytes suggested that it was transported by a two-step process (Izumo *et al*, 1989; Tanabe, 1990). It was suggested that glucose diffuses into the red blood cells through the malaria induced pore and then is actively transported into the parasite by a glucose/H^+ symporter in the malarial membrane (Izumo *et al*, 1989; Tanabe, 1990). These conclusions were based on experiments using 2-deoxy-D-glucose (Izumo *et al*, 1989) or D-glucose itself (Sherman and Tanigoshi, 1974; Tripatara and Yuthathong, 1986). It was found that the levels of 2-deoxy-D-glucose in the parasite were at a concentration 16X higher than in the surrounding mouse erythrocyte and that the metabolic poisons DCCD, and CCCP, inhibited this accumulation (Izumo *et al*, 1989). These inhibitors prevent the production of ATP: DCCD is an inhibitor of the H^+/ATPase, and CCCP is a proton ionophore. However, it could be argued that these poisons exert their effect by preventing the phosphorylation of 2-deoxy-D-glucose and that a H^+ dependent mechanism is not required.

4.1.2.3. Lactate Transport

The efflux of lactate has also been investigated (Kanaani and Ginsburg, 1991). The infected cell has to transport lactate much faster than the host, and it has been shown

that the host's lactate transporter does not have sufficient capacity to export all the lactate produced by the parasite. It has been suggested that lactate leaves the cell through the malaria induced pore. Good evidence for this is that lactate transport in malaria infected erythrocytes does not saturate up to 50mM (Kanaani and Ginsburg, 1991). Lactate is smaller than glucose and negatively charged; it is for these reasons that it is believed that lactate is transported efficiently by the pore (Cramner and Halstrap, 1993).

4.1.2.4. Aim of this Study

The aim of this study is to characterise the transport of glucose into human erythrocytes infected with *Plasmodium falciparum* trophozoites and schizonts. In this study we make extensive use of the glucose analogue 6-deoxy-D-glucose. The advantage with this analogue is that it cannot be metabolised. Unlike 2-deoxy-D-glucose this analogue is not a substrate for hexokinase. For this reason, it is believed that 6-deoxy-D-glucose only measures transport and not uptake. This compound has previously been used successfully to analyse glucose transport into trypanosomes (Eisenthal *et al*, 1989) and *Chlorella* (Komor and Tanner, 1974).

4.2. Methods

4.2.1. Manipulation of *Plasmodium falciparum*

Plasmodium falciparum was cultured in 50ml cultures as described in section 2.2. and purified from contaminating red blood cells to at least 85% parasitaemia using gelatine (section 2.2.4.). The cells were washed in complete medium (section 2.1.3.) and, unless stated otherwise, stored in this buffer until ready to use. The cells were maintained at 37°C throughout, unless otherwise stated. Uninfected cells, which were used as controls, were of the same age as infected cells and treated identically to infected cells before use. For some experiments mature *Plasmodium falciparum* parasites were released from the surrounding red blood cell by N_2-cavitation (section 2.2.7.) and purified from unreleased parasites and uninfected erythrocytes using a Percoll™ gradient (section 2.2.8.). Released parasites were stored in artificial intracellular medium (AIM).

4.2.2. Preparation of Metabolic Inhibitors

Inhibitors of transport were dissolved in ethanol before use. Table 11 shows the concentrations of the inhibitors used, unless otherwise stated.

Inhibitor	Concentration of stock - in ethanol	Working concentration
Cytochalasin B	10mM (200X)	50μM
Phloretin	320mM (1000X)	320μM
Phlorizin	200mM (1000X)	200μM
Niflumic Acid	100mM (200X)	500μM

Table 11. Concentrations of inhibitors. The inhibitors were dissolved in ethanol to the stated concentration and then diluted to the working concentration in buffer. After stirring to dissolve, the temperature of the inhibitor solution was adjusted.

4.2.3. Source of Radiolabelled Sugar Analogues

Although unlabelled 6-deoxy-D-glucose is commercially available (Sigma), a radiolabelled analogue had to be synthesised 'in house'. The synthesis of [C6-^{3}H]-6-deoxy-D-glucose has previously been described (Game, 1988). Briefly, 6-iodo-6-deoxy-α-methyl-D-glucoside was supplied to Amersham, where a halogen/tritium exchange reaction was performed to yield [C6-^{3}H]-6-deoxy-α-methyl-D-glucoside.

The methyl group was removed and the product purified from its precursors using paper chromatography (Game, 1988).

[1-^{14}C]-L-glucose and 1-^{14}C]-2-deoxy-D-glucose were purchased from Amersham.

4.2.4. PROTEIN ESTIMATION

The sample, in a final volume of 5µl, was mixed with 5µl of 0.1M NaOH, and placed in a microtitre plate. The assay was performed in duplicate and a range of BSA concentrations also used to produce a standard curve. The BCA reagent consisted of 1% BCA-Na_2, 2% Na_2CO_3, 0.16% Na_2Tartrate, 0.4% NaOH, 0.95% $NaHCO_3$ and 4% $CuSO_4$, pH 11.25. To each sample 200µl of BCA reagent was added. After incubation at 37°C for 30 minutes the absorbance of each well of the microtitre plate was determined and the standard BSA concentrations used to determine the protein concentration in the sample.

4.2.5. ESTIMATION OF PACKED CELL VOLUME

Cell volume was estimated by taking a haematocrit. A sample of the cell suspension was drawn up through a capillary tube and then the end of the tube plugged with Plasticine. The capillary tube was centrifuged at 500g for 5 minutes, the height of cells measured, and used to calculate the percentage of cells in the suspension and the volume of packed cells.

4.2.6. CELL COUNTING USING A HAEMOCYTOMETER

An aliquot of the cell suspension was diluted appropriately and the number of cells determined using a haemocytometer. The cells were counted using a Zeiss microscope at X40 magnification. The haemocytometer consisted of a grid of 25 squares. The cells in all the squares were counted and this number divided by 25 to give the number of cells per square. This number was multiplied by the dilution factor and divided by the volume of liquid under one square of the haemocytometer (10^{-4} ml) to give the number of cells per ml in the undiluted cell suspension.

4.2.7. Zero-Trans Influx Into Red Blood Cells Infected by Late Stage Parasites

All reactions were performed in 3ml reaction tubes (NUNC) and all centrifugations were performed in an Ole Dich cooling centrifuge set to 4°C. The cells were maintained at 37°C in RPMI before the transport assay. The cells were washed free of glucose by washing 3X with 3ml 37°C PBS (± inhibitor depending on the assay), by centrifugation followed by aspiration with a glass Pasteur pipette attached to a vacuum. The cells were then resuspended in 50µl of prewarmed PBS (± inhibitor). The cells were added to 50µl of the labelled sugar mix (1µCi of label was used per tube for ^{3}H and 0.2µCi for ^{14}C-labelled sugar analogues), which may also contain inhibitor, and left at 37°C to transport the analogue. After the required time the transport was stopped by the addition of 3ml of stop buffer (ice-cold PBS containing 200µM phlorizin and 320µM phloretin). All time-points were performed in duplicate. Non-specific uptake, or background values, were obtained by adding this stopping buffer to the cells before adding labelled substrate. The cells were concentrated by centrifugation at 10 000g for 40 seconds, the supernatant removed, and the cells washed twice with 3ml of stop buffer. The cell pellet was then lysed with 500µl of 0.5% Triton X-100, rotary mixed and the proteins precipitated with 500µl of 5% trichloroacetic acid. After incubation at 4°C for 30 minutes the samples were centrifuged for 2 minutes at 10 000g to pellet the proteins. The supernatant was counted with 5ml of scintillation fluid (optiphase 'safe') using a β-counter (LKB).

4.2.8. Zero-Trans Influx of 6-Deoxy-D-Glucose into Parasites Free of the Host Membrane

The cells were prepared as described (sections 2.2.7. and 2.2.8.) and washed free of glucose by washing three times with 37°C glucose-free AIM (120mM KCl, 20mM NaCl, 10mM Pipes and 1mM $MgCl_2$ pH 6.7) (Mikkelsen *et al*, 1986). The cells were resuspended to a final volume of 20µl in the same buffer. A 20µl sugar stock consisting of 10mM 6-deoxy-D-glucose and 1µCi [^{3}H]-6-deoxy-D-glucose in 37°C, glucose-free, AIM was prepared. At zero time the cells and sugar stock were mixed together. For inhibitor studies the cells were resuspended in AIM containing the inhibitor and mixed with a sugar solution that also contained the inhibitor at the same

concentration. The cells were incubated at 37°C in the sugar solution (± inhibitor) for the required time before the assay was stopped using 3ml of stop solution. The stop solution consisted of ice-cold, glucose free AIM containing 60μM phloretin and 200μ M phlorizin. After washing the cells twice with 3ml of stop solution, the cells were lysed with 500μl 0.5% Triton X-100 and 500μl of 5% trichloroacetic acid. After incubation at 4°C for 30 minutes, the samples were centrifuged for 2 minutes at 10 000g and the 1ml of supernatant removed into a scintillation vial containing 5ml of scintillant. The samples were counted in a LKB β-counter.

4.2.9. Zero-Trans Efflux

For efflux measurements, the influx protocol (section 4.2.8.) was modified to include a pre-incubation step. The cells were washed free of glucose using 3X 3ml PBS and then pre-incubated with 100μl radiolabelled sugar mix for 5 minutes. The cells were then diluted to 1ml with PBS and the efflux terminated with stop buffer (ice-cold PBS containing 320μM phloretin and 200μM phlorizin) at the desired time. Efflux studies using L-glucose were performed at 37°C; studies using 6-deoxy-D-glucose were performed at room temperature due to the rapid efflux of 6-deoxy-D-glucose at 37°C.

4.2.10. Reproduction of Results

Unless otherwise stated, the results presented in the next section are representative of many experiment repetitions.

4.2.11. Modelling using the Computer

The kinetics of the system was modelled iteraratively using the FigP v5.0 computer package on an IBM compatible personal computer.

4.3. Results

4.3.1. The Effect of Pre-Incubation Temperature on the Uptake of 6-Deoxy-D-Glucose

Before the transport of glucose and glucose analogues could be successfully studied, a reliable transport assay was required. The effect of temperature on 6-deoxy-D-glucose uptake was investigated. Many previous studies on transport in malaria-infected erythrocytes have cooled the cells to 4°C (Tripatara and Yuthathong, 1986; Izumo *et al*, 1989; Choi and Mikkelsen, 1990) before use. Adding cold medium to cultures of *P. falciparum* cultures severely inhibits their growth so an experiment was performed to study the precise effect of temperature on *Plasmodium falciparum*. The experiment showed that exposure to 4°C for even 1 minute was detrimental and after 1 hour the cells were non-viable (figure 21).

A zero-trans uptake experiment was performed on malaria-infected red blood cells that had been incubated at either 4°C, 20°C or 37°C for 3 hours before use (figure 22). The experiment showed that the cells could be stored at 20°C or 37°C before use but not at 4°C. If the cells were stored at 4°C, they could not transport glucose at the same rate as cells kept at higher temperatures (figure 22). These results indicate that red blood cells infected with mature *Plasmodium falciparum* parasites should not be placed on ice during or prior to transport assays.

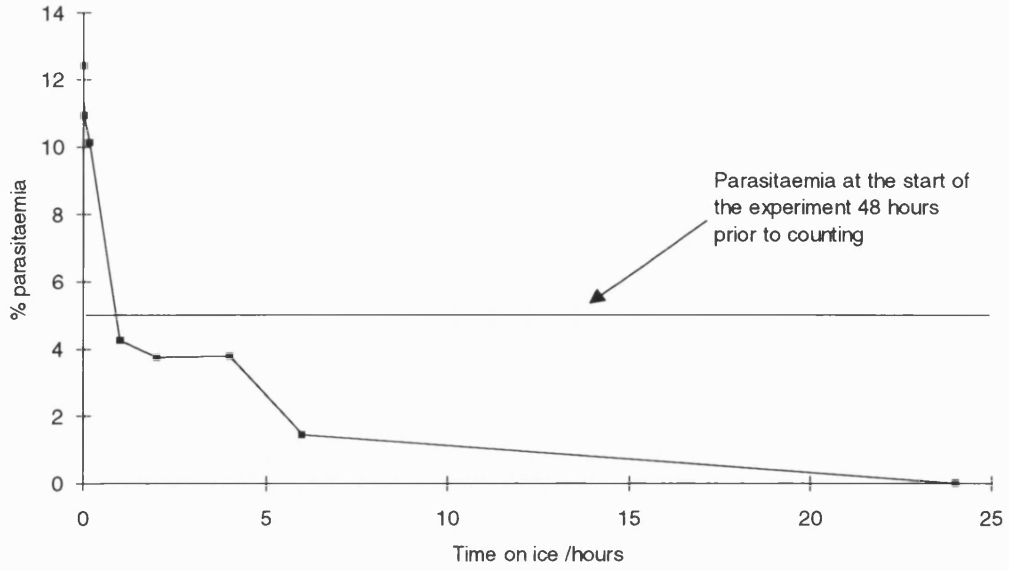

Figure 21. The effect of incubation at 4°C on the viability of *P. falciparum*. A 50ml culture was split into 10x5ml aliquots and left on ice for between 10 seconds and 24 hours before returning to the incubator. After 48 hours Giemsa stained slides of the cultures were made and the percentage parasitaemia calculated. One minute at 4°C inhibits the growth of *Plasmodium falciparum*. After 1 hour the parasitaemia dropped below the level it was at the start of the experiment and the cells could not recover after further incubation.

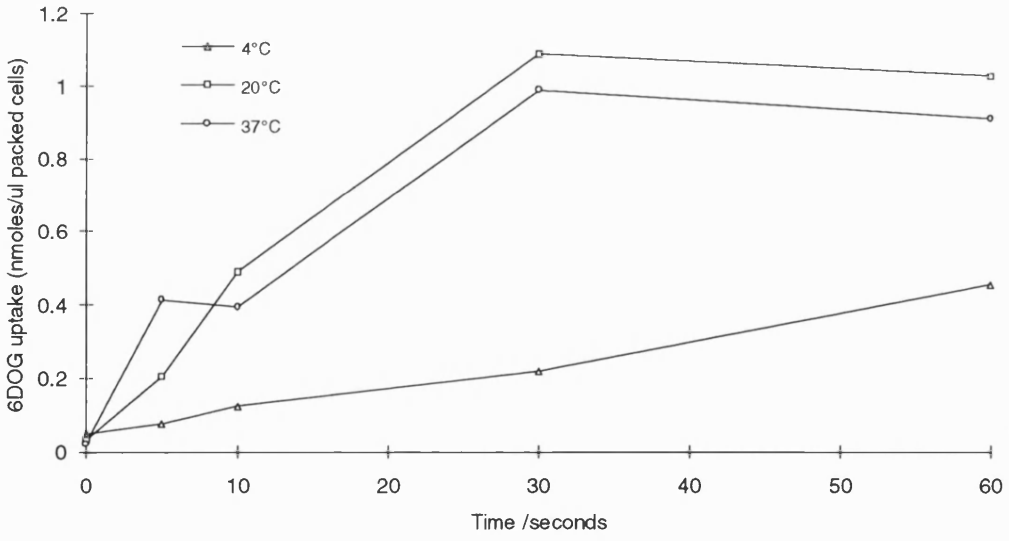

Figure 22. The effect of pre-incubation temperature on the subsequent rate of 20mM 6-deoxy-D-glucose uptake. Trophozoite and schizont-infected human red blood cells were purified using gelatine and then stored in RPMI-1640 medium for 3 hours. The cells were stored at either 4°C, 20°C or 37°C and then returned to 37°C before the uptake experiment was performed. Cells stored at 4°C transport 6-deoxy-D-glucose slower than cells stored at 20°C or 37°C.

4.3.2. The Effect of Glucose Deprivation and Storage on *Plasmodium falciparum*-Infected Erythrocytes

The phenomenon of counterflow means that transport assays must be performed in cells that are free of glucose. Practical considerations also mean that after purification, infected cells must be stored while the transport assay is prepared, and cells used towards the end of the experiment may have been stored for 3 hours. The effect of 6 hours (considered the longest time between purification and completion of the experiment) of glucose starvation and storage was investigated using gelatine purified *P. falciparum*-infected erythrocytes.

Malaria-infected cells stored in glucose-free PBS for 6 hours, at 37°C, showed substantial red blood cell lysis, and the released parasites were clumped at the bottom of the tube. Many trophozoites appeared to have been released prematurely from the erythrocytes and some parasites had begun to shrivel up. This indicated that the cells should be placed in PBS for the minimum time required to perform the transport assay (usually about 20 minutes). After 30 minutes in glucose-free PBS the cells appeared normal and no lysis was observed.

Very little lysis of the erythrocytes was apparent with cells stored in RPMI-1640 (probably due to the release of merozoites) for 6 hours. A Giemsa stained smear revealed that many of the cells had developed to the late schizont stage and stopped growing. These cells looked healthy and were viable if given fresh blood and medium. After 6 hours of storage in RPMI-1640 at 37°C the cells showed normal uptake of 6-deoxy-D-glucose (figure 23).

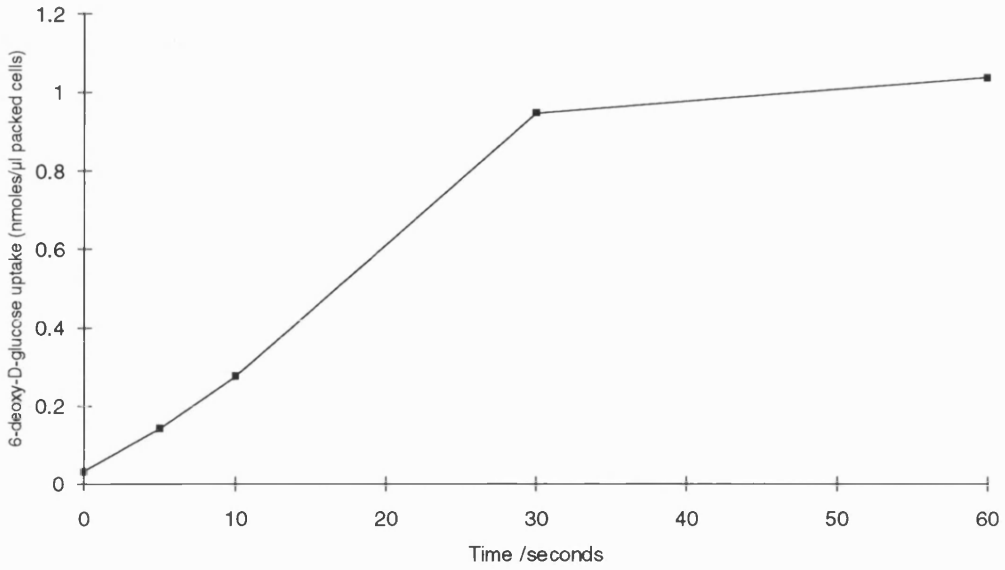

Figure 23. Zero-trans uptake of 20mM 6-deoxy-D-glucose in trophozoite/schizont-infected red blood cells. The infected cells were purified by gelatine flotation and stored in complete RPMI-1640 at 37°C for 6 hours. Examination of the cells revealed a little lysis of the erythrocytes and many cells in the late schizont stage. The graph indicates that cells have normal 6-deoxy-D-glucose uptake characteristics.

4.3.3. The Uptake of 2-deoxy-D-Glucose into Trophozoite-Infected Red Blood Cells

Following the work of Izumo *et al* (1989) 2-deoxy-D-glucose was used as a substrate. These researchers performed their experiments at 4°C and on the mouse malaria *Plasmodium yoelii*. When the zero-trans uptake of 2-deoxy-D-glucose was investigated using *P. falciparum* different results were obtained depending on the pre-incubation and assay temperature. If the experiment was performed at 4°C, which prevents the depletion of ATP but reduces native transport, results similar to those of Izumo *et al* (1989) were obtained (figure 24). The faster metabolism of malaria-infected erythrocytes compared to uninfected cells causes a faster accumulation in infected cells (figure 24). If the cells are maintained at 37°C, ATP depletes quickly in infected cells causing 2-deoxy-D-glucose to equilibrate in trophozoite/schizont-infected cells (figure 25). Due to their slow metabolism, ATP does not deplete in uninfected cells and hence 2-deoxy-D-glucose continues to accumulate (figure 25).

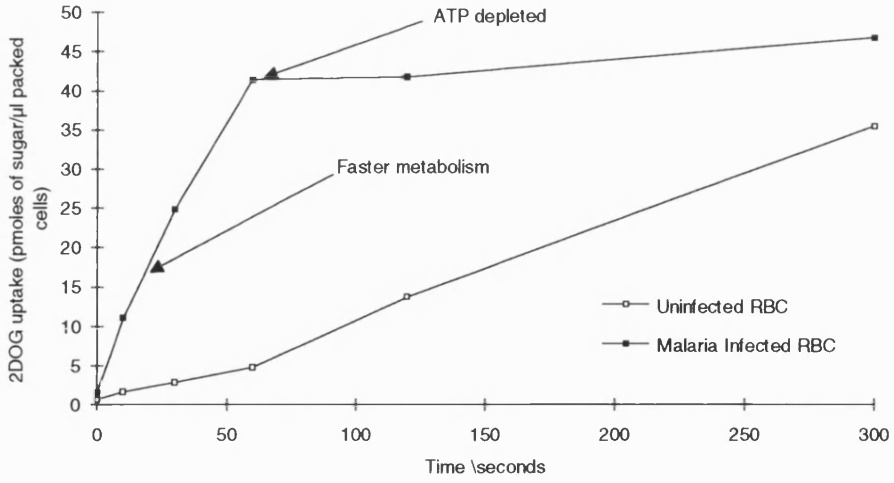

Figure 24. Zero-trans uptake of 100µM 2-deoxy-D-glucose by trophozoite/schizont-infected and uninfected red blood cells. The malaria-infected cells were purified from contaminating uninfected cells using gelatine and stored in RPMI-1640 buffer at 4°C before the transport assay was performed at 4°C. Uninfected cells were also stored at 4° C in RPMI-1640 buffer. The cells were washed 3X in PBS buffer and then resuspended in this buffer. The cells were then mixed with an equal volume of 200µM 2-deoxy-D-glucose.

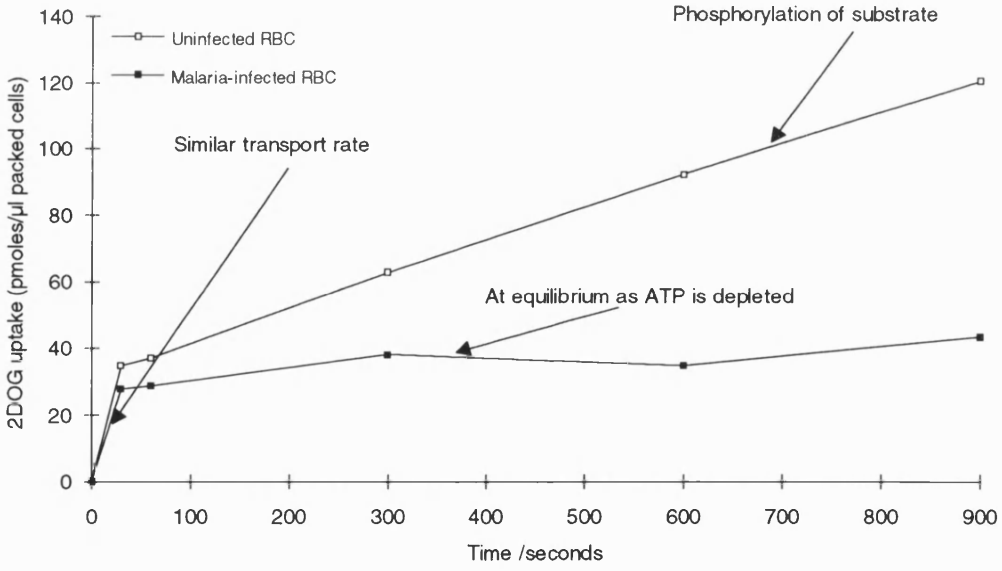

Figure 25. Zero-trans uptake of 100µM 2-deoxy-D-glucose at 37°C by trophozoite/schizont-infected, and uninfected red blood cells. The malaria-infected cells were purified from contaminating uninfected cells using gelatine and stored in RPMI-1640 buffer at 37°C before the transport assay. Uninfected cells were also stored in RPMI-1640 buffer. The cells were washed 3X in glucose-free PBS and then resuspended in this buffer. The cells were added to an equal volume of 200µM 2-deoxy-D-glucose, the transport was allowed to proceed and then stopped with ice-cold stop buffer.

4.3.4. L-Glucose Transport into Trophozoite-Infected and Uninfected Red Blood Cells

Unlike 2-deoxy-D-glucose, L-glucose is not a substrate for hexokinase and does not undergo metabolism within infected or uninfected cells. As L-glucose is the mirror image of D-glucose it is not surprising, using the 'lock and key' hypothesis, that it is not a substrate for hexokinase. This is advantageous in these studies because it allows the transport to be studied in isolation from metabolism. In uninfected cells, L-glucose uptake is very slow due to its relatively poor interaction with the host's glucose transporter, GLUT1, and its impermeability across the lipid bilayer. The uptake of L-glucose into uninfected cells is facilitated completely by GLUT1; this is demonstrated by total inhibition using D-glucose (figure 26). Further evidence for this is provided by work using phloretin, which is a specific inhibitor of facilitative glucose transporters. This inhibitor totally abolishes L-glucose uptake into uninfected cells (figure 27).

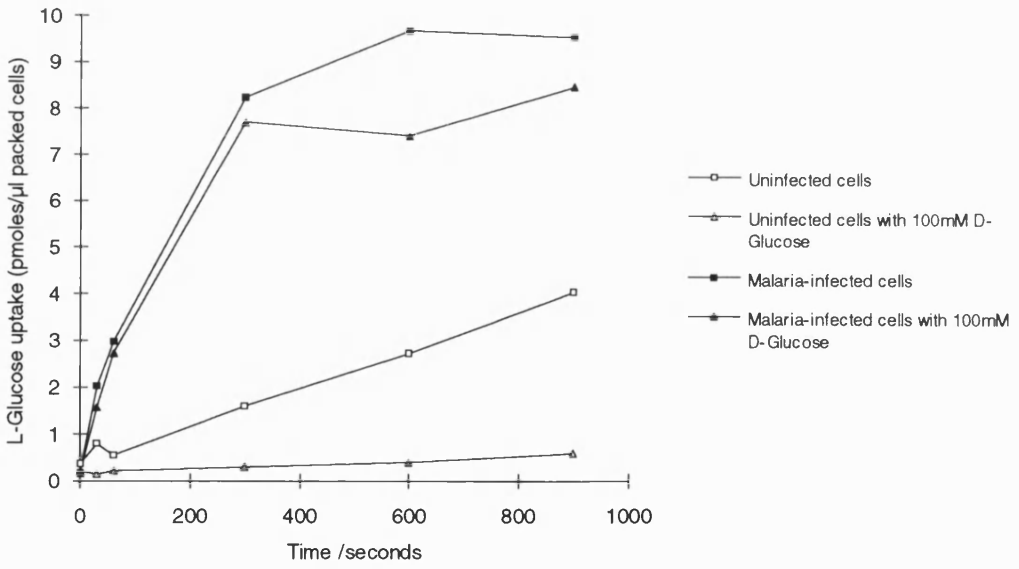

Figure 26. Zero-trans uptake of 35µM L-glucose into malaria-infected and uninfected cells and its inhibition by 100mM D-glucose. Gelatine purified trophozoite and schizont-infected, and uninfected, erythrocytes were stored in RPMI-1640 buffer. Before the uptake experiment, the cells were washed 3X in glucose-free PBS and resuspended in this buffer. The cells were mixed with an equal volume of 70µM L-glucose (± 100mM D-glucose) and left for the required time.

The increased uptake of L-glucose (figures 26 and 27) into malaria-infected cells has been known for some time (Homewood and Neame, 1974; Tripatara and Yuthathong,

1986). In red blood cells infected with mature stage parasites, the transport of L-glucose is much faster than in uninfected cells, and is not inhibited by D-glucose (figure 26). This suggests that L-glucose enters by another route in infected erythrocytes and is consistent with previous reports (Homewood and Neame, 1974; Izumo *et al*, 1989) which state that a second malaria induced pathway for transport of hexoses exists in malaria-infected erythrocytes. Figure 27 shows that this induced pathway is inhibited to an equal extent by 200μM phlorizin and 320μM phloretin. This is in contrast to transport in uninfected erythrocytes where phloretin is a more effective inhibitor at these concentrations (figure 27).

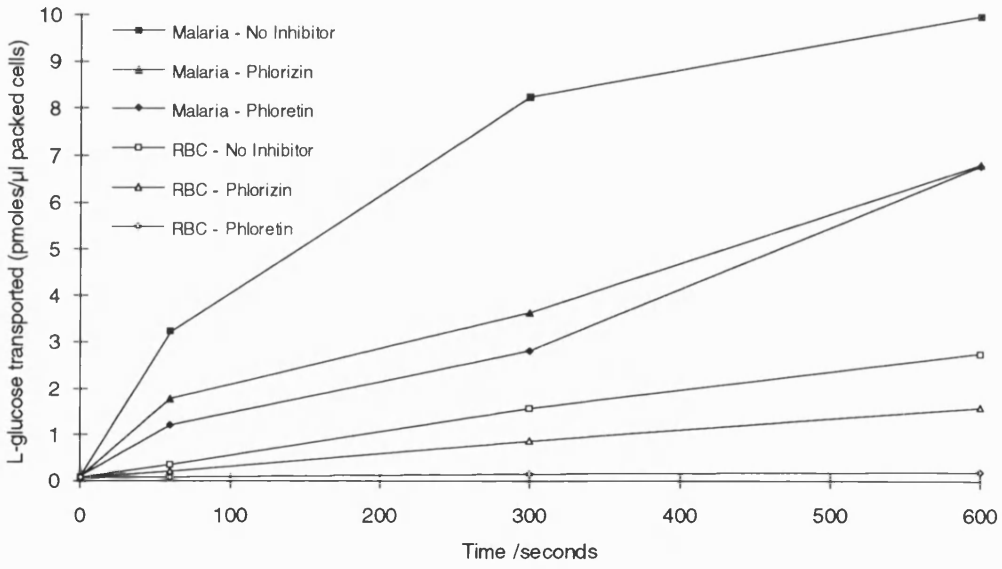

Figure 27. Zero-trans uptake of 35μM L-glucose into uninfected and malaria-infected red blood cells and its inhibition by phloretin and phlorizin. Infected cells were purified to over 90% parasitaemia using gelatine and stored in RPMI-1640 medium before use. Uninfected cells were stored in RPMI-1640 medium before use. To start the experiment the cells were washed 3X with glucose-free PBS (± 320μM phloretin or 200μM phlorizin) and then resuspended in wash solution. The cells were added to an equal volume of 70μM L-glucose (± 320μM phloretin or 200μM phlorizin) and stopped after the required time.

The identity of the malaria induced pathway was elucidated using the chloride channel inhibitor niflumic acid, which has been shown to be an inhibitor of transport through the malaria induced pore (Kirk *et al*, 1992b). Uninfected cells transported L-glucose at the same rate in the presence and absence of 500μM niflumic acid (figure 28). This suggests that niflumic acid does not affect the transport of hexoses through GLUT1.

The increased L-glucose transport rate into trophozoite-infected red blood cells is abolished by niflumic acid, reducing it to the same rate as found in uninfected cells (figure 28).

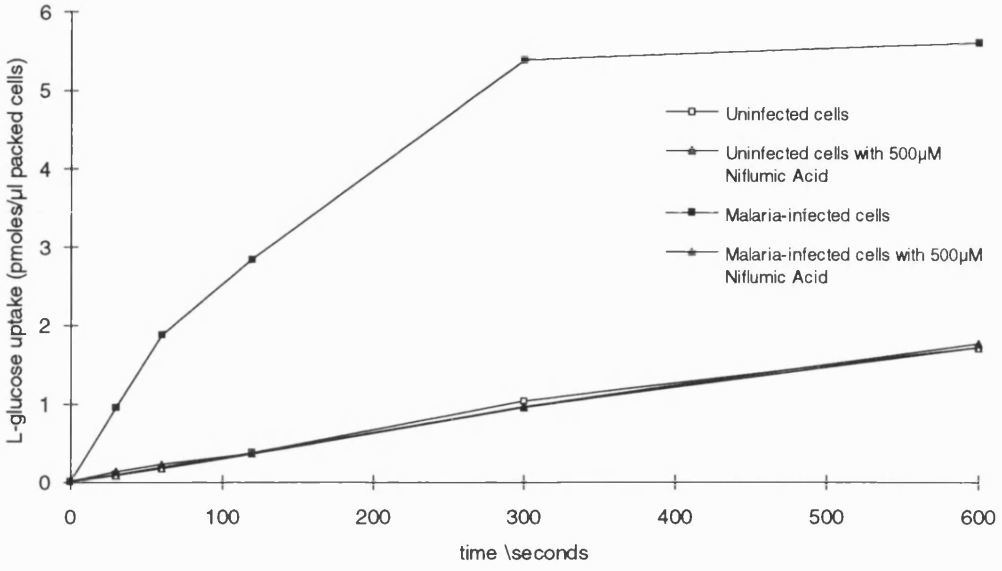

Figure 28. Zero-trans uptake of L-glucose and its inhibition by 500µM niflumic acid. Uninfected and gelatine purified malaria-infected red blood cells were stored in RPMI-1640 medium before use. The cells were washed 3X in glucose-free PBS (± 500µM niflumic acid) and then resuspended in the wash buffer. An equal volume of 70µM L-glucose (± 500µM niflumic acid) was added to the cells and the reaction stopped after the required time.

4.3.5. The Export of L-Glucose from Trophozoite-Infected Red Blood Cells

The export of L-glucose from cells that had already been pre-loaded with L-glucose, and its inhibition by phloretin and phlorizin was also studied. Again phloretin and phlorizin had similar effects in infected cells (figure 29). Both inhibitors caused the partial inhibition of efflux but were unable to inhibit the efflux completely. Niflumic acid, which is a specific inhibitor of transport through the pore, caused total inhibition of L-glucose efflux in malaria-infected red blood cells (figure 30). In uninfected cells L-glucose could not exit in the presence or absence of niflumic acid during the time course of the experiment (figure 30).

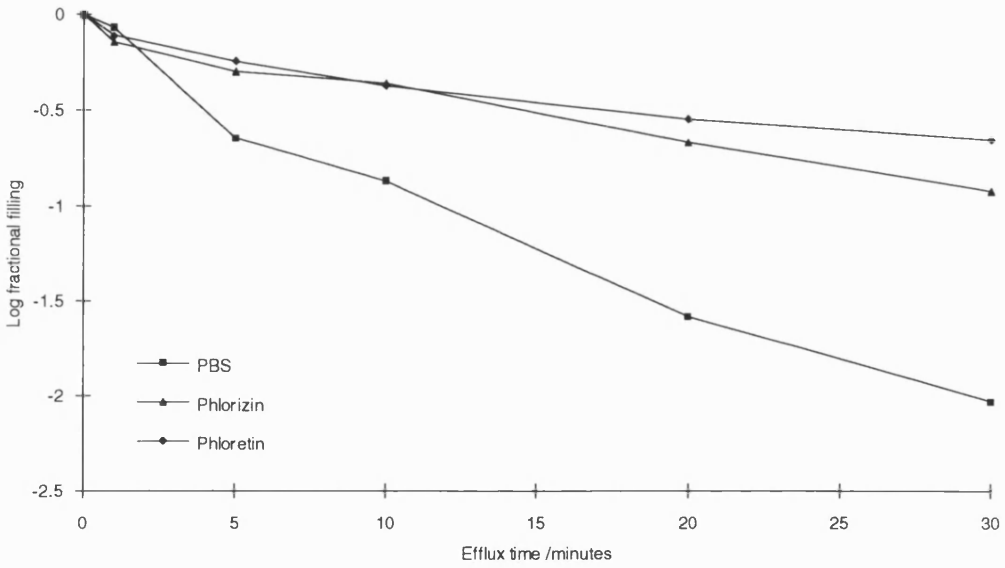

Figure 29. The efflux of 35μM L-glucose and its inhibition by 200μM phlorizin or 320 μM phloretin. Gelatine purified, trophozoite-infected, red blood cells were washed free of glucose using 37°C glucose-free PBS and incubated in 35μM L-glucose for 15 minutes. The cells were then incubated in 35μM L-glucose (± the inhibitor) and incubated for a further 5 minutes. The cells were then diluted 10 fold with 37°C PBS (± phloretin or phlorizin) and the efflux monitored with time.

4.3.6. 6-Deoxy-D-Glucose Transport into Trophozoite-Infected Red Blood Cells

As mentioned previously, 6-deoxy-D-glucose is an ideal substrate for these studies: it is non-metabolisable, and indistinguishable to D-glucose by the human facilitative transporter. The rate of influx of 6-deoxy-D-glucose into malaria-infected and uninfected cells is identical (figures 31, 32 and 34). This is in contrast to L-glucose

where the transport is much faster into infected cells (figures 26, 27 and 28). L-glucose uptake into uninfected red blood cells was inhibited more by phloretin than in infected cells (figure 27). In contrast, the transport of 6-deoxy-D-glucose into infected and uninfected cells are similar in the presence of phloretin (figure 31). The inhibitor seems to be equally effective at inhibiting 6-deoxy-D-glucose uptake into both malaria-infected and uninfected red blood cells suggesting that 6-deoxy-glucose enters both infected and uninfected cells by the same route.

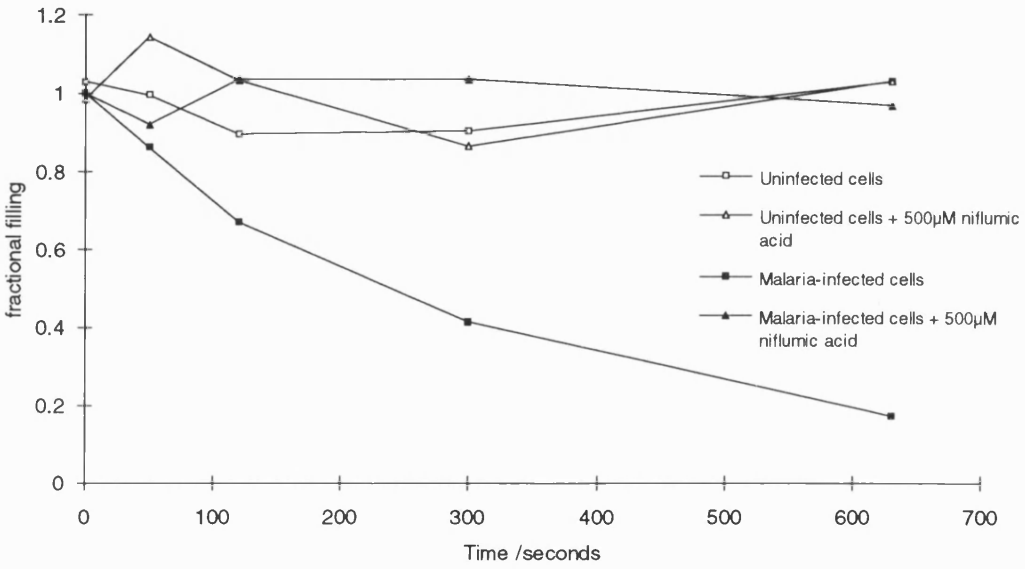

Figure 30. The efflux of 35µM L-glucose and its inhibition by 500µM niflumic acid. Malaria-infected erythrocytes were gelatine purified and stored in RPMI-1640 medium before use. Uninfected cells were also stored in RPMI-1640 medium. The cells were washed free of glucose and incubated, at 37°C, in 35µM L-glucose for 15 minutes. The cells were then incubated in 37°C, 35µM L-glucose (± niflumic acid) and incubated for a further 5 minutes. The cells were then diluted 10 fold with 37°C PBS (± niflumic acid), and the efflux monitored.

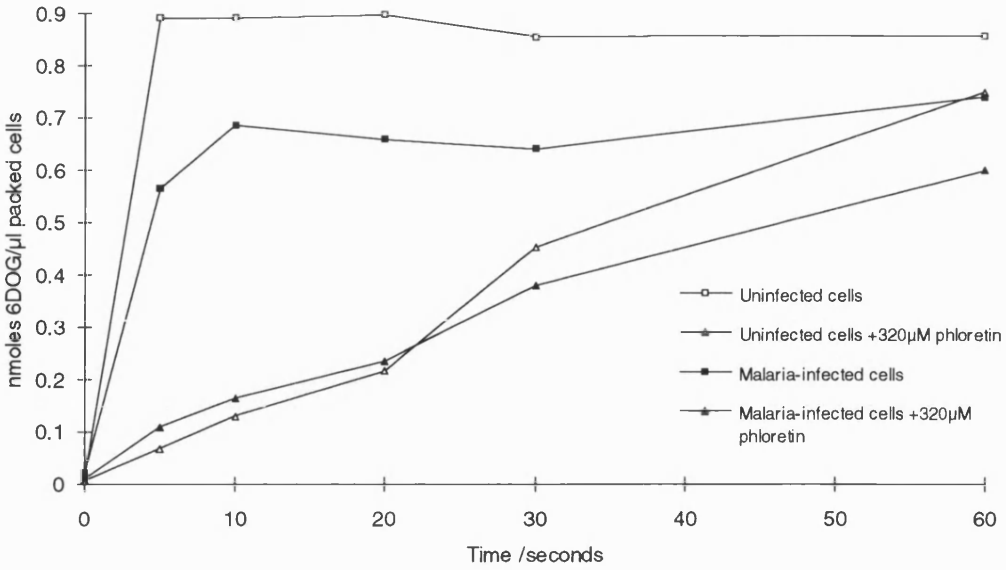

Figure 31. Zero-trans uptake of 5mM 6-deoxy-D-glucose in the presence and absence of 320µM phloretin. Gelatine purified, malaria-infected, and uninfected red blood cells were stored in RPMI-1640 before use. The cells were then washed 3X in 37°C, glucose-free PBS (± 320µM phloretin) and resuspended in the wash buffer. The cells were added to an equal volume of 37°C, 10mM 6-deoxy-D-glucose (± 50µM cytochalasin B) and left for up to 60 seconds before stopping the reaction.

Cytochalasin B is a non-competitive inhibitor of sugar uptake, acting on the inside of the cell (Taverna and Langdon, 1973; Lin and Spudich, 1974). At a concentration of 50µM this compound inhibited uptake of 6-deoxy-D-glucose by over 99.9% in uninfected erythrocytes (figure 32). In parasitised cells cytochalasin B was also an effective inhibitor of 6-deoxy-D-glucose uptake achieving inhibition of 87.0% over the first 5 seconds of transport (figure 32). As cytochalasin B was less effective at inhibiting 6-deoxy-D-glucose uptake into malaria-infected cells than into uninfected cells the Ki for cytochalasin B was determined for both cell populations (figure 33). Uninfected cells have an apparent Ki for cytochalasin B of 3.1×10^{-8}M; infected cells have a Ki_{app} of 10^{-6}M.

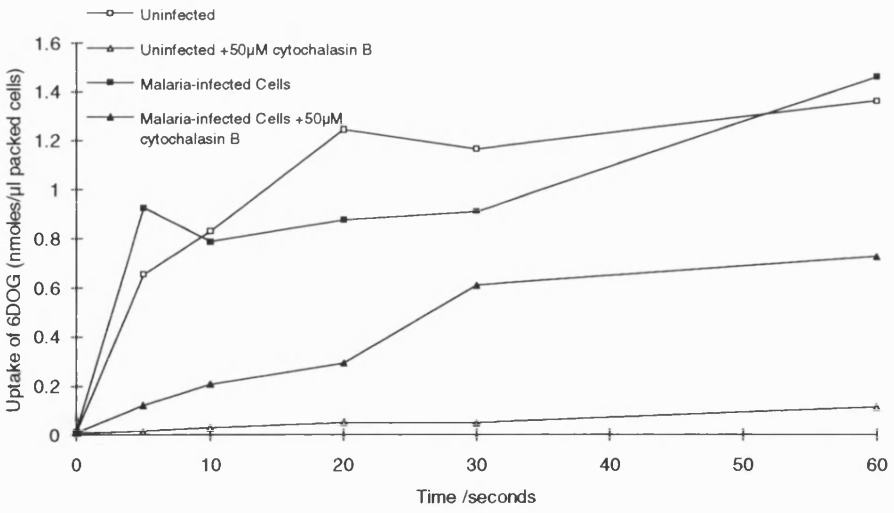

Figure 32. Zero-trans uptake of 20mM 6-deoxy-D-glucose in the presence and absence of 50μM cytochalasin B. Gelatine purified, malaria-infected, and uninfected red blood cells were stored in RPMI-1640 before use. The cells were washed 3X in glucose-free, 37°C PBS (± 50μM cytochalasin B) and resuspended in the wash buffer. The cells were added to an equal volume of 37°C, 40mM 6-deoxy-D-glucose (± 50μM cytochalasin B) and left for up to 60 seconds before stopping the reaction.

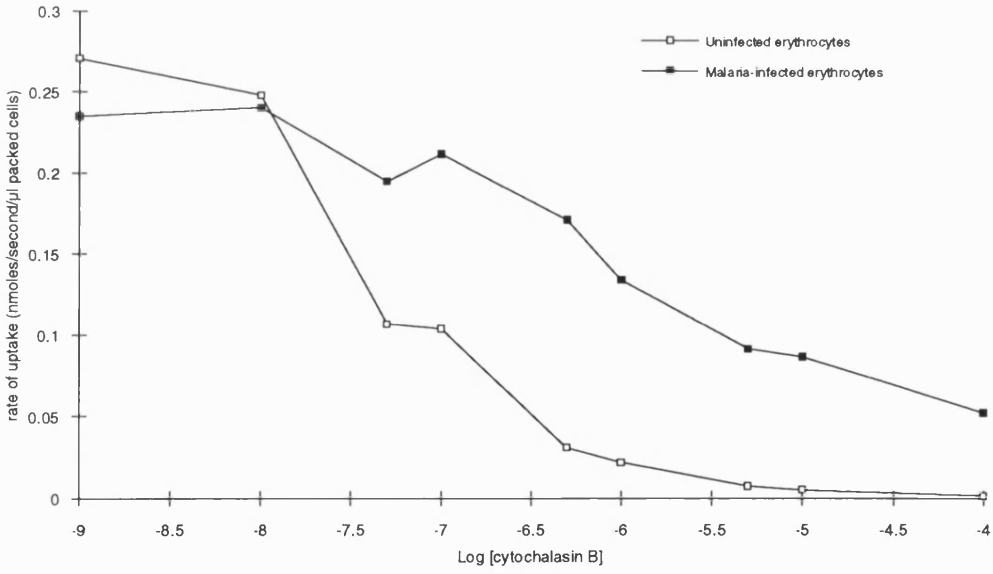

Figure 33. Differential inhibition of zero-trans uptake of 20mM 6-deoxy-D-glucose by cytochalasin B. Gelatine purified, malaria-infected, and uninfected red blood cells were stored in RPMI-1640 before use. The cells were then washed 3X in 20°C, glucose-free PBS (± cytochalasin B at 10^{-9}M→10^{-4}M concentration) and resuspended in the wash buffer. The cells were added to an equal volume of 20°C, 40mM 6-deoxy-D-glucose (± cytochalasin B at 10^{-9}M→10^{-4}M concentration) and left for 5 seconds before stopping the reaction. We have assumed that the transport rate is linear over the first 5 seconds to obtain the initial rate estimates. Uninfected cells have a Ki for cytochalasin B of 3.1×10^{-8}M; infected cells have a Ki of 10^{-6}M.

It has been reported that the malaria induced pathway has characteristics similar to chloride channels, and is inhibited by niflumic acid and furosemide, both potent specific inhibitors of this channel (Kirk *et al*, 1992b). Niflumic acid was used in conjunction with cytochalasin B to inhibit uptake into infected cells further (figure 34). This suggests that some 6-deoxy-D-glucose enters the parasite through the malaria induced pore. Niflumic acid alone has no inhibitory effect on the uptake confirming that a large proportion of the hexose is transported through the host's, niflumic acid insensitive, hexose transporter.

Malaria-infected red blood cells were subjected to N_2-cavitation (section 2.2.7.) to release parasites (and the parasitophorous vacuolar membrane) from the red blood cells. Zero-trans uptake of 6-deoxy-D-glucose into these released parasites revealed that cytochalasin B was only partially effective at inhibiting the uptake of the sugar (figure 35).

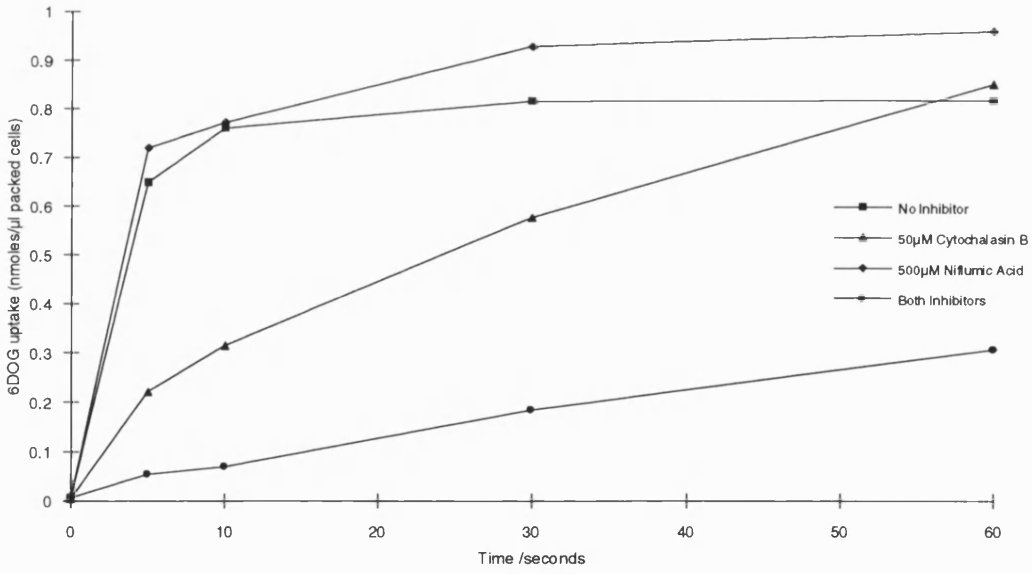

Figure 34. Zero-trans uptake of 5mM 6-deoxy-D-glucose into trophozoite and schizont-infected red blood cells in the presence and absence of 50µM cytochalasin B and/or 500µM niflumic acid. Gelatine purified, malaria-infected, red blood cells were stored in RPMI-1640 before use. The cells were then washed 3X in 37°C, glucose-free PBS (± cytochalasin B and/or niflumic acid) and resuspended in the wash buffer. The cells were added to an equal volume of 37°C, 10mM 6-deoxy-D-glucose (± cytochalasin B and/or niflumic acid) and left for up to 60 seconds before stopping the transport.

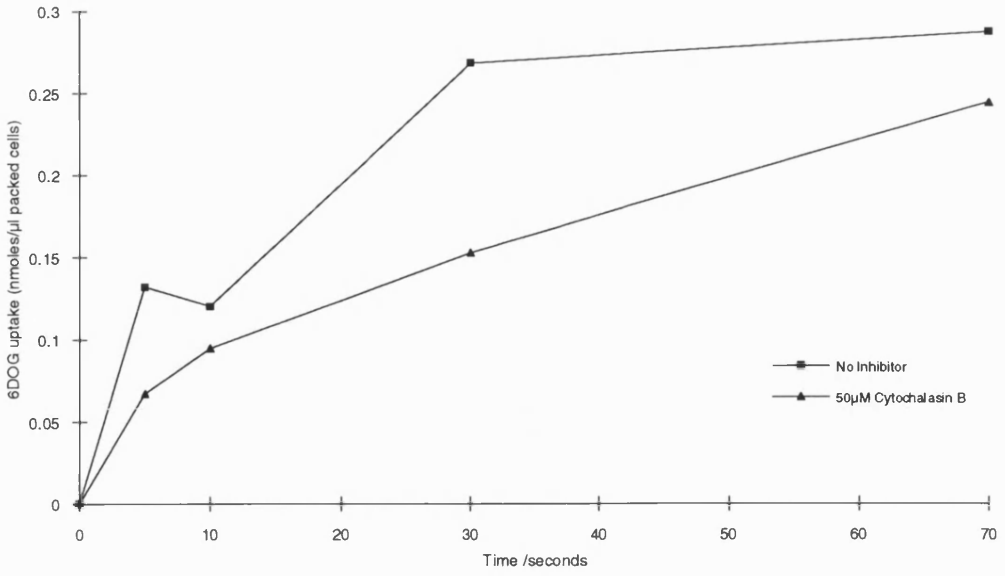

Figure 35. Zero-trans uptake of 5mM 6-deoxy-D-glucose into *Plasmodium falciparum* parasites that were released from erythrocytes. Malaria-infected red blood cells were purified using gelatine and then the red blood cells were lysed by N_2-cavitation. The cells were stored in 37°C, AIM containing 5mM D-glucose before use. When ready the cells were washed 3X in glucose-free AIM and resuspended in the wash buffer. An equal volume of 37°C, 10mM 6-deoxy-D-glucose in AIM (± 50µM cytochalasin B) was added to the cells and the uptake of sugar allowed to proceed. The transport was stopped by the addition of stop buffer. Due to the technical difficulties of performing this assay this experiment was not repeated.

4.3.7. The Export of 6-Deoxy-D-Glucose from Trophozoite-Infected Red Blood Cells

By studying the export of 6-deoxy-D-glucose from infected and uninfected red blood cells the transport characteristics of the parasite can be investigated. The hexose left uninfected red blood cells faster than infected cells (figure 36). Cytochalasin B is a competitive inhibitor of efflux (Taverna and Langdon, 1973; Lin and Spudich, 1974). When cytochalasin B was used at 50µM it was able effectively to inhibit efflux of 20mM 6-deoxy-D-glucose (figure 37). This indicates that cytochalasin B is a good inhibitor of 6-deoxy-D-glucose efflux and that most of the sugar leaves on a facilitative carrier. Further evidence for this is provided by the effect of phloretin and phlorizin on the efflux of glucose from malaria-infected cells. Unlike L-glucose efflux, 6-deoxy-D-glucose efflux is totally inhibited by 320µM phloretin and only partially inhibited by phlorizin (figure 38).

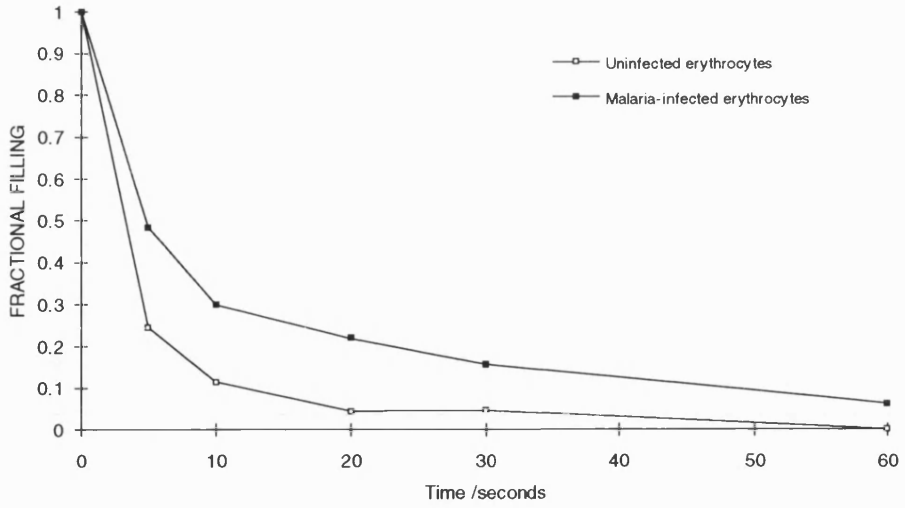

Figure 36. Zero-trans efflux of 6-deoxy-D-glucose in malaria-infected and uninfected red blood cells. Gelatine purified, malaria-infected, and uninfected red blood cells were stored in RPMI-1640 before use. The cells were then washed 3X in glucose-free PBS and resuspended in the wash buffer. The cells were incubated at 20°C in 20mM 6-deoxy-D-glucose for 15 minutes to pre-load the cells with the hexose. The cells were then incubated for 2 minutes in 20mM 6-deoxy-D-glucose. The cells were diluted 10 fold with 20°C, glucose-free PBS and the egress of the sugar followed. The results are expressed as fractional filling [$(S_i-S_\infty)/(S_o-S_\infty)$] versus time.

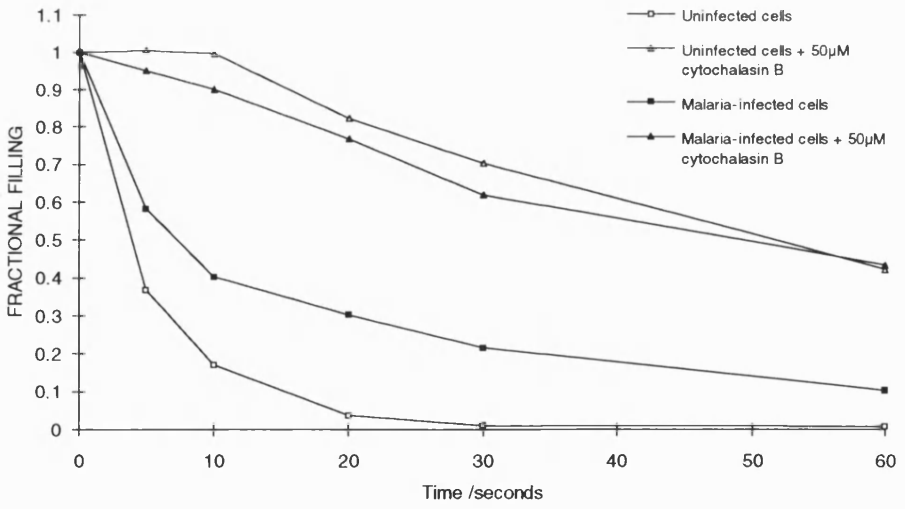

Figure 37. Zero-trans efflux of 20mM 6-deoxy-D-glucose in malaria-infected and uninfected red blood cells in the presence of absence of cytochalasin B. Gelatine purified, malaria-infected, and uninfected red blood cells were stored in RPMI-1640 before use. The cells were then washed 3X in glucose-free PBS and resuspended in the wash buffer. The cells were incubated in 20mM 6-deoxy-D-glucose for 10 minutes to pre-load the cells with the hexose. The cells were then incubated for 2 minutes in 20mM 6-deoxy-D-glucose (± 50µM cytochalasin B). The cells were diluted 10 fold with 20°C, glucose-free PBS (± 50µM cytochalasin B) and the egress of the sugar followed. The results are expressed as fractional filling [$(S_i-S_\infty)/(S_o-S_\infty)$] versus time.

The export of 6-deoxy-glucose is completely inhibited by phloretin and partially inhibited by phlorizin in malaria-infected cells. In uninfected cells phlorizin does not inhibit export at all whereas phloretin can totally block its export.

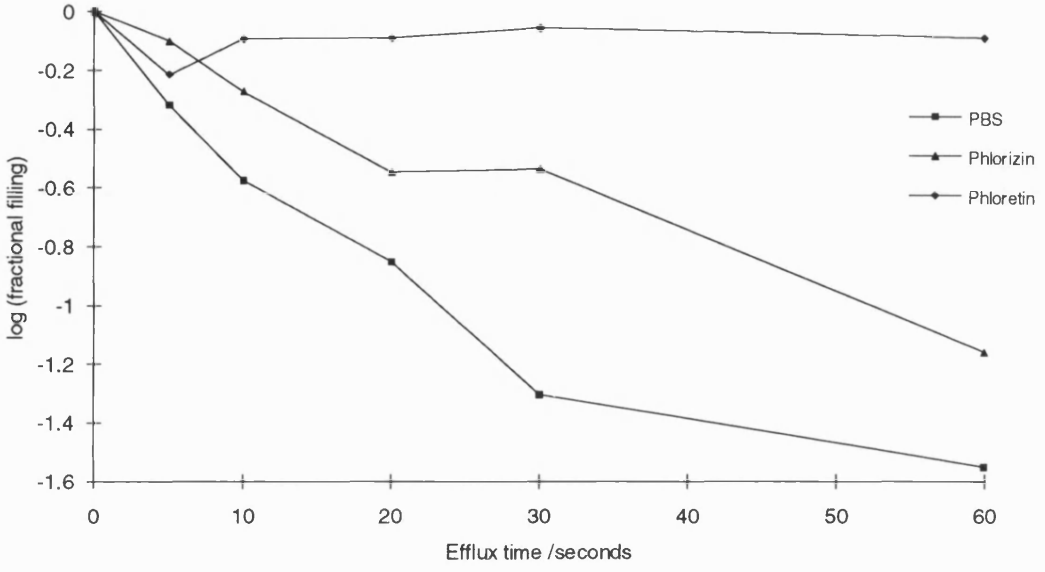

Figure 38. Zero-trans efflux of 20mM 6-deoxy-D-glucose in malaria-infected red blood cells in the presence of phloretin or phlorizin. Gelatine purified, malaria-infected, red blood cells were stored in RPMI-1640 before use. The cells were then washed 3X in 20 °C, glucose-free PBS and resuspended in the wash buffer. The cells were then incubated at 20°C in 20mM 6-deoxy-D-glucose for 10 minutes to pre-load the cells with the hexose. The cells were then incubated in 20mM 6-deoxy-D-glucose (± 200μM phlorizin or 320μM phloretin). The cells were diluted 10 fold with glucose-free, 20°C PBS (± 200μM phlorizin or 320μM phloretin) and the egress of the sugar followed. The results are expressed as log fractional filling [log $(S_i-S_\infty)/(S_o-S_\infty)$] versus time.

The efflux of 6-deoxy-D-glucose was studied at different concentrations of 6-deoxy-D-glucose in an attempt to estimate the kinetic parameters and to help determine the mechanisms involved in malarial transport (figure 39).

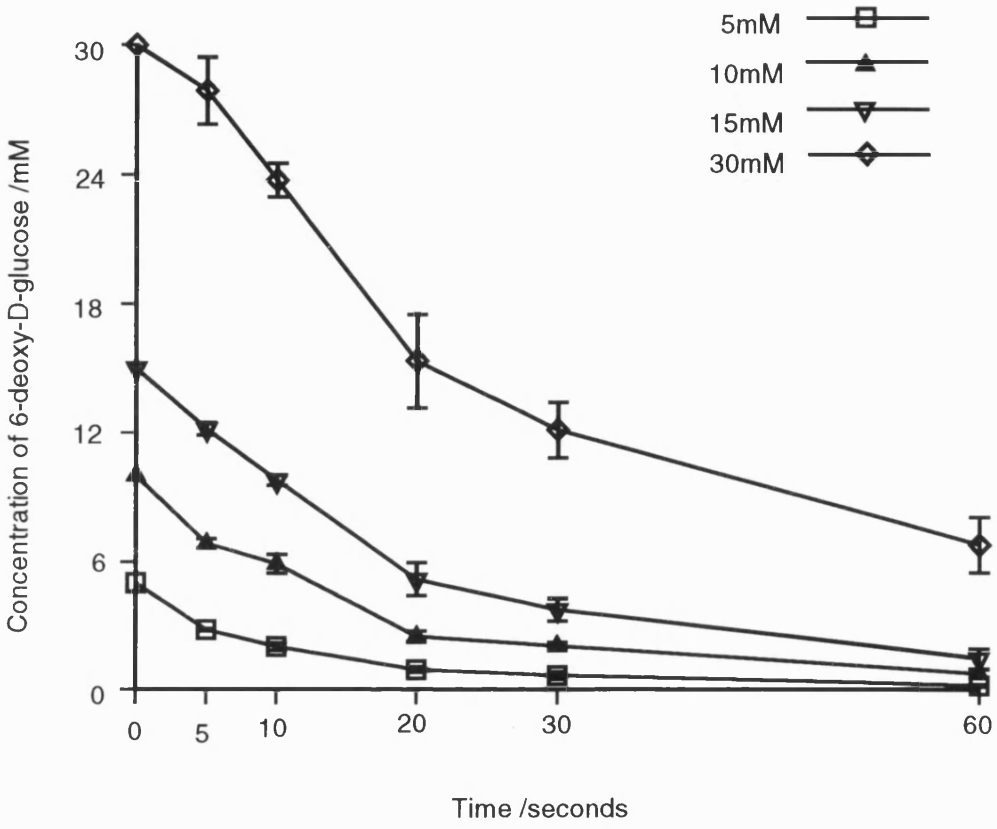

Figure 39. Zero-trans efflux of 6-deoxy-D-glucose in malaria-infected red blood cells. Gelatine purified, malaria-infected, red blood cells were stored in RPMI-1640 before use. The cells were then washed 3X in 20°C, glucose-free PBS and resuspended in the wash buffer. The cells were then incubated in 6-deoxy-D-glucose (at 5, 10, 15 or 30mM) for 5 minutes to pre-load the cells with the hexose. The cells were diluted 10 fold with 20°C, glucose-free PBS and the egress of the sugar followed. The results are expressed as intracellular concentration versus time. Each point is the average of 3 experiments and the SEM are displayed.

4.4. Discussion

The observations that cooling malaria-infected red cells inhibits its growth (figure 21) and its uptake of 6-deoxy-D-glucose (figure 22) is consistent with the results of Wunderlich *et al* (1982). He studied the protein distribution in *Plasmodium*-infected erythrocytes. About 30% less protein is found on the periplasmic side of the red blood membrane after incubation at 4°C for 10 minutes (Wunderlich *et al*, 1982), suggesting specific translocation of proteins from the outside face of the membrane at low temperatures. If these proteins normally transport 6-deoxy-D-glucose it would explain the reduced transport after incubation at 4°C. This mechanism may have biological significance as a rapid drop in temperature in the wild would mean the parasites have left the human host and entered the cold blooded *Anopheles* mosquito. Under these conditions the asexual stages of the parasite (the merozoites, rings, trophozoites and schizonts) do not survive. The sudden reduction in metabolism may prevent the mosquito from starving under the increased demand from the asexual parasite.

It has also been reported that synchronised *P. falciparum* trophozoites that were maintained at 28°C matured slowly. These cultures also appeared to reinvade inefficiently at later stages in the life-cycle (Rojas and Wasserman, 1993).

To gain insight into how D-glucose is transported into the cell, a D-glucose analogue must be used. One of the main investigations into glucose transport in malaria-infected erythrocytes used 2-deoxy-D-glucose (Izumo *et al*, 1989) and the mouse parasite *P. yoelii*. This analogue is far from ideal because 2-deoxy-D-glucose is also a substrate for hexokinase (Sols and Crane, 1954; Sols *et al*, 1958). On entering the cell, 2-deoxy-D-glucose is phosphorylated to 6-phospho-2-deoxy-D-glucose, which is not transported by the glucose transporter, and becomes trapped within the cell. Because many studies on glucose transport to date have used this analogue, the conclusion that transport into malarial parasites is active requires further investigation. The use of D-glucose to study transport (Sherman and Tanigoshi, 1974; Tripatara and Yuthathong, 1986) suffers from similar limitations to using 2-deoxy-D-glucose. The sugar is rapidly

metabolised, being converted to lactate and expelled, or incorporated into high molecular weight compounds.

Using metabolisable analogues of D-glucose means that the results that are obtained depend on the metabolic state of the cell. Zero-trans influx experiments require the use of starved parasites as glucose must not be present inside the cell. This means that ATP is likely to deplete in the metabolically active parasite during the transport assay. If the assay is performed at 4°C, the malaria-infected cell has ATP available for some time but the cells are not viable. At 37°C, ATP is depleted while the cells are being washed free of glucose and before the uptake experiment is commenced.

At 4°C, *Plasmodium falciparum*-infected red blood cells accumulate 2-deoxy-D-glucose for about 60 seconds, after which time the cell's ATP stores deplete and the unphosphorylated 2-deoxy-D-glucose equilibrates across the erythrocyte membrane (figure 24). At 37°C 2-deoxy-D-glucose equilibrates at a lower level in infected cells because the cellular ATP levels were depleted before the assay was started (figure 25). In uninfected cells similar results are obtained at 4°C and at 37°C (except that the transport and subsequent metabolism is faster at 37°C); the sugar equilibrates quickly and then sugar levels increase steadily as the hexose is phosphorylated.

L-glucose has one main advantage over 2-deoxy-D-glucose: it is not modified. L-glucose diffuses into the cell and diffuses out again. It is not phosphorylated and does not accumulate in the cell. Another property of L-glucose is its slow transport rate by the host's glucose transporter. This allows the malaria induced pathways to be studied in isolation from the host's transporter.

The transport of L-glucose into uninfected cells is totally inhibitable by both D-glucose and phloretin (figures 26 and 27). This indicates that L-glucose enters into uninfected red blood cells exclusively through the D-glucose mammalian transporter GLUT1. Phlorizin, a specific inhibitor of the Na^+/glucose intestinal symporter protein (Koepsell and Madrala, 1987), does inhibit the uptake of L-glucose into uninfected cells, but not to the same extent as phloretin. Compared to 6-deoxy-D-glucose, which reaches equilibrium at 5mM in 10 seconds (figures 31 and 34), the transport rate of L-glucose

is very slow. After 10 minutes 35μM L-glucose does not reach equilibrium (figures 26, 27 and 28).

The increased uptake of L-Glucose into infected red blood cells has been reported before. Previous work (Homewood and Neame, 1974) suggests that the space available for L-glucose increases in samples containing *P. berghei*-infected mouse cells, i.e. L-glucose enters the cells. These authors were unable to detect a similar increase in space using uninfected cells. Tripatara and Yuthathong (1986) reported that phloretin was ineffective as an inhibitor of L-glucose uptake into both *P. berghei*-infected and uninfected erythrocytes.

We have found that in trophozoite-infected human red blood cells, L-glucose is transported faster than in uninfected cells and shows different inhibition characteristics (figures 26, 27 and 28). The uptake of L-glucose into infected cells is almost totally insensitive to D-glucose (figure 26) and less sensitive to phloretin (figure 27). In *P. falciparum*-infected human erythrocytes we have found that the inhibition by phloretin is only partial in infected cells but complete in uninfected erythrocytes (figures 27). The discrepancy between our results and those of other authors is probably due to the high L-glucose concentration used in their experiments (5mM), and to the use of mouse instead of human erythrocytes. These results suggest that L-glucose is imported into malaria-infected erythrocytes by a pathway that is not present in uninfected cells. As this transport is totally inhibited by 500μM niflumic acid (figure 28) and significantly inhibited by 200μM phlorizin (figure 27) it is likely that the majority of the L-glucose enters the infected cells through the malaria induced pore (Kutner *et al*, 1987; Kirk *et al*, 1992b). It is unclear from zero-trans uptake studies whether L-glucose enters the parasite or only enters the infected red blood cell.

The export of L-glucose is similarly inhibited by phloretin and phlorizin (figure 29). In infected cells both 320μM phloretin and 200μM phlorizin are equally effective at inhibiting L-glucose efflux (figure 29). In uninfected cells the efflux is so slow that after 10 minutes none had appeared to leave the cells (figure 30). In infected cells the efflux is inhibited totally by niflumic acid. Efflux from the parasite does not appear to

follow a simple first-order mechanism (figure 30). This would suggest that L-glucose is leaving from two compartments and suggests that L-glucose has access to the parasite as well as the erythrocyte.

Although a useful tool for studying the properties of the pore, L-glucose is not transported as efficiently as D-glucose in either infected or uninfected cells. To gain insight into how D-glucose is transported into the cell, a D-glucose analogue must be used. Although 2-deoxy-D-glucose is commercially available and appears to have similar Km and Vmax values for the mammalian transporters, this analogue is far from ideal. As mentioned earlier, not only does the glucose transporter protein recognise it as glucose but so does hexokinase (Sols and Crane, 1954; Sols *et al*, 1958). Once phosphorylated it cannot leave the cell on the transporter and accumulates within the cell. The rate of accumulation is proportional to the rate of phosphorylation and not related to the rate of transport so it is very hard to study transport using these analogues. A solution to the problem is to use a D-glucose analogue where the C6 OH group has been replaced with a hydrogen atom. This analogue, called 6-deoxy-D-glucose (or epifucose), cannot be phosphorylated by hexokinase making the study of transport possible in the absence of metabolism.

An unexpected result was that the 6-deoxy-D-glucose transport rate into malaria-infected and uninfected cells was the same (figures 31, 32 and 34). This result was unexpected because it is known that infected cells use much more glucose than uninfected cells (Sherman and Tanigoshi, 1974; Sherman, 1979) and it has been suggested that a malaria induced pathway (the pore) is required to supply the infected cell with enough glucose to grow (Tanabe, 1990). This result indicates that the native mammalian glucose transporter has a sufficient capacity to supply the mature parasite with glucose, and that in uninfected mature human erythrocytes glucose is transported below capacity.

Cytochalasin B, at a concentration of 50μM, which is a specific inhibitor of facilitative sugar transporters (Taverna and Langdon, 1973; Lin and Spudich, 1974), inhibited uptake of 6-deoxy-D-glucose by over 99.9% in uninfected erythrocytes (figure 32). In

parasitised cells, cytochalasin B was also an effective inhibitor of 6-deoxy-D-glucose uptake, achieving inhibition of about 87.0% over the first 5 seconds of transport (figure 32). These results confirm that the majority of 6-deoxy-D-glucose is transported into malaria-infected red blood cells through GLUT1. This is in contrast to L-glucose where the majority is transported through the malaria induced pore. As cytochalasin B appears to be a less potent inhibitor of 6-deoxy-D-glucose transport in infected cells (figure 33), it seems likely that either the kinetics of the host transporter is modified or some of the hexose enters through a cytochalasin B insensitive pathway.

The suggestion of a modified host transporter is consistent with several reports of modified transporters in *Plasmodium*-infected erythrocytes, e.g. glucose (Tripatara and Yuthathong, 1986), adenosine (Gero *et al*, 1988), lactate (Kanaani and Ginsburg, 1991), tryptophan (Ginsburg and Krugliak, 1983) and choline (Ancelin *et al*, 1985).

The theoretical results obtained for differential inhibition by a competitive inhibitor are depicted in figure 40. A graph of initial velocity against log inhibitor concentration gives a sigmoid curve (see figure 40A) and the difference between these two curves gives a peak (figure 40B).

Plasmodium falciparum is known to place its own proteins in the red blood cell membrane (Howard, 1988; Gormley *et al*, 1992) and to alter the lipid composition of this membrane (Hsiao *et al*, 1991). Either of these processes could, in theory, alter the host's transporter. Although our results initially confirm that the malaria parasite has changed the sensitivity of GLUT1 to cytochalasin B (compare figure 40B and figure 41) it appears unlikely that this is true. Altering GLUT1 would not be of any advantage to the malarial parasite. The native transporter is sufficient to supply all the D-glucose that the parasite requires, and the parasite does not increase the rate of 6-deoxy-D-glucose transport into infected cells (figures 31, 32 and 34). Furthermore, the results that we have obtained can be explained using a simple hypothesis that does not involve modification of the host's native transporter. The small amount of 6-deoxy-D-glucose that enters through the parasite induced pore competes with cytochalasin B for its internal binding site lowering its effectiveness. Some hexose

might also enter the parasite directly through the parasitophorous duct and the less cytochalasin B sensitive parasite transporter. This hypothesis is depicted in figure 42.

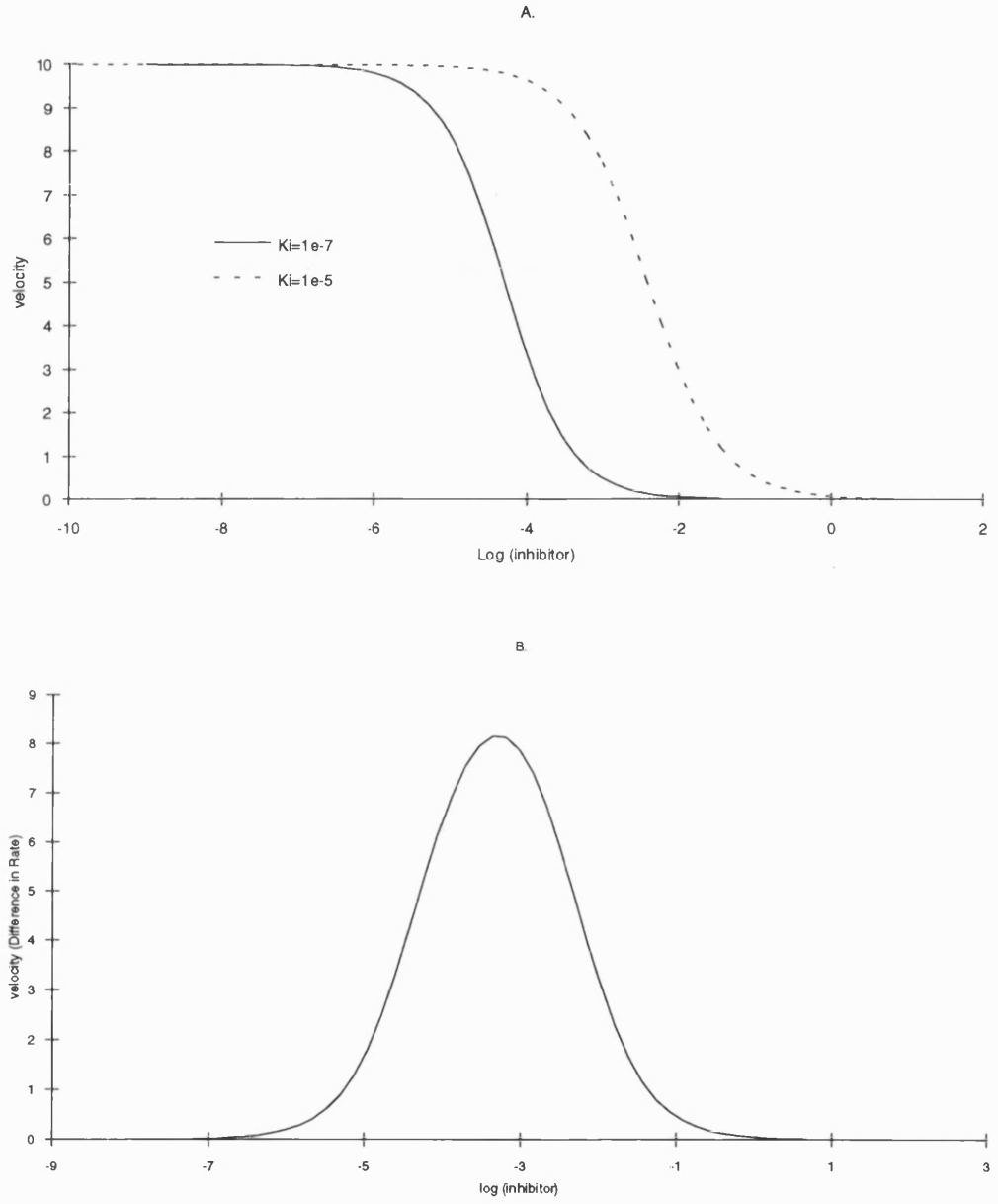

Figure 40. Theoretical graphs showing differential inhibition of two systems by a competitive inhibitor. The equation for competitive inhibition is $v = (Vmax.S)/(Km(1+(I/Ki))+S))$. The following values were set: Vmax = 10, Km = 10^{-5}M and S = $5x10^{-3}$M and then the inhibitor concentration was varied between 10^{-9}M→1M for two different systems (with Ki values of 10^{-7}M and 10^{-5}M). A.) A graph of velocity against log inhibitor concentration produced a sigmoid curve. The larger the Ki the further the curve is shifted to the left. B.) The difference in the two velocities against log inhibitor concentration. The difference peaks and then falls off rapidly. The curve is symmetrical.

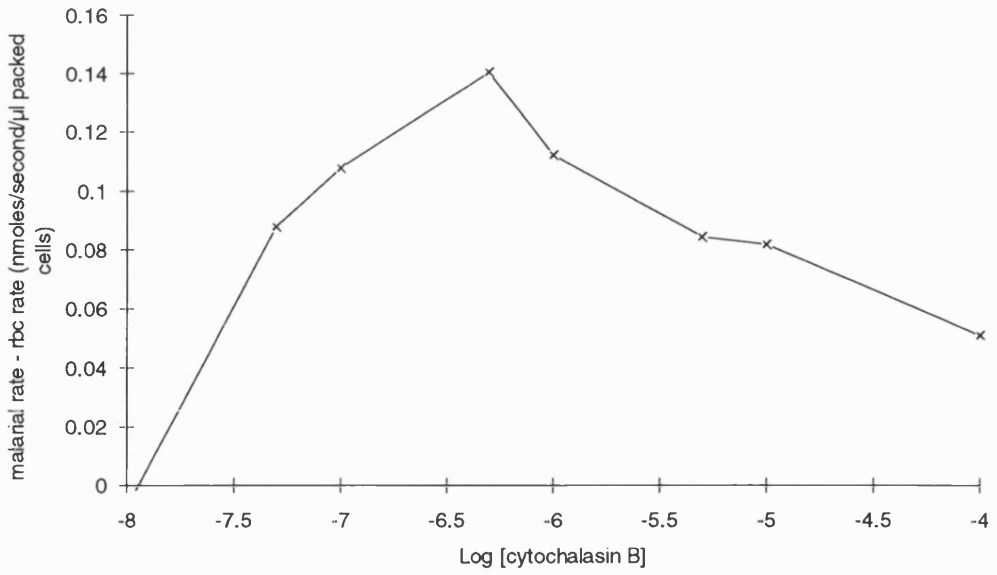

Figure 41. Inhibition of 6-deoxy-D-glucose uptake by cytochalasin B. Difference between transport rate in malaria-infected cells and uninfected cells using data shown in figure 33.

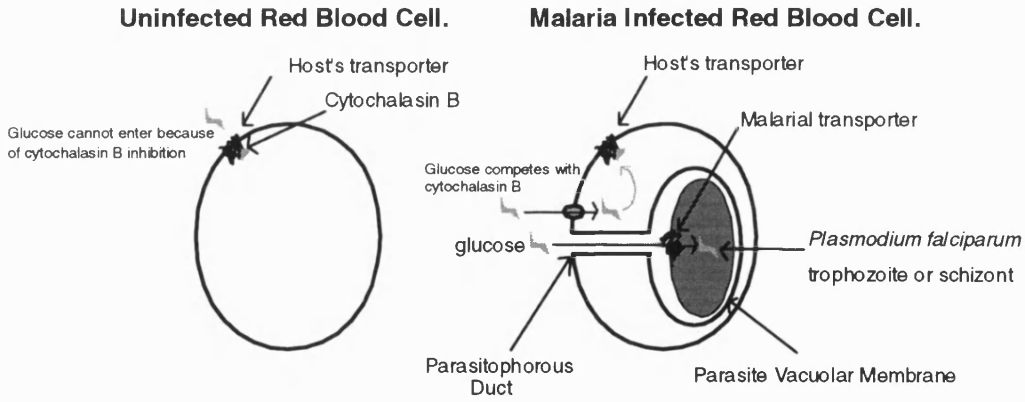

Figure 42. A hypothesis to explain the lowering of the Ki_{app} of cytochalasin B on 6-deoxy-D-glucose uptake. This hypothesis does not predict the modification of the host's constitutive transporter. The small amount of 6-deoxy-D-glucose that enters through the pore in infected cells competes with cytochalasin B lowering its effectiveness. The situation is further complicated by some 6-deoxy-D-glucose entering the parasite directly through the duct and the parasite's less cytochalasin B sensitive transporter.

It has been reported that the malaria induced pathway has characteristics similar to chloride channels and is inhibited by niflumic acid and furosemide - both potent specific

inhibitors of these channels (Kirk *et al*, 1992b). Niflumic acid was used in conjunction with cytochalasin B to inhibit uptake into infected cells further (figure 34). This suggests that some 6-deoxy-D-glucose enters the parasite through the malaria induced pore. Figure 34 also shows that niflumic acid in the absence of cytochalasin B has no inhibitory effect on the uptake. This confirms that much of the hexose is transported through the host's niflumic acid insensitive hexose transporter, and that the main function of the malaria induced pore is not to transport glucose. The host's glucose transporter supplies all the glucose the intraerythrocytic parasite requires.

Although niflumic acid has a synergistic effect on the inhibition of 6-deoxy-D-glucose uptake when used in conjunction with cytochalasin B, the inhibition is not complete (figure 34). At 50μM cytochalasin B completely inhibits transport through the host's transporter (figure 32) and at 500μM niflumic acid completely stops transport through the malaria induced pore (figure 28). This raises the possibility that some of the hexose enters through a pathway that is either insensitive or less sensitive to cytochalasin B or niflumic acid. It is possible that some of this sugar has direct access to the parasite by way of the parasitophorous duct (Pouvelle *et al*, 1991). This model is displayed is figure 43.

This hypothesis is supported by data on adenosine influx into *P. falciparum*-infected red blood cells. It has been reported (Gati *et al*, 1987) that nucleosides enter infected cells through one of three pathways: the host's constitutive transporter, a non-saturable, non-inhibitable transporter (the pore), and a pathway that is weakly inhibited by nucleoside specific inhibitors (like NBMPR). When this paper was published the presence of the duct was unknown and so it was assumed that all these three transporters were located in the erythrocyte's plasma membrane. It is possible that the mildly inhibited pathway is located in the parasite membrane in a similar way to the mildly cytochalasin B sensitive glucose transport system found in this study. Similar results have been obtained when studying lactate efflux. It was found (Kanaani and Ginsburg, 1991) that *Plasmodium falciparum* infection induced another saturatable, CAD (cinnamic acid derivative) sensitive pathway in human red blood cells. This

transporter could also be located in the parasite's plasma membrane (Cramner and Halstrap, 1993).

Further evidence for this is provided by work on parasites released from their erythrocyte. Transport into these cells showed incomplete inhibition by cytochalasin B and suggests that the parasite's hexose transporter may be less sensitive to cytochalasin B. This agrees with work by other authors who reported that 2-deoxy-D-glucose uptake into released parasites was only weakly inhibited by cytochalasin B (Izumo *et al*, 1989; Tanabe, 1990). It seems likely that some glucose enters directly into the parasite through the malaria induced parasitophorous duct and its native transporter. This would explain the incomplete inhibition of 6-deoxy-D-glucose uptake by 500μM niflumic acid together with 50μM cytochalasin B (figure 34).

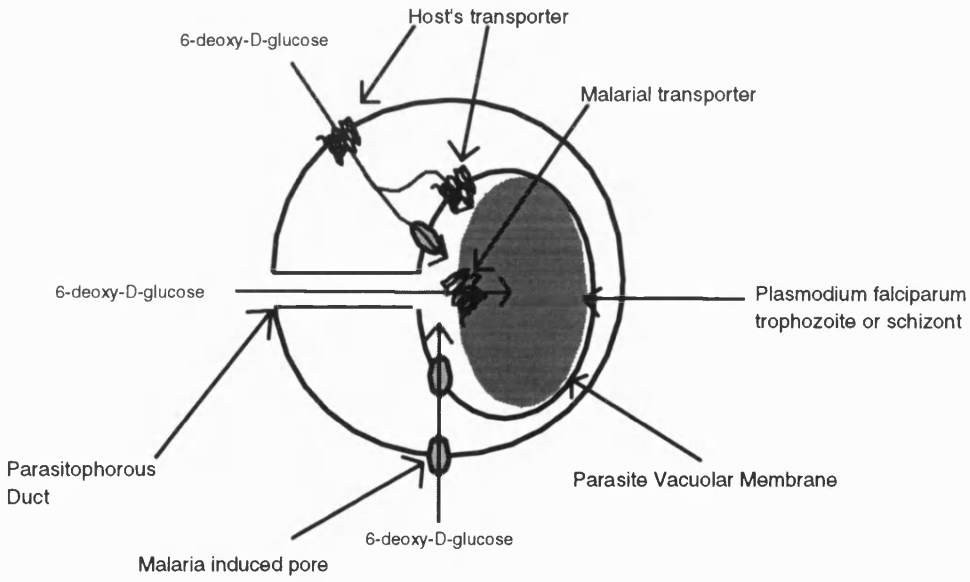

Figure 43. A model to explain the kinetics of 6-deoxy-D-glucose uptake into a human erythrocyte infected by a mature *Plasmodium falciparum* parasite. The sugar can enter the cell by three methods i) through the host's constitutive transporter, ii) through the malaria induced pore and iii) through the parasitophorous duct and a transporter in the malarial parasite. Due to the large number of OH groups it is unlikely that the sugar enters the parasite by non-Stokesian diffusion (Ginsburg and Stein, 1987)

Technically, the zero-trans uptake experiments on released parasites were hard to perform. Many workers have expressed difficulty in obtaining intact free parasites

suitable for transport studies (Sherman, 1988; Gero and O'Sullivan, 1990). There are many reasons for this including:

i) The released parasites are hard to obtain in a form that have their membranes intact. It has been reported that lysis of erythrocytes using saponin or antiserum complement, alters the membrane properties of the released parasite and has been shown to cause protein leakage (Sherman, 1979, 1988; Gero and O'Sullivan, 1990). Instead of using saponin a high pressure method was employed where the cells were placed under 30 atmospheres of N_2, and the erythrocytes lysed on returning the cells to atmospheric pressure (Mikkelsen *et al*, 1986; Choi and Mikkelsen, 1990). This method has been reported to release the parasites gently and not disrupt their delicate membranes as detergent is not used. Unfortunately, many of the malaria-infected erythrocytes remained intact and some of the parasites lysed using this method. The released parasites were purified from contaminating erythrocytes using Percoll ™. Although partially successful, and despite many attempts, most of the free parasites clumped in the Percoll™.

ii) The low yield of free parasites and the small internal volume meant that the radioactive counts in the assay were low (~300cpm) and errors high.

It is unfortunate that obtaining free parasites suitable for transport studies proved so troublesome because many further experiments could have been performed on them. Uptake could have been studied at different substrate concentrations to obtain estimates of Km and Vmax for the malarial transporter, and the effect of phloretin, phlorizin and niflumic acid on malarial transport could also have been investigated. There is some evidence that phlorizin directly affects the parasite, as well as inhibiting transport through the pore in the erythrocyte membrane (Silfen *et al*, 1988).

A criticism of the work on free parasites is that the parasites are still surrounded by an intact parasitophorous membrane (Choi and Mikkelsen, 1990). The volume of the vacuolar space is however very small compared to the intraparasitic volume. Also, the presence of a very high concentration of pores in the parasitophorous vacuolar

membrane (Desai *et al*, 1993), and presumably comparable levels of GLUT1 to the host's plasma membrane, suggests that the transport of sugar across the parasitophorous vacuolar membrane is not rate limiting.

Other useful experiments in the area could be performed using Sendai virus treated red blood cells (Silfen *et al*, 1988). These cells have large holes punched in the erythrocyte plasma membrane and the parasitophorous vacuolar membrane that are large enough to allow the entry of small proteins. This treatment induces cell-cell fusion, the almost complete release of haemoglobin and permeabilisation of the host cell membrane to 10 000 Dalton FITC-dextran (Silfen *et al*, 1988) but does not interfere with parasite maturation and release of merozoites. Malaria-infected cells treated with this virus will essentially behave like free parasites. These cells could be obtained in large quantities and would allow the characteristics of the parasite transporter to be determined.

When the zero-trans efflux of 6-deoxy-D-glucose into infected erythrocytes was studied it was found that export from uninfected cells was faster than export from malaria-infected cells (figures 36 and 37). This would be expected if the transport across the red blood cell membrane was faster than the export across the parasitic membrane (or the parasite vacuolar membrane). All the 6-deoxy-D-glucose left the uninfected cell through a fast route (GLUT1). This means that the export of hexose from uninfected cells follows first order kinetics, and that a graph of log fractional filling vs. time gives a straight line (figure 44).

In erythrocytes infected with a mature *Plasmodium falciparum* parasite the efflux is more complex. Assuming that the volume of space around the parasite (parasitophorous vacuolar space and the duct) is very small, the hexose will be in two main compartments: the red blood cell, and the parasite. From uptake studies it is likely that the 6-deoxy-D-glucose located in the erythrocyte will leave at a similar rate to the efflux from uninfected cells (figures 31, 32 and 34). The hexose located in the parasite can leave through 2 main routes: i) through the malarial transporter and the duct, ii) through the malarial transporter and across the parasitophorous and red blood

cell membranes via the pore or the host's transporter (figure 45). This complicated situation means that the efflux from infected cells is not first order (figure 44).

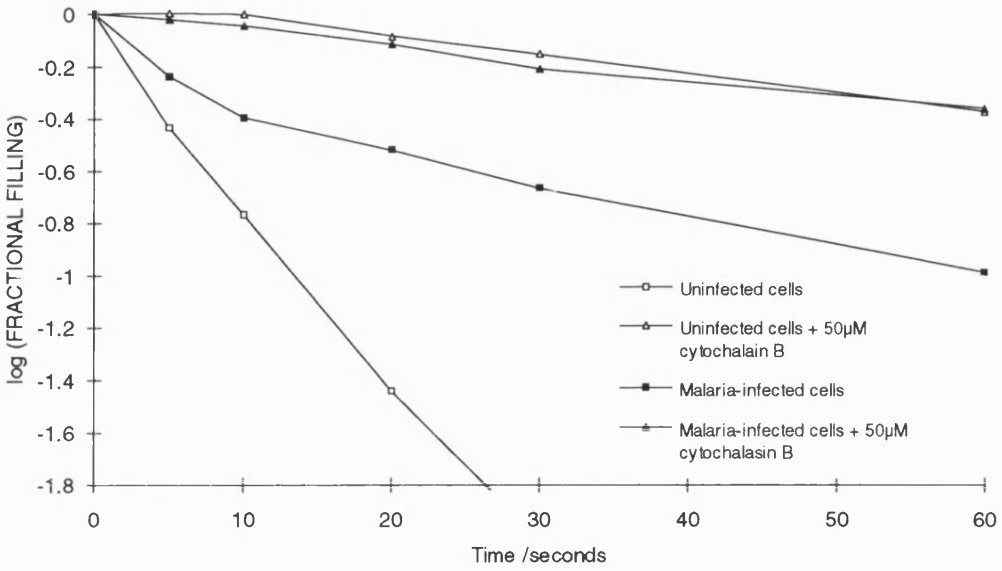

Figure 44. Zero-trans efflux of 20mM 6-deoxy-D-glucose in malaria-infected and uninfected red blood cells in the presence or absence of cytochalasin B. The data in figure 37 is expressed as log fractional filling [log $(S_i-S_\infty)/(S_o-S_\infty)$] versus time. The hexose leaves uninfected cells faster than infected cells. The efflux from uninfected cells appears to be first order (as it is reasonably close to linearity) and the efflux from infected cells a higher order. Cytochalasin B appears to be a good inhibitor in both malaria-infected and uninfected erythrocytes.

It is interesting to note that the 6-deoxy-D-glucose is not retained within the parasite. It can diffuse freely into and out of infected cells. This is further evidence that the malarial glucose transporter is facilitative and not a proton symporter as suggested by other workers (Izumo *et al*, 1989; Tanabe, 1990).

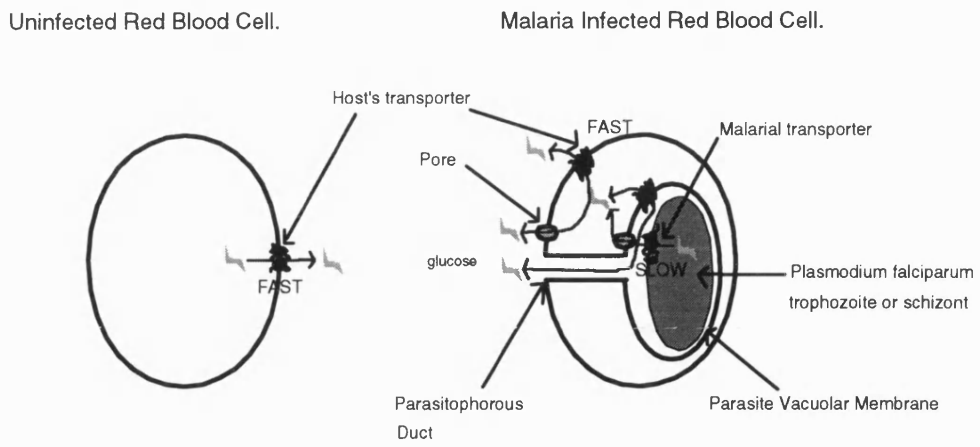

Figure 45. A model showing the predicted efflux of 6-deoxy-D-glucose from uninfected and malaria-infected red blood cells. In uninfected cells the sugar leaves the cells through the host's transporter. In infected cells the 6-deoxy-D-glucose in the erythrocyte leaves the cell either through the pore or through the host's transporter and the 6-deoxy-D-glucose in the parasite leaves through the parasite's transporter and then either through the duct or via the erythrocyte. The uptake experiments and the effective inhibition by 50μM cytochalasin B suggests that the pore has a minimal effect on the flux of sugar in infected cells; most leaves through GLUT1. The egress from the parasite is slower than from the red blood cell.

It has been attempted to model the efflux of 6-deoxy-D-glucose. The data from 6-deoxy-D-glucose efflux experiments (figure 39) was fitted to a two compartment, biphasic efflux model. This model is shown is figure 46.

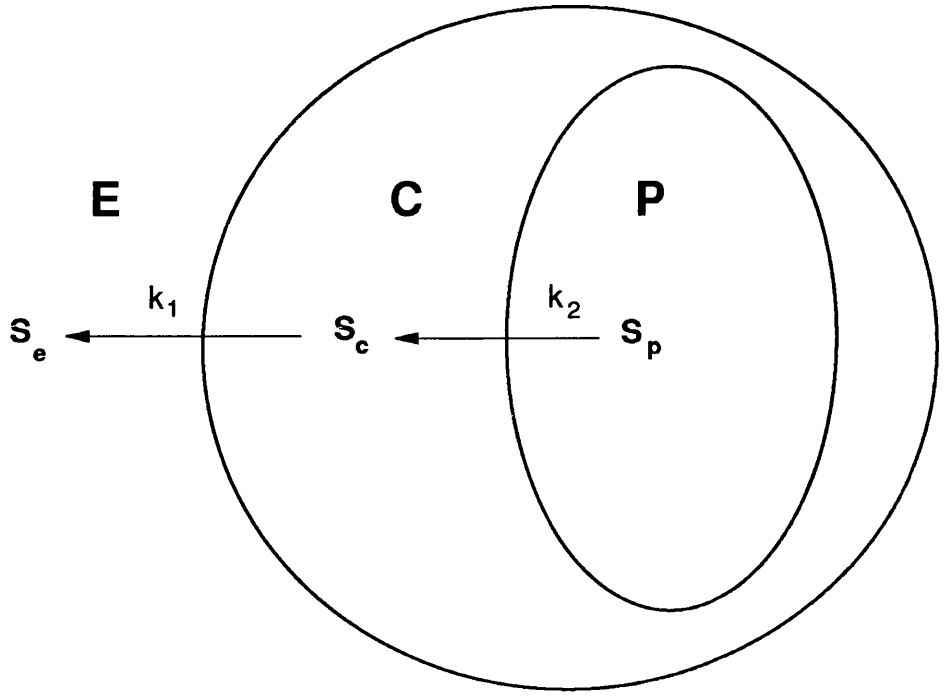

Figure 46. A simplified model of the flux of substrate out of a malaria infected cell. In the model there are three compartments called E (extracellular), C (red blood cell) and P (parasite). Initially sugar concentration in compartments C and P are at 1 unit and the concentration in compartment E is 0 units. As time proceeds sugar leaves C and P and enters E. In the model there will eventually be no sugar remaining in C or P. The rate at which sugar leaves C is described by the rate constant k_1 and the rate at which sugar leaves P is described by the rate constant k_2. Sugar leaves C faster than it leaves P.

The amount of sugar inside the malaria infected cell in figure 46 (i.e. S_c+S_p) is described by the biexponential equation:

$$S_i = ae^{-k_1 t} + be^{-k_2 t} + c$$

where S_i is the amount of sugar at time t, a, b, and c are constants that depend on the substrate concentration, and k_1 and k_2 are the rate constants. This approach has been used previously to model the flux of 3-O-methyl-D-glucose into intracellular compartments in *Xenopus* oocytes expressing GLUT1 or GLUT4 (Nishimura *et al*, 1993).

In malaria infected erythrocytes this equation is a necessary simplification. Not only is it possible that some 6-deoxy-D-glucose has direct access to the parasite through the

duct (i.e. the two compartments are in parallel as well as in series) but also the sugar leaves at least one of the compartments through two systems (through GLUT1 and the pore). When the initial concentration is standardised (i.e. the fractional filling is used) the constants a and b are proportional to the relative volumes of the compartments. At time = 0 S_i will be 1 and so a+b+c will equal 1. It can be shown that in this case that c will be zero and the constants a and b will be the fraction of the cells volume occupied by the compartments P and C respectively. The model also assumes that there is no flux of sugar back into the cell (i.e. that compartment E is very large compared to C and P). Using the data shown in figure 39 and the FigP iterative model solving program estimates of a=0.64 and b=0.42 were obtained at each substrate concentration. The constancy of a and b gives some confidence that a model of this nature has some validity. The estimates for a and b are in good agreement with the quoted volumes of the parasite and red blood cytosol from the literature (Choi and Mikkelsen,1990).

Using these values of a and b the rate constants shown in table 12 were obtained. These constants were used to graphically display the excellent fit obtained (figure 47).

Substrate concentration	k_1	k_2
5mM	0.204	0.039
10mM	0.128	0.025
15mM	0.085	0.022
30mM	0.054	0.011

Table 12. Values of constants (to 3 decimal places) obtained for the biexponential model using values of a=0.64 and b=0.42 in the equation shown above. The values are obtained using an iterative program and the data displayed in figure 39. Using these values the data is shown to be in good agreement with the model (figure 47).

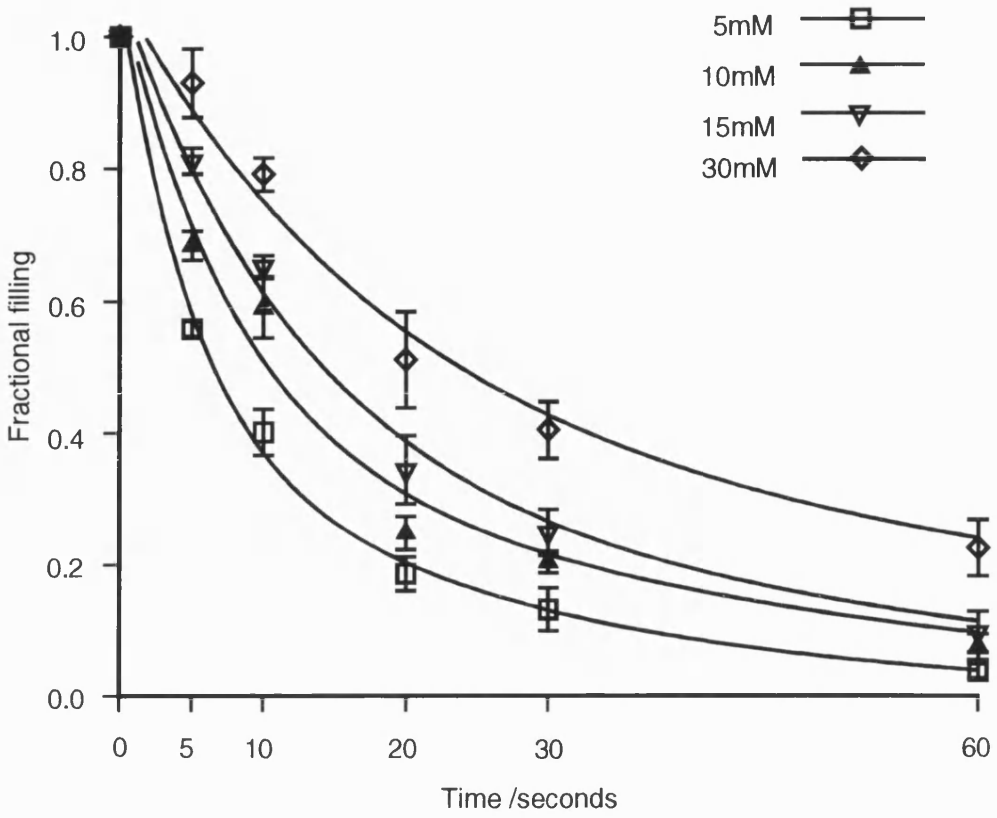

Figure 47. Replot of data shown in figure 39. The data is plotted as fractional filling $[(S_i-S_\infty)/(S_o-S_\infty)]$ versus time. The lines were generated using the FigP iterative program and the model shown in figure 46. The SEM are shown.

The data was insufficient to propose the involvement of saturatable carriers, and hence estimates for the Km and Vmax of the malaria transporter could not be accurately determined. More data points both at these concentrations and other concentrations would be required in order to calculate these constants. Released parasite studies could confirm the estimates obtained by this method.

Since the discovery of the parasitophorous duct (Pouvelle *et al*, 1991) the function of the malaria induced pores has become unclear. Our work shows that the pore is not important in the transport of glucose, which it transports relatively inefficiently. It has been suggested that the pore has specificity for small negatively charged molecules (Kirk *et al*, 1992b) like lactate (Kanaani and Ginsburg, 1991; Cramner and Halstrap, 1993). However, with the discovery of the duct it is clear that the parasite has direct

access to the periplasm and that transporters of these molecules are not required in the red blood cell membrane.

Treatment of normal red blood cells with the oxidising agent diamide has been shown to induce pores in the red blood cell membrane that have similar properties to those found in malaria-infected cells (Deuticke *et al*, 1983). Diamide cross-links spectrin, produces pores of diameter ~6.5Å, and allows the entry of molecules smaller than sucrose (Deuticke *et al*, 1983). Diamide induced pores do not increase the rate of D-glucose transport into uninfected human erythrocytes and it appears that the uptake is equally sensitive to cytochalasin B whether or not the cells are treated with diamide (Gero *et al*, 1991).

It has been suggested (Moulder, 1962) that in the course of evolution, the malarial parasite has lost many of the specific transporters in its plasma membrane. Moulder goes on to suggest that the parasite loses its specificity and becomes permeable to 'all sorts' of molecules. This suggestion has also been made by Sherman (1979) who suggested that the difficulty associated with obtaining released parasites that were not leaky was due to an inherent leakiness of plasmodial cells. If this were so, it would appear that some D-glucose would gain direct entry into the parasite, travelling down the duct and into the parasite through its leaky membrane. The mechanism of leakage may have been recently elucidated (Desai *et al*, 1993). These workers have studied a pore found in the parasitophorous vacuolar membrane of malaria-infected erythrocytes. They have suggested that these pores must exist in one of two configurations. In the first, the pore only spans the PVM; in the second, the pore has properties similar to a gap junction and joins both the PVM and the parasite's plasma membrane (Desai *et al*, 1993). If the second configuration is closer to the truth it would explain the leakage from malarial parasites. However, there is also some evidence against this hypothesis. First, inhibition data on freed parasites suggests that 6-deoxy-D-glucose uptake is inhibited by the glucose transporter specific cytochalasin B, albeit weakly. If the cells were leaky the effects of cytochalasin B on released parasites would have to be non-specific. A second piece of evidence is the different ion concentration in the parasite and the erythrocyte (Tanabe *et al*, 1983; Nillni *et al*, 1985). The pore in the PVM has

been reported to be an efficient mediator of both cation and anion flux (Desai *et al*, 1993). If the pore spans both these membranes it would be difficult to envisage a mechanism by which these different ion concentrations could be maintained. An increased leak of potassium ions from malaria infected red blood cells has been reported (Kirk *et al*, 1992a).

Other useful experiments in this area could be performed using *P. yoelii*-infected mouse erythrocytes. Unlike their human counterparts, mouse erythrocytes have a low basal glucose transport rate. This may partly explain the increased rate of transport of 2-deoxy-D-glucose into *P. yoelii*-infected erythrocytes when compared to uninfected control cells observed by Izumo *et al* (1989). Our results showed similar accumulation characteristics when the experiment was performed at 4°C, but using human cells the initial transport rate was unaltered by a malarial parasite. Experiments using 6-deoxy-D-glucose and *P. yoelii*-infected mouse erythrocytes would allow the malaria induced pathways (the pore and the duct) to be studied in more detail because the transport would be unlikely to be masked by the host's transport system.

The following conclusions on glucose transport into *P. falciparum*-infected red blood cells may be drawn from the present work.

- Incubation of the cells at 4°C before the experiment lowers the transport rate.
- The results of 2-deoxy-D-glucose uptake depends on the pre-incubation temperature.
- L-glucose is transported into uninfected cells through the host's transporter.
- Most L-glucose is transported into infected cells through the malaria induced pore. Some enters through the host's transporter.
- Most L-glucose leaves infected cells through the malaria induced pore.

- The majority of 6-deoxy-D-glucose enters the infected cell through the host's native transporter. Some enters through the malaria induced pore and some may enter through the parasitophorous duct.
- It is unlikely that the parasite modifies the host's native glucose transporter.
- 6-deoxy-D-glucose is transported into the cell at the same rate in infected and uninfected cells.
- Glucose is not actively transported either into the erythrocyte or the parasite.
- The parasite transporter is sensitive to cytochalasin B but is not inhibited to the same extent as the host's transporter.
- The efflux of 6-deoxy-D-glucose from uninfected cells is faster than from infected cells. Cytochalasin B is a good inhibitor of efflux from both cell types.
- The efflux of 6-deoxy-D-glucose from uninfected cells is first order. The efflux from infected cells is more complicated.

5. References

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Appendix 1. Alignment of Glucose Transporters from Various Species

HUMGLUT1	Human GLUT1 - Erythrocyte type	A_THALIANA	Arabidopsis glucose transporter - higher plant
RATGLUT1	Rat GLUT1 - Erythrocyte type	CKESS_GLUT	Chlorella - Glucose/H^+ transporter
MUSGLUT1	Mouse GLUT1 - Erythrocyte type	SYNECHOCYS	Synechocystis glucose transporter
RABGLUT1	Rabbit GLUT1 - Erythrocyte type	SCERE_SNF3	Yeast high affinity (SNF3) glucose transporter
PIGGLUT1	Pig GLUT1 - Erythrocyte type	Z_MOB_GLUT	Zymomonas mobilis glucose transporter
HUMGLUT2	Human GLUT2 - Liver type	ECOARAB/H+	E. coli Arabidopsis/H+ transporter
RATGLUT2	Rat GLUT2 - Liver type	ECOXYLO/H+	E. coli xylose/H+ transporter
MUSGLUT2	Mouse GLUT2 - Liver type	SCERE_GAL2	Yeast galactose transporter
HUMGLUT3	Human GLUT3 - Brain type	KLACT_RAG1	Kluyveromyces glucose transporter
HUMGLUT4	Human GLUT4 - Insulin regulatable	KLACT_LACP	Kluyveromyces lactose permease
RATGLUT4	Rat GLUT4 - Insulin regulatable	LENRI_GLUT	Leishmania glucose transporter
MUSGLUT4	Mouse GLUT4 - Insulin regulatable	N_CRASSQTR	Neurospora quinate transporter
HUMGLUT5	Human GLUT5 - Fructose transporter	A_NIGQPERM	Aspergillus quinate transporter

```
                1       .       .       .       .       .       .
HUMGLUT1  :     ------------------------------------------------------------
RATGLUT1  :     ------------------------------------------------------------
MUSGLUT1  :     ------------------------------------------------------------
RABGLUT1  :     ------------------------------------------------------------
PIGGLUT1  :     ------------------------------------------------------------
HUMGLUT2  :     ------------------------------------------------------------
RATGLUT2  :     ------------------------------------------------------------
MUSGLUT2  :     ------------------------------------------------------------
HUMGLUT3  :     ------------------------------------------------------------
HUMGLUT4  :     ------------------------------------------------------------
RATGLUT4  :     ------------------------------------------------------------
MUSGLUT4  :     ------------------------------------------------------------
HUMGLUT5  :     ------------------------------------------------------------
A_THALIANA:     ------------------------------------------------------------
CKESS_GLUT:     ------------------------------------------------------------
SYNECHOCYS:     ------------------------------------------------------------
SCERE_SNF3:     MDPNSNSSSETLRQEKQGFLDKALQRVKGIALRRNNSNKDHTTDDTTGSIRTPTSLQRQN
Z_MOB_GLUT:     ------------------------------------------------------------
ECOARAB/H+:     ------------------------------------------------------------
ECOXYLO/H+:     ------------------------------------------------------------
SCERE_GAL2:     ----------------------------MAVEENNVPVVSQQPQAGEDVISSLSKDSHL
KLACT_RAG1:     ----------------------------------MSNQMTDSTSAGSGTEHSVDTNT
KLACT_LACP:     --------------------------MADHSSSSSSLQKKPINTIEHKDTLGNDRDHK
LENRI_GLUT:     ------------------------------------------------------MSDR
N_CRASSQTR:     ------------------------------------------------------------
A_NIGQPERM:     ------------------------------------------------------------
```

```
                                                          //////////M1/////////
            61        .         .         .         .         .         .
HUMGLUT1  :  ------------------------MEPSSKKLTGRL---MLAVGGAVL-GSLQFGYNTGV
RATGLUT1  :  ------------------------MEPSSKKVTGRL---MLAVGGAVL-GSLQFGYNTGV
MUSGLUT1  :  ------------------------MDPSSKKVTGRL---MLAVGGAVL-GSLQFGYNTGV
RABGLUT1  :  ------------------------MEPSSKKVTGRL---MLAVGGAVL-GSLQFGYNTGV
PIGGLUT1  :  ------------------------------------------------------------
HUMGLUT2  :  --------------------------MTEDKVTGTL---VFTVITAVL-GSFQFGYDIGV
RATGLUT2  :  --------------------------MSEDKITGTL---AFTVFTAVL-GSFQFGYDIGV
MUSGLUT2  :  --------------------------MSEDKITGTL---AFTVFTAVL-SSFQFGYDIGV
HUMGLUT3  :  --------------------------MGTQKVTPAL---IFAITVATI-GSFQFGYNTGV
HUMGLUT4  :  ------------MPSGFQQIGSEDGEPPQQRVTGTL---VLAVFSAVL-GSLQFGYNIGV
RATGLUT4  :  ------------MPSGFQQIGSEDGEPPQQRVTGTL---VLAVFSAVL-GSLQFGYNIGV
MUSGLUT4  :  ----------MPSGFQQIGSDVKDGEPPRQRVTGTL---VLAVFSAVL-GSLQFGYNIGV
HUMGLUT5  :  -------------------MEQQDQSMKEGRLTLVL---ALATLIAAFGSSFQYGYNVAA
A_THALIANA:  -----------------MPAGGFVVGDGQKAYPGKLTPFVLFTCVVAAMGGLIFGYDIGI
CKESS_GLUT:  -------------MAGGGVVVVSGRGLSTGDYRGGLTVYVVMVAFMAACGGLLLGYDNGV
SYNECHOCYS:  --------------------MNPSSSPSQSTANVKF---VLLISGVAALGGFLFGFDTAV
SCERE_SNF3:  SDRQSNMTSVFTDDISTIDDNSILFSEPPQKQSMMM---SICVGVFVAVGGFLFGYDTGL
Z_MOB_GLUT:  --------------------------MSSESSQGL----VTRLALIAAIGGLLFGYDSAV
ECOARAB/H+:  ---------------MVTINTESALTPRSLRDTRRM---NMFVSVAAAVAGLLFGLDIGV
ECOXYLO/H+:  ---------------------------MNTQYNSSY---IFSITLVATLGGLLFGYDTAV
SCERE_GAL2:  SAQSQKYSNDELKAGESGPEGSQSVPIEIPKKPMSEYVTVSLLCLCVRFGGFMFGWDTST
KLACT_RAG1:  ALKAGSPNDLKVSHEEDLNDLEKTAEETLQQKPAKEYIFVSLCCVMVAFGGFVFGWDTGT
KLACT_LACP:  EALNSDNDNTSGLKINGVPIEDAREEVLLPGYLSKQYYKLYGLCFITYLCATMQGYDGAL
LENRI_GLUT:  VEVNERRSDSVSEKEPARDDARKDVTDDQEDAPPFMTANNARVMLVQAIGGSLNGYSIGF
N_CRASSQTR:  -------------------MTLLALKEDRPTPKAVYNWRVYTCAAIASFASCMIGYDSAF
A_NIGQPERM:  -------------------MSILALVEDRPTPREVYNWRVYLLAAVASFTSCMIGYDSAF

             /
           121        .         .         .         .         .         .
HUMGLUT1  :  INAPQKV---IEEFYNQTW---------VHRY----------------------------
RATGLUT1  :  INAPQKV---IEEFYNQTW---------NHRY----------------------------
MUSGLUT1  :  INAPQKV---IEEFYNQTW---------NHRY----------------------------
RABGLUT1  :  INAPQKV---IEEFYNQTW---------IHRY----------------------------
PIGGLUT1  :  ------------EFYNQTW---------LHRY----------------------------
HUMGLUT2  :  INAPQQV---IISHYRHVLGVPLDDRKAINNYVINSTDELPTISYSMNPKPTPW------
RATGLUT2  :  INAPQEV---IISHYRHVLGVPLDDRRATINYDINGTDTPLIVTPAHTTPDAW-------
MUSGLUT2  :  INAPQEV---IISHYRHVLGVPLDDRKAAINYDVNGTDTPLTVTPAYTTPAPW-------
HUMGLUT3  :  INAPEKI---IKEFINKTL---------TDK----------------------------
HUMGLUT4  :  INAPQKV---IEQSYNETW---------LGRQGP-------------------------
RATGLUT4  :  INAPQKV---IEQSYNATW---------LGRQGP-------------------------
MUSGLUT4  :  INAPQKV---IEQSYNATW---------LGRQGP-------------------------
HUMGLUT5  :  VNSPALL---MQQFYNETY---------YGR----------------------------
A_THALIANA:  SGGVTSMPSFLKRFFPSVY--------RKQQEG-------------------------
CKESS_GLUT:  TGGVVSLEA-FEKFFPDVWAKKQE-----------------------------------
SYNECHOCYS:  INGAVAA---LQKHFQ-------------------------------------------
SCERE_SNF3:  INSITSM------NYVKS-----------------------------------------
Z_MOB_GLUT:  IAAIGTP---VDIHFI-------------------------------------------
ECOARAB/H+:  IAGALPF---ITDHF--------------------------------------------
ECOXYLO/H+:  ISGTVES---LNTVFV-------------------------------------------
SCERE_GAL2:  ISGFV-----LQTDFLRRFG----------------------------------------
KLACT_RAG1:  ISGFVN-----QTDFLRRFG----------------------------------------
KLACT_LACP:  MGSIYT-----EDAYLKYY----------------------------------------
LENRI_GLUT:  VGVYSTLFGYSTNCASFLQENSCTTVPNADCKWFVSPTGSSYCGWPEVTCRKEYAYSSPA
N_CRASSQTR:  IGTTLAL-----PSFTKEF----------------------------------------
A_NIGQPERM:  IGTTLSL-----QSFQNEF----------------------------------------
```

```
                                                    ////////M2/////////
          181       .         .         .         .         .         .
HUMGLUT1  :  ---------------------GESILPTTLTT-LWSLSVAIFSVGGMIGSFSVGLFVNRF
RATGLUT1  :  ---------------------GESIPSTTLTT-LWSLSVAIFSVGGMIGSFSVGLFVNRF
MUSGLUT1  :  ---------------------GEPIPSTTLTT-LWSLSVAIFSVGGMIGSFSVGLFVNRF
RABGLUT1  :  ---------------------GERILPTTLTT-LWSLSVAIFSVGGMIGSFSVGLFVNRF
PIGGLUT1  :  ---------------------GESISPATLTT-LWSLSVAIFSVGGMIGSFSVGLFVNRF
HUMGLUT2  :  -------------------AEEETVAAAQLITMLWSLSVSSFAVGGMTASFFGGWLGDTL
RATGLUT2  :  --------------------EEETEGSAHIVTMLWSLSVSSFAVGGMVASFFGGWLGDKL
MUSGLUT2  :  -------------------DEEETEGSAHIVTMLWSLSVSSFAVDGMVASFFGGWLGDKL
HUMGLUT3  :  ---------------------GNAPPSEVLLTSLWSLSVAIFSVGGMIGSFSVGLFVNRF
HUMGLUT4  :  --------------------EGPSSIPPGTLTTLWALSVAIFSVGGMISSFLIGIISQWL
RATGLUT4  :  --------------------GGPDSIPQGTLTTLWALSVAIFSVGGMISSFLIGIISQWL
MUSGLUT4  :  --------------------GGPDSIPQGTLTTLWALSVAIFSVGGMISSFLIGIISQWL
HUMGLUT5  :  ---------------------TQEFMEDFPLTLLWSVTVSMFPFGGFIGSLLVGPLVNKF
A_THALIANA:  --------------------ASTNQYCQYDSPTL-TMFTSSLYLAALISSLVASTVTRKF
CKESS_GLUT:  --------------------VHEDSPYCTYDNAKLQLFVSSLFLAGLVSCLFASWITRNM
SYNECHOCYS:  --------------------------TDSLLT---GLSVSLALLGSALGAFGAGPIADRH
SCERE_SNF3:  ---------------------HVAPNHDSFTAQQMSILVSFLSLGTFFGALTAPFISDSY
Z_MOB_GLUT:  ---------------------APRHLSATAAASLSGMVVVAVLVGCVTGSLLSGWIGIRF
ECOARAB/H+:  ----------------------------VLTSRLQEWVVSSMMLGAAIGALFNGWLSFRL
ECOXYLO/H+:  ---------------------APQNLSESAANSLLGFCVASALIGCIIGGALGGYCSNRF
SCERE_GAL2:  --------------------MKHKDGTHYLSNVRTGLIVAIFNIGCAFGGIILSKGGDMY
KLACT_RAG1:  --------------------QEKADGSHYLSNVRTGLIVSIFNIGCAVGGIVLSNIGDRW
KLACT_LACP:  -------------------------HLDINSSSGTGLVFSIFNVGQICGAFFVPLMD-WK
LENRI_GLUT:  EMPGALARCEADSRCRWSYSDEECQNPSGYSSSESGIFAGSMIAGCLIGSVFAGPLASK-
N_CRASSQTR:  ---------------------DFASYTPGALALLQSNIVSVYQAGAFFGCLFAYATSYFL
A_NIGQPERM:  ------------------------NWESLNTDLISANIVSLYQRGAFFGALFAYPIGHFW

                ////////M3/////////                          ////M4//
          241       .         .         .         .         .         .
HUMGLUT1  :  -GRRNSMLMMNLLAFVSAVLMGFSKLGKS---------------FEMLILGRFIIGVYCG
RATGLUT1  :  -GRRNSMLMMNLLAFVSAVLMGFSKLGKS---------------FEMLILGRFIIGVYCG
MUSGLUT1  :  -GRRNSMLMMNLLAFVAAVLMGFSKLGKS---------------FEMLILGRFIIGVYCG
RABGLUT1  :  -GRRNSMLMMNLLAFVSAVLMGFSKLAKS---------------FEMLILGRFIIGVYCG
PIGGLUT1  :  -GRRNSMLMMNLLAFISAVLMGFSKLGKS---------------FEMLILGRFIIGVYCG
HUMGLUT2  :  -GRIKAMLVANILSLVGALLMGFSKLGPS---------------HILIIAGRSISGLYCG
RATGLUT2  :  -GRIKAMLAANSLSLTGALLMGCSKFGPA---------------HALIIAGRSVSGLYCG
MUSGLUT2  :  -GRIKAMLAANSLSLTGALLMGCSKFGPA---------------HALIIAGRSVSGLYCG
HUMGLUT3  :  -GRRNSMLIVNLLAVTGGCFMGLCKVAKS---------------VEMLILGRLVIGLFCG
HUMGLUT4  :  -GRKRAMLVNNVLAVLGGSLMGLANAAAS---------------YEMLILGRFLIGAYSG
RATGLUT4  :  -GRKRAMLANNVLAVLGGALMGLANAAAS---------------YEILILGRFLIGAYSG
MUSGLUT4  :  -GRKRAMLANNVLAVLGGALMGLANAVAS---------------YEILILGRFLIGAYSG
HUMGLUT5  :  -GRKGALLFNNIFSIVPAILMGCSRVATS---------------FELIIISRLLVGICAG
A_THALIANA:  -GRRLSMLFGGILFCAGALINGFAKHVW-----------------MLIVGRILLGFGIG
CKESS_GLUT:  -GRKVTMGIGGAFFVAGGLVNAFAQDM-----------------AMLIVGRVLLGFGVG
SYNECHOCYS:  -GRIKTMILAAVLFTLSSIGSGLPFTIWD---------------F---IFWRVLGGIGVG
SCERE_SNF3:  -GRKPTIIFSTIFIFSIGNSLQVGAGG-----------------ITLLIVGRVISGIGIG
Z_MOB_GLUT:  FGRRGGLLMSSICFVAAGFGAALTEKLFGTGGSA----------LQIFCFFRFLAGLGIG
ECOARAB/H+:  -GRKYSLMAGAILFVLGSIGSAFATSV-----------------EMLIAARVVLGIAVG
ECOXYLO/H+:  -GRRDSLKIAAVLFFISGVGSAWPELGFTSINPDNTVPVYLAGYVPEFVIYRIIGGIGVG
SCERE_GAL2:  -GRKKGLSIVVSVYIVGIIIQIASINKW----------------YQYFI-GRIISGLGVG
KLACT_RAG1:  -GRRIGLITVIIIYVIGIIIQIASVDKWY-----------------QYFIGRIISGLGVG
KLACT_LACP:  -GRKPAILIGCLGVVIGAIISSLTTTKSA-----------------LIGGRWFVAFFAT
LENRI_GLUT:  IGARLSFLLVGLVGVVASVMYHASCAA---------------DEFWVLIVGRFVIGLFLG
N_CRASSQTR:  -GRRKSLIAFSVVFIIGAAIMLAADGQGRG--------------IDPIIAGRVLAGIGVG
A_NIGQPERM:  -GRRWGLMFSALIFFLGAGMMLGANGDRG---------------LGLIYGGRVLAGIGVG
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                 /////M4//////          //////////M5/////////
              301         .           .           .           .           .
HUMGLUT1   :  LTTGFVPMYVGEVSPTAFRGALGTLHQLGIVVGILIAQVFGL-DSIM--GNKD---L---
RATGLUT1   :  LTTGFVPMYVGEVSPTALRGALGTLHQLGIVVGILIAQVFGL-DSIM--GNAD---L---
MUSGLUT1   :  LTTGFVPMYVGEVSPTALRGALGTLHQLGIVVGILIAQVFGL-DSIM--GNAD---L---
RABGLUT1   :  LTTGFVPMYVGEVSPTALRGALGTLHQLGIVVGILIAQVFGL-DSIM--GNED---L---
PIGGLUT1   :  LTTGFVPMYVGEVSPTALRGALGTLHQLGIVVGILIAQVFGL-DSIM--GNEE---L---
HUMGLUT2   :  LISGLVPMYIGEIAPTALRGALGTFHQLAIVTGILISQIIGL-EFIL--GNYD---L---
RATGLUT2   :  LISGLVPMYIGEIAPTTLRGALGTLHQLALVTGILISQIAGL-SFIL--GNQD---Y---
MUSGLUT2   :  LISGLVPMYIGEIAPTTLRGALGTLHQLALVTGILISQIAGL-SFIL--GNQD---H---
HUMGLUT3   :  LCTGFVPMYIGEISPTALRGAFGTLNQLGIVVGILVAQIFGL-EFIL--GSEE---L---
HUMGLUT4   :  LTSGLVPMYVGEIAPTHLRGALGTLNQLAIVIGILIAQVLGL-ESLL--GTAS---L---
RATGLUT4   :  LTSGLVPMYVGEIAPTHLRGALGTLNQLAIVIGILVAQVLGL-ESML--GTAT---L---
MUSGLUT4   :  LTSGLVPMYVGEIAPTHLRGALGTLNRLAIVIGILVAQVLGL-ESML--GTAT---L---
HUMGLUT5   :  VSSNVVPMYLGELAPKNLRGALGVVPQLFITVGILVAQIFGL-RNLL--ANVD---G---
A_THALIANA:   FANQAVPLYLSEMAPYKYRGALNIGFQLSITIGILVAEVLNY-FFAK--IKGG---W---
CKESS_GLUT:   LGSQVVPQYLSEVAPFSHRGMLNIGYQLFVTIGILIAGLVNYAVRDWENG----------
SYNECHOCYS:   AASVIAPAYIAEVSPAHLRGRLGSLQQLAIVSGIFIALLSNWFIALMAGGSAQNPWLFGA
SCERE_SNF3:   AISAVVPLYQAEATHKSLRGAIISTYQWAITWGLLVSSAVSQGTHARNDASS--------
Z_MOB_GLUT:   VVSTLTPTYIAEIRPPDKRGQMVSGQQMAIVTGALTGYIFTWLLAHFGSIDWV-----NA
ECOARAB/H+:   IASYTAPLYLSEMASENVRGKMISMYQLMVTLGIVLAFLSDTAFSYS--GN---------
ECOXYLO/H+:   LASMLSPMYIAELAPAHIRGKLVSFNQFAIIFGQLLVYCVNYFIARS--GDAS---WLNT
SCERE_GAL2:   GIAVLCPMLISEIAPKHLRGTLVSCYQLMITAGIFLGYCTNYGTKSYSNSVQ--------
KLACT_RAG1:   GITVLSPMLISETAPKHLRGTLVSCYQLMITFGIFLGYCTNYGTKNYSNSVQ--------
KLACT_LACP:   IANAAAPTYCAEVAPAHLRGKVAGLYNTLWSVGSIVAAFSTYGTNKNFPNSSK-------
LENRI_GLUT:   VICVACPVYTDQNAHPKWKRTIGVMFQVFTTLGIFVAALMGLALGQSIRFDHDGDQKVMA
N_CRASSQTR:   GASNMVPIYISELAPPAVRGRLVGIYELGWQIGGLVGFWINYGVNTTMAPTRS-------
A_NIGQPERM:   AGSNICPIYISEMAPSAIRGRLVGVYELGWQIGGVVGFWINYGVDETLAPSHK-------

                  //////////M6/////////
              361         .           .           .           .           .
HUMGLUT1   :  --WPLLLSIIFIPALLQCIVLPFCPESPRFLL-INRNEENRAKSV--LKKLRGTAD-VTH
RATGLUT1   :  --WPLLLSVIFIPALLQCILLPFCPESPRFLL-INRNEENRAKSV--LKKLRGTAD-VTR
MUSGLUT1   :  --WPLLLSVIFIPALLQCILLPFCPESPRFLL-INRNEENRAKSV--LKKLRGTAD-VTR
RABGLUT1   :  --WPLLLSVIFVPALLQCIVLPLCPESPRFLL-INRNEENRAKSV--LKKLRGNAD-VTR
PIGGLUT1   :  --WPLLLSVIFIPALLQCVLLPFCPESPRFLL-INRNEENRAKSV--LKKLRGTAD-VTR
HUMGLUT2   :  --WHILLGLSGVRAILQSLLLFFCPESPRYLY-IKLDEEVKAKQS--LKRLRGYDD-VTK
RATGLUT2   :  --WHILLGLSAVPALLQCLLLLFCPESPRYLY-LNLEEEVRAKKS--LKRLRGTED-ITK
MUSGLUT2   :  --WHILLGLSAVPALLQCLLLLFCPESPRYLY-IKLEEEVRAKKS--LKRLRGTED-VTK
HUMGLUT3   :  --WPLLLGFTILPAILQSAALPFCPESPRFLL-INRKEEENAKQI--LQRLWGTQD-VSQ
HUMGLUT4   :  --WPLLLGLTVLPALLQLVLLPFCPESPRYLY-IIQNLEGPARKS--LKRLTGWAD-VSG
RATGLUT4   :  --WPLLLAITVLPALLQLLLLPFCPESPRYLY-IIRNLEGPARKS--LKRLTGWAD-VSD
MUSGLUT4   :  --WPLLLALTVLPALLQLILLPFCPESPRYLY-IIRNLEGPARKS--LKPLTGWAD-VSD
HUMGLUT5   :  --WPILLGLTGVPAALQLLLLPFFPESPRYLL-IQKKDEAAAKKA--LQTLRGWDS-VDR
A_THALIANA:   -GWRLSLGGAVVPALIITIGSLVLPDTPNSM--IERGQHEEAKTK--LRRIRGVDD-VSQ
CKESS_GLUT:   --WRLSLGLAAAPGAILFLGSLVLPESPNFL--VEKGKTEKGREV--LQKLRGTSEVDAE
SYNECHOCYS:   AAWRWMFWTELIPALLYGVCAFLIPESPRYL--VAQGQGEKAAAI--LWKVEG-GD-VPS
SCERE_SNF3:   --YRIPIGLQYVWSSFLAIGMFFLPESPRYYV-LKDKLDEAAKSLSFLRGVPVHDSGLLE
Z_MOB_GLUT:   SGWCWSPASEGLIGIAFLLLLLTAPDTPHWL--VMKGRHSEASKI--LARLEPQAD-PNL
ECOARAB/H+:   --WRAMLGVLALPAVLLIILVVFLPNSPRWL--AEKGRHIEAEEV--LRMLRDTSE-KAR
ECOXYLO/H+:   DGWRYMFASECIPALLFLMLLYTVPESPRWL--MSRGKQEQAEGI--LRKIMGNTL-ATQ
SCERE_GAL2:   --WRVPLGLCFAWSLFMIGALTLVPESPRYLC-EVNKVEDAKRSIAKSNKVSPEDPAVQA
KLACT_RAG1:   --WRVPLGLCFAWAIFMVLGMMFVPESARFLV-ETDQIEEARKSLAKTNKVSIDDPVVKY
KLACT_LACP:   -AFKIPLYLQMMFPGLVCIFGWLIPESPRWLVGVGREEEAREFIIKYHLNGDRTHPLLDM
LENRI_GLUT:   RMQGLCVFSTLFSLLTVVLGIVT-RESRAKFDGGEEGRAELNPSEYGYVEMIPR------
N_CRASSQTR:   -QWLIPFAVQLIPAGLLFLGSFWIPESPRWLY-ANGKREEAMKVLCWIRNLEPTDRYIVQ
A_NIGQPERM:   -QWIIPFAVQLIPAGLLIIGALLIRESPRWLF-LRGNREKGIETLAWIRNLPADHIYMVE
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                                                  //////////M7/////////
           421        .         .         .         .         .         .
HUMGLUT1   :  DLQEMKEESRQMMREKKVTILELF----RSPAYRQPILIAVVLQLSQQLSGINAVFYYST
RATGLUT1   :  DLQEMKEEGRQMMREKKVTILELF----RSPAYRQPILIAVVLQLSQQLSGINAVFYYST
MUSGLUT1   :  DLQEMKEEGRQMMREKKVTILELF----RSPAYRQPILIAVVLQLSQQLSGINAVFYYST
RABGLUT1   :  DLQEMKEESRQMMREKKVTILELF----RSPAYRQPILSAVVLQLSQQLSGINAVFYYST
PIGGLUT1   :  DLQEMKEESRQMMREKKVTILELF----RSAAYRQPILIAVVLQLSQQLSGINAVFYYST
HUMGLUT2   :  DINEMRKEREEASSEQKVSIIQLF----TNSSYRQPILVALMLHVAQQFSGINGIFYYST
RATGLUT2   :  DINEMRKEKEEASTEQKVSVIQLF----TDPNYRQPIVVALMLHLAQQFSGINGIFYYST
MUSGLUT2   :  DINEMKKEKEEASTEQKVSVIQLF----TDANYRQPILVALMLHMAQQFSGINGIFYYST
HUMGLUT3   :  DIQEMKDESARMSQEKQVTVLELF----RVSSYRQPIIISIVLQLSQQLSGINAVFYYST
HUMGLUT4   :  VLAELKDEKRKLERERPLSLLQLL----GSRTHRQPLIIAVVLQLSQQLSGINAVFYYST
RATGLUT4   :  ALAELKDEKRKLERERPLSLLQLL----GSRTHRQPLIIAVVLQLSQQLSGINAVFYYST
MUSGLUT4   :  ALAELKDEKRKLERERPMSLLQLL----GSRTHRQPLIIAVVLQLSQQLSGINAVFYYST
HUMGLUT5   :  EVAEIRQEDEAEKAAGFISVLKLF----RMRSLRWQLLSIIVLMGGQQLSGVNAIYYYAD
A_THALIANA:  EFDDLVAASKESQSIEH-PWRNLL----R-RKYRPHLTMAVMIPFFQQLTGINVIMFYAP
CKESS_GLUT:  FADIVAAVEIARPITMRQSWASLF-----TRRYMPQLLTSFVIQFFQQFTGINAIIFYVP
SYNECHOCYS:  RIEEI-QATVSLDHKPRFSDL-LS----RRGGLLPIVWIGMGLSALQQFVGINVIFYYSS
SCERE_SNF3:  ELVEIKATYDYEASFGSSNFIDCFISSKSRPKQTLRMFTGIALQAFQQFSGINFIFYYGV
Z_MOB_GLUT:  TIQKIKAGFDKAMDKSSAGLFAF---------GITVVFAGVSVAAFQQLVGINAVLYYAP
ECOARAB/H+:  E--ELNEIRESLKLKQGGWAL-FK----INRNVRRAVFLGMLLQAMQQFTGMNIIMYYAP
ECOXYLO/H+:  AVQEIKHSLDHGR--KTGGRLLMF----GVGV----IVIGVMLSIFQQFVGINVVLYYAP
SCERE_GAL2:  ELDLIMAGIEAEKLAGNASWGELF---STKTKVFQRLLMGVFVQMFQQLTGNNYFFYYGT
KLACT_RAG1:  ELLKIQSSIELEKAAGNASWGELI---TGKPSMFRRTLMGIMIQSLQQLTGDNYFFYYGT
KLACT_LACP:  EMAEIIESFHGTDLSNPLEMLDVR-SLFRTRSDRYRAMLVILMAWFGQFSGNNVCSYYLP
LENRI_GLUT:  -------------------------------------LLMGCVMAGTLQLTGINAVMNYAP
N_CRASSQTR:  EVSFIDADLERYTRQVGNGFWKPF-LSLKQRKVQWRFFLGGMLFFWQNGSGINAINYYSP
A_NIGQPERM:  EINMIEQSLEQQRVKIGLGFWKPFKAAWTNKRILYRLFLGSMLFLWQNGSGINAINYYSP

                          ////////M8/////////           ////////M9////
           481        .         .         .         .         .         .
HUMGLUT1   :  SIFEKAGV---QQPVYATIGSGIVNTAFTVVSLFV-VERAGRRTLHLIGLAGMAGCAILM
RATGLUT1   :  SIFEKAGV---QQPVYATIGSGIVNTAFTVVSLFV-VERAGRRTLHLIGLAGMAGCAVLM
MUSGLUT1   :  SIFEKAGV---QQPVYATIGSGIVNTAFTVVSLFV-VERAGRRTLHLIGLAGMAGCAVLM
RABGLUT1   :  SIFEKAGV---QQPVYATIGSGIVNTAFTVVSLFV-VERAGRRTLHLIGLAGMAACAVLM
PIGGLUT1   :  SIFEKAGV---QQPVYATIGSGIVNTAFTVVSLFV-VERAGRRTLHLIGLAGMAGCAVLM
HUMGLUT2   :  SIFQTAGI---SKPVYATIGVGAVNMVFTAVSVFL-VEKAGRRSLFLIGMSGMFVCAIFM
RATGLUT2   :  SIFQTAGI---SQPVYATIGVGAINMIFTAVSVLL-VEKAGRRTLFLAGMIGMFFCAVFM
MUSGLUT2   :  TIFQTAGI---SQPVYATIGVGAINMIFTAVSVLL-VEKAGRRTLFLTGMIGMFFCTIFM
HUMGLUT3   :  GIFKDAGV---QEPIYATIGAGVVNTIFTVVSLFL-VERAGRRTLHMIGLGGMAFCSTLM
HUMGLUT4   :  SIFETAGV---GQPAYATIGAGVVNTVFTLVSVLL-VERAGRRTLHLLGLAGMCGCAILM
RATGLUT4   :  SIFELAGV---EQPAYATIGAGVVNTVFTLVSVLL-VERAGRRTLHLLGLAGMCGCAILM
MUSGLUT4   :  SIFESAGV---GQPAYATIGAGVVNTVFTLVSVLL-VERAGRRTLHLLGLAGMCGCAILM
HUMGLUT5   :  QIYLSAGVPE-EHVQYVTAGTGAVNVVMTFCAVFV-VELLGRRLLLLLGFSICLIACCVL
A_THALIANA:  VLFNTIGFTT-DASLMSAVVTGSVNVGATLVSIYG-VDRWGRRFLFLEGGTQMLICQAVV
CKESS_GLUT:  VLFSSLGSAN-SAALLNTVVVGAVNVGSTLIAVMF-SDKFGRRFLLIEGGIQCCLAMLTT
SYNECHOCYS:  VLWRSVGFTE-EKSLLITVITGFINILTTIVAIAF-VDKFGRKPLLLMGSIGMTITLGIL
SCERE_SNF3:  NFFNKTGV---SNSYLVSFITYAVNVVFNVPGLFF-VEFFGRRKVLVVGGVIMTIANFIV
Z_MOB_GLUT:  QMFQNLGFGA-DTALLQTISIGVVNFIFTMIASRV-VDRFGRKPLLIWGALGMAAMMAVL
ECOARAB/H+:  RIFKMAGFTTTEQQMIATLVVGLTFMFATFIAVFT-VDKAGRKPALKIGFSVMALGTLVL
ECOXYLO/H+:  EVFKTLGAST-DIALLQTIIVGVINLTFTVLAIMT-VDKFGRKPLQIIGALGMAIGMFSL
SCERE_GAL2:  VIFKSVGL---DDSFETSIVIGVVNFASTFFSLWT-VENLGRRKCLLLGAATMMACMVIY
KLACT_RAG1:  TIFQSVGM---DDSFETSIVLGIVNFASTFFALYT-VDHFGRRNCLLYGCVGMVACYVVY
KLACT_LACP:  TMLRNVGMKSVSLNVLMNGVYSIVTWISSICGAFF-IDKIGRREGFLGSISGAALALTGL
LENRI_GLUT:  TIMGSLGL----APLVGNFVVMLWNFVTTLASIPLSYVFTMRHVFLFGSIFTSCMCLFMC
N_CRASSQTR:  TVFRSIGITGTDTGFLTTGIFGVVKMVLTIIWLLWLVDLVGRRRILFIGAAGGSLCMWFI
A_NIGQPERM:  RVFKSIGVSGGNTSLLTTGIFGVVTAVITFVWLLYLIDHFGRRNLLLVGAAGGSVCLWIV
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            //M9///                    /////////M10/////////
          541        .         .         .         .         .
HUMGLUT1  :  TIALALL-------E-QLP--WMSYLSIVAIFGFVAFFEVGPGPIPWFIVAELFSQGPRP
RATGLUT1  :  TIALALL-------E-QLP--WMSYLSIVAIFGFVAFFEVGPGPIPWFIVAELFSQGPRP
MUSGLUT1  :  TIALALL-------E-RLP--WMSYLSIVAIFGFVAFFEVGPGPIPWFIVAELFSQGPRP
RABGLUT1  :  TIALALL-------E-QLP--WMSYLSIVAIFGFVAFFEVGPGPIPWFIVAELFSQGPRP
PIGGLUT1  :  TIALALL-------E-QLP--WMSYLSIVAIFGFVAFFEVGPGPIPWFIVAELFSQGPRP
HUMGLUT2  :  SVGLVLL-------N-KFS--WMSYVSMIAIFLFVSFFEIGPGPIPWFMVAEFFSQGPRP
RATGLUT2  :  SLGLVLL-------D-KFT--WMSYVSMTAIFLFVSFFEIGPGPIPWFMVAEFFSQGPRP
MUSGLUT2  :  SVGLVLL-------D-KFA--WMSYVSMTAIFLFVSFFEIGPGPIPWFMVAEFFSQGPRP
HUMGLUT3  :  TVSLLLK-------D-NYN--GMSFVCIGAILVFVAFFEIGPGPIPWFIVAELFSQGPRP
HUMGLUT4  :  TVALLLL-------E-RVP--AMSYVSIVAIFGFVAFFEIGPGPIPWFIVAELFSQGPRP
RATGLUT4  :  TVALLLL-------E-RVP--SMSYVSIVAIFGFVAFFEIGPGPIPWFIVAELFSQGPRP
MUSGLUT4  :  TVALLLL-------E-RVP--AMSYVSIVAIFGFVAFFEIGPGPIPWF-VAELFSQGPRP
HUMGLUT5  :  TAALALQ-------D-TVS--WMPYISIVCVISYVIGHALGPSPIPALLITEIFLQSSRP
A_THALIANA:  AACIGAKFGVDGTPG-ELP-KWYAIVVVTFICIYVAGFAWSWGPLGWLVPSEIFPLEIRS
CKESS_GLUT:  GVVLAIEF---AKYGTDPLPKAVASGILAVICIFISGFAWSWGPMGWLIPSEIFTLETRP
SYNECHOCYS:  SVVFGGATVVNG-QP-TLT-GAAGIIALVTANLYVFSFGFSWGPIVWVLLGEMFNNKIRA
SCERE_SNF3:  AIVGCSL-------K-TVA---AAKVMIAFICLFIAAFSATWGGVVWVISAELYPLGVRS
Z_MOB_GLUT:  GCCFWFK--------------VGGVLPLASVLLYIAVFGMSWGPVCWVVLSEMFPSSIKG
ECOARAB/H+:  GYCLMQFD-----NG-TAS-SGLSWLSVGMTMMCIAGYAMSAAPVVWILCSEIQPLKCRD
ECOXYLO/H+:  GTAFYTQ--------------APGIVALLSMLFYVAAFAMSWGPVCWVLLSEIFPNAIRG
SCERE_GAL2:  ASVGVTRLYPHGKSQ-PSS-KGAGNCMIVFTCFYIFCYATTWAPVAWVITAESFPLRVKS
KLACT_RAG1:  ASVGVTRLWPDGPDHPDISSKGAGNCMIVFACFYIFCFATTWAPIAYVVISESYPLRVKG
KLACT_LACP:  SICTARY-------E-KTKKKSASNGALVFIYLFGGIFSFAFTPMQSMYSTEVSTNLTRS
LENRI_GLUT:  GIPVYPG--------VSKKLEAKNGVAITGILLFILGFEVCVGPCYYVLTQDMFPPSFRP
N_CRASSQTR:  GAYIKIADPGSNKAE-DAKLTSGGIAAIFFFYLWTAFYTPSWNGTPWVINSEMFDQNTRS
A_NIGQPERM:  GGYIKIAKPENN-PE-GTQLDSGGIAAIFFFYLWTAFYTPSWNGTPWVINSEMFDPTVRS

            ////////M11//////////                   ////////M12////////
          601        .         .         .         .         .
HUMGLUT1  :  AAIAVAGFSNWTSNFIVGMCFQ-------YVEQL------CGPYVFIIFTVLLVLFFIFT
RATGLUT1  :  AAVAVAGFSNWTSNFIVGMCFQ-------YVEQL------CGPYVFIIFTVLLVLFFIFT
MUSGLUT1  :  AAIAVAGFSNWTSNFIVGMCFQ-------YVEQL------CGPYVFIIFTVLLVLFFIFT
RABGLUT1  :  AAVAVAGFSNWTSNFIVGMCFQ-------YVEQL------CGPYVFIIFTVLLVLFFIFT
PIGGLUT1  :  AAIAVAGFSNWTSNFIVGMCFQ-------YVEQL------CGPYVFIIFTVLLVLFFIFT
HUMGLUT2  :  AALAIAAFSNWTCNFIVALCFQ-------YIADF------CGPYVFFLFAGVLLAFTLFT
RATGLUT2  :  TALALAAFSNWVCNFIIALCFQ-------YIADF------LGPYVFFLFAGVVLVFTLFT
MUSGLUT2  :  TALALAAFSNWVCNFVIALCFQ-------YIADF------LGPYVFFLFAGVVLVFTLFT
HUMGLUT3  :  AAMAVAGCSNWTSNFLVGLLFP-------SAAHY------LGAYVFIIFTGFLITFLAFT
HUMGLUT4  :  AAMAVAGFSNWTSNFIIGMGFQ-------YVAEA------MGPYVFLLFAVLLLGFFIFT
RATGLUT4  :  AAMAVAGFSNWTCNFIVGMGFQ-------YVADA------MGPYVFLLFAVLLLGFFIFT
MUSGLUT4  :  AAMAVAGFSNWTCNFIVGMGFQ-------YVADR------MGPYVFLLFAVLLLGFFIFT
HUMGLUT5  :  SAFMVGGSVHWLSNFTVGLIFP-------FIQEG------LGPYSFIVFAVICLLTTIYI
A_THALIANA:  AAQSITVSVNMIFTFIIAQIFL-------TMLCH------LKFGLFLVFAFFVVVMSIFV
CKESS_GLUT:  AGTAVAVVGNFLFSFVIGQAFV-------SMLCA------MEYGVFLFFAGWLVIMVLCA
SYNECHOCYS:  AALSVAAGVQWIANFIISTTFPP------LLDTV------GLGPAYGLYATSAAISIFFI
SCERE_SNF3:  KCTAICAAANWLVNFICALITP-------YIVDTGSHTSSLGAKIFFIWGSLNAMGVIVV
Z_MOB_GLUT:  AAMPIAVTGQWLANILVNFLFKVADGSPALNQTF------NHGFSYLVFAALSILGGLIV
ECOARAB/H+:  FGITCSTTTNWVSNMIIGATFLT------LLDSI------GAAGTFWLYTALNIAFVGIT
ECOXYLO/H+:  KALAIAVAAQWLANYFVSWTFPMMDKNSWLVAHF------HNGFSYWIYGCMGVLAALFM
SCERE_GAL2:  KCMALASASNWVWGFLIAFFTP-------FITSA------INFYYAYVFMGCLVAMFFYV
KLACT_RAG1:  KAMAIASASNWIWGFLIGFFTP-------FITSA------IHFYYGYVFMGCMVFAFFYV
KLACT_LACP:  KAQLLNFVVSGVAQFVNQFATP-------KAMKN------IKYWFYVFYVFFDIFEFIVI
LENRI_GLUT:  RGASFTQVAQFIFNLIINVCYP-----IATESISGGPSGNQDKGQAVAFIFFGGLGLICF
N_CRASSQTR:  LGQASAAANNWFWNFIISRFTP-------QMFIK------MEYGVYFFFASLMLLSIVFI
A_NIGQPERM:  LAQACAAASNWLWNFLISRFTP-------QMFTS------MGYGVYFFFASLMILSIVFV
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            //
           661        .         .         .         .         .         .
HUMGLUT1   :  YFKVPETKGRTFDEIASGF--R---QGGAS---------QSDKTPEELFHPLGADSQV--
RATGLUT1   :  YFKVPETKGRTFDEIASGF--R---QGGAS---------QSDKTPEELFHPLGADSQV--
MUSGLUT1   :  YFKVPETKGRTFDEIASGF--R---QGGAS---------QSDKTPEELFHPLGADSQV--
RABGLUT1   :  YFKVPETKGRTFDEIASGF--R---QGGAS---------QSDKTPEELFHPLGADSQV--
PIGGLUT1   :  YFKVPETKGRTFDEIASGF--R---QGGAS---------QSDKTPEELFHPLGADSQV--
HUMGLUT2   :  FFKVPETKGKSFEEIAAEF--Q---KKSGS---------AHRPKAAVEMKFLGATETV--
RATGLUT2   :  FFKVPETKGKSFDEIAAEF--R---KKSGS---------APPRKATVQMEFLGSSETV--
MUSGLUT2   :  FFKVPETKGKSFEEIAAEF--R---KKSGS---------APPRKAAVQMEFLASSESV--
HUMGLUT3   :  FFKVPETRGRTFEDITRAFEGQ---AHGAD---------RSGKDGVMEMNSIEPAKETTT
HUMGLUT4   :  FLRVPETRGRTFDQISAAFH-R---TPSLL---------EQEVKPSTELEYLGPDEND--
RATGLUT4   :  FLRVPETRGRTFDQISATFR-R---TPSLL---------EQEVKPSTELEYLGPDEND--
MUSGLUT4   :  FLKVPETRGRTFDQISAAFR-R---TPSLL---------EQEVKPSTELEYLGPDEND--
HUMGLUT5   :  FLIVPETKAKTFIEINQIF--T---KMNKV---------SEVYPEKEELKELPPVTSEQ-
A_THALIANA:   YIFLPETKGIPIEEMGQVWRSHWYWSRFVE---------DGEYGNALEMGKNSNQAGTKH
CKESS_GLUT:   IFLLPETKGVPIERVQALYARHWFWNRVMGPAAAEVIAEDEKRVAAASAIIKEEELSKAM
SYNECHOCYS:   WFFVKETKGKTLEQM-----------------------------------------------
SCERE_SNF3:   YLTVYETKGLTLEEIDELY--------------------IKSSTGVVSPKFNKDIR---
Z_MOB_GLUT:   ARFVPETKGRSLDEIEEMWRSK---------------------------------------
ECOARAB/H+:   FWLIPETKNVTLEHIERKL------------------------MAGEKLRNIGV------
ECOXYLO/H+:   WKFVPETKGKTLEELEALW--------------------EPETKKTQQTATL--------
SCERE_GAL2:   FFFVPETKGLSLEEIQELWEEGVLPWKSEGWIP------SSRRGNNYDLEDLQHDDKPWY
KLACT_RAG1:   YFFVPETKGLTLEEVNEMYSEGVLPWKSSSWVP------SSRRGAEYDVDALQHDDKPWY
KLACT_LACP:   YFFFVETKGRSLEELEVVFEAPNPRKASVDQAFLAQVRATLVQRNDVRVANAQNLKEQEP
LENRI_GLUT:   VIQVFFLHPWDEERDGKKVVAPAIGKKELSEESIGNRAE---------------------
N_CRASSQTR:   YFFLPVTKSIPLEAMDRLFEIKPVQNANKNLMAELNFDRNPEREESSSLDDKDRVTQTEN
A_NIGQPERM:   FFLIPETKGVPLESMETLFDKKPVWHAHSQLIRELRENEEAFRADMGASGKGGVTKEYVE

           721        .
HUMGLUT1   :  ----------------
RATGLUT1   :  ----------------
MUSGLUT1   :  ----------------
RABGLUT1   :  ----------------
PIGGLUT1   :  ----------------
HUMGLUT2   :  ----------------
RATGLUT2   :  ----------------
MUSGLUT2   :  ----------------
HUMGLUT3   :  NV--------------
HUMGLUT4   :  ----------------
RATGLUT4   :  ----------------
MUSGLUT4   :  ----------------
HUMGLUT5   :  ----------------
A_THALIANA:   V---------------
CKESS_GLUT:   K---------------
SYNECHOCYS:   ----------------
SCERE_SNF3:   ----------------
Z_MOB_GLUT:   ----------------
ECOARAB/H+:   ----------------
ECOXYLO/H+:   ----------------
SCERE_GAL2:   KAMLE-----------
KLACT_RAG1:   KAML------------
KLACT_LACP:   LKSDADHVEKLSEAESV
LENRI_GLUT:   ----------------
N_CRASSQTR:   AV--------------
A_NIGQPERM:   EA--------------
```

Appendix 2. Sequences of Cloned PCR Products

Sequence of Product A.

FRONT OF GENE

```
                    P  V  G  H  L  L  T  N  T  S  A  N  H  S
  E  S  P  R  Y  F  Q  *  A  I  F  *  Q  T  P  Q  P  T  I  Q
 OLIGONUCLEOTIDE  S  S  R  P  S  F  D  K  H  L  S  Q  P  F  K
TGAAAGTCCTAGATATTTCCAGTAGGCCATCTTTTGACAAACACCTCAGCCAACCATTCA
        10        20        30        40        50        60

 N  K  L  L  F  *  H  L  F  *  L  C  E  L  P  Y  *  T  *  N
  T  N  F  C  F  D  T  F  F  N  Y  A  S  F  R  I  K  P  R  I
   Q  T  F  V  L  T  P  F  L  T  M  R  A  S  V  L  N  L  E  S
AACAAACTTTTGTTTTGACACCTTTTTTAACTATGCGAGCTTCCGTATTAAACCTAGAAT
        70        80        90       100       110       120

 L  Y  S  E  D  P  C  Q  *  T  Q  P  A  L  V  L  C  F  L  P
  S  T  Q  R  T  H  V  N  K  L  S  L  L  *  S  Y  V  S  F  H
   L  L  R  G  P  M  S  I  N  S  A  C  S  S  L  M  F  P  S  T
CTCTACTCAGAGGACCCATGTCAATAAACTCAGCCTGCTCTAGTCTTATGTTTCCTTCCA
       130       140       150       160       170       180

 P  L  S  H  T  L  K  I  H  S  H  T  Y
  P  Y  P  T  P  L  K  S  I  P  T  H  I
   L  I  P  H  P  *  N  P  F  P  H  I  F
CCCTTATCCCACACCCTTAAAATCCATTCCCACACATATT
       190       200       210
```

BACK OF GENE

```
 H  H  F  H  P  P  H  L  V  E  S  F  V  H  *  I  T  *  F  I
  I  T  F  I  L  P  I  L  *  K  A  L  C  T  E  S  P  N  S  L
   S  L  S  S  S  P  S  C  R  K  L  C  A  L  N  H  L  I  H  *
CATCACTTTCATCCTCCCCATCTTGTAGAAAGCTTTGTGCACTGAATCACCTAATTCATT
        10        20        30        40        50        60

 K  K  I  K  G  I  S  F  F  K  F  G  I  *  F  Q  P  W  V  F
  R  K  S  R  A  L  A  S  L  S  S  E  Y  S  F  N  H  G  S  S
   E  N  Q  G  H  *  L  L  *  V  R  N  I  V  S  T  M  G  L  Q
AAGAAAATCAAGGGCATTAGCTTCTTTAAGTTCGGAATATAGTTTCAACCATGGGTCTTC
        70        80        90       100       110       120

 K  I  L  *  A  P  R  R  E  R  I  W  R  G  F  N  S OLIGONUC
  K  F  S  E  L  P  G  G  R  E  S  G  E  V  S  I  V  E  R  A
   N  S  L  S  S  Q  E  G  E  N  L  E  R  F  Q  *
AAAATTCTCTGAGCTCCCAGGAGGGAGAGAATCTGGAGAGGTTTCAATAGTAGAAAGAGC
       130       140       150       160       170       180

              -- MULTIPLE CLONING SITE--
   R  R  T   SmaI     XbaI
               BamHI
TCGAAGAACAACGGGGATCCTCTAGAGTCGACCTGCAGGCA
      190       200       210       220
```

Sequence of Product B

COMPLETE SEQUENCE.

```
                                H  W  T  W  I  Q  S  M  R  Y
 Multiple     OLIGONUCLEOTIDE   I  G  H  G  S  N  L  *  D  I
Cloning SiteE  R  A  R  R  R  T  L  D  M  D  P  I  Y  E  I  F
AGAGGAGCCCCGAAAGAGCTCGAAGAAGAACATTGGACATGGATCCAATCTATGAGATAT
        10        20        30        40        50        60

S  E  Y  H  *  E  S  F  W  R  V  C  L  H  V  H  H  V  *  F
 Q  N  I  I  R  N  H  F  G  G  C  V  C  M  S  I  M  F  S  S
  R  I  S  L  G  I  I  L  E  G  V  F  A  C  P  S  C  L  V  L
TCAGAATATCATTAGGAATCATTTTGGAGGGTGTGTTTGCATGTCCATCATGTTTAGTTC
            75            90           105           120

Y  I  Q  V  P  G  H  P  N  F  S  S  W  Q  L  C  I  V  S  L
 T  S  R  S  L  D  I  Q  I  S  V  P  G  S  C  A  L  S  V  L
  H  P  G  P  W  T  S  K  F  Q  F  L  A  A  V  H  C  Q  S  W
TACATCCAGGTCCCTGGACATCCAAATTTCAGTTCCTGGCAGCTGTGCATTGTCAGTCTT
            135           150           165           180

G  L  L  *  C  L  Q  S  Q  V  H  H  T  L  V  D  H  S  N  K
 G  Y  S  S  V  F  N  L  K  Y  T  T  H  W  L  T  T  P  T  S
  V  T  L  V  S  S  I  S  S  T  P  H  I  G  *  P  L  Q  Q  A
GGGTTACTCTAGTGTCTTCAATCTCAAGTACACCACACATTGGTTGACCACTCCAACAAG
            195           210           225           240

L  *  A  N  I  A  P  A  H  L  A  D  R  L  *  V  K  G  F  V
 S  E  P  T  L  P  Q  H  I  L  Q  T  G  C  E  *  K  V  L  C
  L  S  Q  H  C  P  S  T  S  C  R  Q  V  V  S  K  R  F  C  V
CTCTGAGCCAACATTGCCCCAGCACATCTTGCAGACAGGTTGTGAGTAAAAGGTTTTGTG
            255           270           285           300

S  G  F  M  S  V  P  P  M  E  V  P  Y  L  P  L  L  A  V  L
 L  G  S  C  Q  S  H  Q  W  K  F  L  I  F  H  Y  *  Q  S  S
  W  V  H  V  S  P  T  N  G  S  S  L  S  S  I  T  S  S  P  H
TCTGGGTTCATGTCAGTCCCACCAATGGAAGTTCCTTATCTTCCATTACTAGCAGTCCTC
            315           330           345           360

T  R  Y  R  A
 L  G  T  E
  *  V  P  S
ACTAGGTACCGAGCT
            375
```

Sequence of Product C.

FRONT OF SEQUENCE

```
    BamHI   E  R  A  R  R  R  T  L  Q  A  H  T  H  K  E  S
       SmaI                          C  K  H  T  H  T  K  N  H
--- MCS --- ---OLIGONUCLEOTIDE---  A  S  T  H  T  Q  R  I  T
AGAGGATCCCCAGAAAGAGCACGAAGAAGAACATTGCAAGCACACACACACAAAGAATCA
             15             30             45             60

H  Y  N  I  L  I  S  H  I  N  *  F  C  D  G  Q  *  N  *  Y
 T  I  I  F  *  *  A  T  *  I  N  S  V  M  D  N  E  T  N  I
  L  *  Y  F  D  K  P  H  K  L  I  L  *  W  T  M  K  L  I  S
CACTATAATATTTTGATAAGCCACATAAATTAATTCTGTGATGGACAATGAAACTAATAT
             75             90            105            120

H  N  H  I  S  *  Y  D  S  D  V  L
 I  I  T  S  V  N  M  T  A  M  F
  *  S  H  Q  L  I  *  Q  R  C  S
 CATAATCACATCAGTTAATATGACAGCGATGTTCTT
           135            150
```

BACK OF SEQUENCE.

```
D  *  R  V  G  Q  E  P  L  G  N  K  T  T  W  Y  L  L  H  R
 T  R  E  L  D  K  S  H  L  G  T  R  P  H  G  I  C  Y  I  G
  L  E  S  W  T  R  A  T  W  E  Q  D  H  M  V  F  V  T  *  V
GACTAGAGAGTTGGACAAGAGCCACTTGGGAACAAGACCACATGGTATTTGTTACATAGG
             15             30             45             60

F  C  P  R  L  R  T  P  V  I  T  V  A  E  G  S  W  L  N  I
 S  V  L  D  *  E  H  Q  L  S  Q  W  Q  K  A  H  G  S  T  L
  L  S  *  T  K  N  T  S  Y  H  S  G  R  R  L  M  A  Q  H  C
TTCTGTCCTAGACTAAGAACACCAGTTATCACAGTGGCAGAAGGCTCATGGCTCAACATT
             75             90            105            120

 V  L  D  R  K  H  A  V  G  A  L  L
 S  L  I  E  N  M  Q  L  E  H  C  C
  P  *  *  K  T  C  S  W  S  T  A  A
GTCCTTGATAGAAAACATGCAGTTGGAGCACTGCTGCC
           135            150
```

Sequence of Product D.

FRONT OF GENE

```
 A  R  R  R  T  F  A  I  T  R  C  G  R  L  P  Q  C  L  M  P
                   P  S  Q  D  A  A  G  F  R  S  A  L  C  H
 OLIGONUCLEOTIDE      H  H  K  M  R  P  A  S  A  V  P  Y  A  T
GCACGTAGAAGAACATTTGCCATCACAAGATGCGGCCGGCTTCCGCAGTGCCTTATGCCA
        10        20        30        40        50        60

 P  K  Q  R  T  S  C  V  Q  E  *  C  A  P  S  H  K  P  T  L
  L  N  K  E  Q  A  V  C  K  S  N  A  R  Q  V  I  S  P  P  W
   *  T  K  N  K  L  C  A  R  V  M  R  A  K  S  *  A  H  P  G
CCTAAACAAAGAACAAGCTGTGTGCAAGAGTAATGCGCGCCAAGTCATAAGCCCACCCTG
        70        80        90       100       110       120

 G  V  S  M  R  S  W  V  S  D  Q  S  G  V  D  T  D  A  G  V
  A  C  L  *  D  L  G  *  A  T  S  Q  A  W  T  P  T  R  V  Y
   R  V  Y  E  I  L  G  K  R  P  V  R  R  G  H  R  R  G  C  I
GGCGTGTCTATGAGATCTTGGGTAAGCGACCAGTCAGGCGTGGACACCGACGCGGGTGTA
       130       140       150       160       170       180

 Y  K  Q  R  L  S  E  A  L  V  F  L  I  N  M  Q  *  R  L  A
  I  S  S  A  F  L  R  H  L  S  S  S  S  I  C  N  K  D  W  L
   *  A  A  P  F  *  G  T  C  L  P  H  Q  Y  A  I  K  I  G  C
TATAAGCAGCGCCTTTCTGAGGCACTTGTCTTCCTCATCAATATGCAATAAAGATTGGCT
       190       200       210       220       230       240

 A  E  D  C  V  V  C  F  C  R  R  K  R  T  D  V  T  H  T
  Q  K  T  V  S  C  A  S  A  A  G  N  A  R  T  *  H  T  L
   R  R  L  C  R  V  L  L  P  Q  E  T  H  G  R  N  T  H  -
GCAGAAGACTGTGTCGTGTGCTTCTGCCGCAGGAAACGCACGGACGTAACACACACTG
       250       260       270       280       290
```

BACK OF PRODUCT

```
 T  S  S  F  L  S  S  D  *  R  Q  M  S  L  C  S  H  G  C  P
  H  P  L  S  C  L  L  I  R  D  R  C  H  C  V  A  M  A  V  L
   I  L  F  L  V  F  *  L  E  T  D  V  T  V  *  P  W  L  S  *
ACATCCTCTTTCTTGTCTTCTGATTAGAGACAGATGTCACTGTGTAGCCATGGCTGTCCT
        10        20        30        40        50        60

 R  T  C  Y  V  D  Q  T  S  L  E  L  T  E  I  S  C  L  Y  F
  E  L  A  M  *  I  R  L  V  L  N  S  Q  R  S  P  A  S  T  S
   N  L  L  C  R  S  D  *  S  *  T  H  R  D  L  L  P  L  L  P
AGAACTTGCTATGTAGATCAGACTAGTCTTGAACTCACAGAGATCTCCTGCCTCTACTTC
        70        80        90       100       110       120

 P  S  N  R  V  D  N  I  Y  Y  H  I  W  L  F  P  L  F  L  F
  Q  V  I  G  L  T  I  Y  T  T  T  Y  G  F  F  P  S  S  S  S
   K  *  *  G  *  Q  Y  I  L  P  H  M  A  F  S  P  L  P  L  R
CCAAGTAATAGGGTTGACAATATATACTACCACATATGGCTTTTTCCCCTCTTCCTCTTC
       130       140       150       160       170       180

 A  L  L  S  L  L  S  S  S  W  Q  T
  L  S  S  P  S  F  L  L  L  G  K  H  --Multiple Cloning site--
```

```
   S  P  L  P  P  F  F  F  L  A  N  M
GCTCTCCTCTCCCTCCTTTCTTCTTCTTGGCAAACATGGGGATCCTCTAGAGTCGACCTG
       190        200        210        220        230        240
```

Sequence of Product E.

COMPLETE SEQUENCE

```
 --- Multiple cloning site ---   V  G  R  D  L  K  *  V  G
                                  W  A  G  I  *  S  R  W  V
                                  G  Q  G  F  E  V  G  G  *
TGCATGCCTGCAGGTCGACTCTAGAGGATCCCCGTGGGCAGGGATTTGAAGTAGGTGGGT
             15             30             45             60

E  G  G  R  V  V  I  Q  E  T  N  P  Y  C  H  S  E  S  Q  M
 K  E  A  E  *  *  Y  R  K  L  T  H  I  A  T  V  S  L  K  W
  R  R  Q  S  S  N  T  G  N  *  P  I  L  P  Q  *  V  S  N  G
GAAGGAGGCAGAGTAGTAATACAGGAAACTAACCCATATTGCCACAGTGAGTCTCAAATG
             75             90            105            120

D  G  W  S  S  D  G  K  V  W  G  P  L  L  A  F  I  Q  C  I
 M  G  G  V  A  M  E  R  C  G  G  L  S  W  L  S  S  S  A  F
  W  V  E  *  R  W  K  G  V  G  A  S  P  G  F  H  P  V  H  L
GATGGGTGGAGTAGCGATGGAAAGGTGTGGGGGCCTCTCCTGGCTTTCATCCAGTGCATT
            135            150            165            180

*  R  K  E  *  L  K  L  T  A  C  H  A  S  L  C  V  L  H  L
 E  G  R  S  D  *  N  S  L  H  A  M  L  H  S  V  C  C  T  W
  K  E  G  V  I  E  T  H  C  M  P  C  F  T  L  C  A  A  P  G
TGAAGGAAGGAGTGATTGAAACTCACTGCATGCCATGCTTCACTCTGTGTGCTGCACCTG
            195            210            225            240

A  F  P  P  M  P  V  S  P  V  S  M  L  P  C  T  I  C  K  S
 P  S  H  P  C  P  Y  H  P  F  P  C  F  H  A  R  S  A  R  V
  L  P  T  H  A  R  I  T  R  F  H  A  S  M  H  D  L  Q  E  S
GCCTTCCCACCCATGCCCGTATCACCCGTTTCCATGCTTCCATGCACGATCTGCAAGAGT
            255            270            285            300

L  S  F  G  L  P  T  G  P  Q  D  P  C  A  R  Y  S  T  K  Q
 S  P  L  G  Y  P  Q  A  L  R  I  P  V  L  D  I  P  Q  N  K
  L  L  W  V  T  H  R  P  S  G  S  L  C  *  I  F  H  K  T  S
CTCTCCTTTGGGTTACCCACAGGCCCTCAGGATCCCTGTGCTAGATATTCCACAAAACAA
            315            330            345            360

A  F  P  L  S  C  M  C  L  V  *  Y  L  C  H  L  P  C  P  W
 R  S  L  F  H  A  C  A  *  F  S  I  C  V  I  C  L  A  P  G
  V  P  S  F  M  H  V  P  S  L  V  S  V  S  F  A  L  P  L  A
GCGTTCCCTCTTTCATGCATGTGCCTAGTTTAGTATCTGTGTCATTTGCCTTGCCCCTGG
            375            390            405            420

L  *  E  P  Q  A  H  K  E  *  F  F  F  E  S  I  R  V  T  E
 F  E  S  P  R  H  I  K  N  S  S  S  S  S  L  F  G  *  P  R
  L  R  A  P  G  T  *  R  I  V  L  L  R  V  Y  S  G  N  R
CTTTGAGAGCCCCAGGCACATAAAGAATAGTTCTTCTTCGAGTCTATTCGGGTAACCGAG
            435            450            465            480
```

Sequence of Product H - Human GLUT4

FRONT OF SEQUENCE

```
                    A  S  S  R  Q  Q  G  *  A  S  L  P  M  P
    M. C. S.     Q  H  L  R  D  S  R  G  R  P  A  C  L  C  H
                T  S  I  F  E  T  A  G  V  G  Q  P  A  Y  A  T
TAGAGGATCCCCCGACCAGCATCTTCGAGACAGCAGGGGTAGGCCAGCCTGCCTATGCCA
               15             30             45             60

P  *  E  L  V  W  S  T  Q  S  S  P  W  S  R  C  C  W  W  S
 H  R  S  W  C  G  Q  H  S  L  H  L  G  L  G  V  V  G  G  A
  I  G  A  G  V  V  N  T  V  F  T  L  V  S  V  L  L  V  E  R
CCATAGGAGCTGGTGTGGTCAACACAGTCTTCACCTTGGTCTCGGTGTTGTTGGTGGAGC
               75             90            105            120

G  R  G  A  D  C  H  S  W  A  W  R  A  C  V  A
 G  G  A  R  T  V  T  P  G  P  G  G  H  V  W  L
  A  G  R  G  L  S  L  L  G  L  A  G  M  C  G  C
GGGCGGGGCGCGGACTGTCACTCCTGGGCCTGGCGGGCATGTGTGGCTGT
              135            150            165
```

BACK OF SEQUENCE

```
P  D  P  Q  P  W  L  W  L  V  S  P  I  D  E  Q  T  S  S  L
 P  T  R  S  H  G  C  G  W  F  L  Q  S  T  S  K  L  H  H  W
  R  P  A  A  M  A  V  A  G  F  S  N  R  R  A  N  F  I  I  G
CCCGACCCGCAGCCATGGCTGTGGCTGGTTTCTCCAATCGACGAGCAAACTTCATCATTG
               15             30             45             60

A  W  V  S  S  M  L  Q  E  L  W  A  L  L  F  L  L  F  A  V
 H  G  F  P  V  C  C  R  S  Y  G  P  Y  S  S  F  Y  L  R  S
  M  G  F  Q  Y  V  A  G  A  M  G  P  T  L  P  S  I  C  G  P
GCATGGGTTTCCAGTATGTTGCAGGAGCTATGGGCCCTACTCTTCCTTCTATTTGCGGTC
               75             90            105            120

L  L  L  G  F  F  I  F  T  F  L
 S  C  W  A  S  S  S  S  P  S  *  -- OLIGO --
  P  A  G  L  L  H  L  H  L  L  R  V  P  E
CTCCTGCTGGGCTTCTTCATCTTCACCTTCTTAGAGTACCTGAAAC
              135            150            165
```

Sequence of Product I.

```
G  R  D  L  K  *  V  G  E  G  G  R  V  V  I  Q  E  T  N  P
 A  G  I  *  S  R  W  V  K  E  A  E  *  *  Y  R  K  L  T  H
  Q  G  F  E  V  G  G  *  R  R  Q  S  S  N  T  G  N  *  P  I
GGCAGGGATTTGAAGTAGGTGGGTGAAGGAGGCAGAGTAGTAATACAGGAAACTAACCCA
            15            30            45            60

Y  C  H  S  E  L  *  M  D  G  W  R  C  D  G  K  V  W  G  L
 I  A  T  V  S  S  K  W  M  G  G  D  A  M  E  R  C  G  A  S
  L  P  Q  *  A  L  N  G  W  V  E  M  R  W  K  G  V  G  P  L
TATTGCCACAGTGAGCTCTAAATGGATGGGTGGAGATGCGATGGAAAGGTGTGGGGCCTC
            75            90            105           120

S  M  L  S  S  V  H  *  R  R  S  D  *  T  H  C  M  P  C  S
 P  C  F  H  Q  C  I  E  G  G  V  I  E  L  T  A  C  H  A  H
  H  A  F  I  S  A  L  K  E  E  *  L  N  S  L  H  A  M  L  I
TCCATGCTTTCATCAGTGCATTGAAGGAGGAGTGATTGAACTCACTGCATGCCATGCTCA
            135           150           165           180

S  R  V  L  H  L  P  P  T  H  A  R  P  H  Y  G  L  P  D  F
 L  V  C  C  T  C  L  P  P  M  P  V  L  T  T  D  S  L  T  S
  S  C  A  A  P  A  S  H  P  C  P  S  S  L  R  T  P  *  L  P
TCTCGTGTGCTGCACCTGCCTCCCACCCATGCCCGTCCTCACTACGGACTCCCTGACTTC
            195           210           225           240

P  D  S  I  C  K  M  S  L  L  W  V  T  H  R  P  S  G  S  L
 L  T  A  S  A  R  C  L  S  F  G  L  P  T  G  P  Q  D  P  C
  *  Q  H  L  Q  D  V  S  P  L  G  Y  P  Q  A  L  R  I  P  V
CCTGACAGCATCTGCAAGATGTCTCTCCTTTGGGTTACCCACAGGCCCTCAGGATCCCTG
            255           270           285           300

C  *  I  F  H  K  T  S  V  P  S  F  M  H  V  P  V  C  I  C
 A  R  Y  S  T  K  Q  A  F  P  L  S  C  M  C  L  F  V  S  V
  L  D  I  P  Q  N  K  R  S  L  F  H  A  C  A  C  L  Y  L  C
TGCTAGATATTCCACAAAACAAGCGTTCCCTCTTTCATGCATGTGCCTGTTTGTATCTGT
            315           330           345           360

V  I  C  L  A  P  G  F  E  S  P  G
 *  F  A  L  P  L  A  L  R  A  Q  A
  D  L  P  C  P  W  L  *  E  P  R
GTGATTTGCCTTGCCCCTGGCTTTGAGAGCCCAGGCC
            375           390


G  L  G  S  Q  S  Q  G  Q  G  K  S  H  R  Y  K  Q  A  H  A
 A  W  A  L  K  A  R  G  K  A  N  H  T  D  T  N  R  H  M  H
  P  G  L  S  K  P  G  A  R  Q  I  T  Q  I  Q  T  G  T  C  M
GGCCTGGGCTCTCAAAGCCAGGGGCAAGGCAAATCACACAGATACAAACAGGCACATGCA
            15            30            45            60

*  K  R  E  R  L  F  C  G  I  S  S  T  G  I  L  R  A  C  G
 E  R  G  N  A  C  F  V  E  Y  L  A  Q  G  S  *  G  P  V  G
  K  E  G  T  L  V  L  W  N  I  *  H  R  D  P  E  G  L  W  V
TGAAAGAGGGAACGCTTGTTTTGTGGAATATCTAGCACAGGGATCCTGAGGGCCTGTGGG
            75            90            105           120
```

```
*  P  K  G  E  T  S  C  R  C  C  Q  G  S  Q  G  V  R  S  E
 N  P  K  E  R  H  L  A  D  A  V  R  E  V  R  E  S  V  V  R
  T  Q  R  R  D  I  L  Q  M  L  S  G  K  S  G  S  P  *  *  G
TAACCCAAAGGAGAGACATCTTGCAGATGCTGTCAGGGAAGTCAGGGAGTCCGTAGTGAG
            135            150            165            180

D  G  H  G  W  E  A  G  A  A  H  E  M  S  M  A  C  S  E  F
 T  G  M  G  G  R  Q  V  Q  H  T  R  *  A  W  H  A  V  S  S
  R  A  W  V  G  G  R  C  S  T  R  D  E  H  G  M  Q  *  V  Q
GACGGGCATGGGTGGGAGGCAGGTGCAGCACACGAGATGAGCATGGCATGCAGTGAGTTC
            195            210            225            240

N  H  S  S  F  N  A  L  M  K  A  W  R  G  P  T  P  F  H  R
 I  T  P  P  S  M  H  *  *  K  H  G  E  A  P  H  L  S  I  A
  S  L  L  L  Q  C  T  D  E  S  M  E  R  P  H  T  F  P  S  H
AATCACTCCTCCTTCAATGCACTGATGAAAGCATGGAGAGGCCCCACACCTTTCCATCGC
            255            270            285            300

I  S  T  H  P  F  R  A  H  C  G  N  M  G  *  F  P  V  L  L
 S  P  P  I  H  L  E  L  T  V  A  I  W  V  S  F  L  Y  Y  Y
  L  H  P  S  I  *  S  S  L  W  Q  Y  G  L  V  S  C  I  T  T
ATCTCCACCCATCCATTTAGAGCTCACTGTGGCAATATGGGTTAGTTTCCTGTATTACTA
            315            330            345            360

L  C  L  L  H  P  P  T  S  N  P  C
 S  A  S  F  T  H  L  L  Q  I  P  A
  L  P  P  S  P  T  Y  F  K  S  L
CTCTGCCTCCTTCACCCACCTACTTCAAATCCCTGCC
            375            390
```

Sequence of Product J.

FRONT OF CLONE

```
*  R  G  S  V  W  A  W  E  G  S  G  L  *  C  L  H  K  H  M
 D  E  A  A  C  G  R  G  R  G  V  G  C  D  A  C  T  S  I  C
  T  R  Q  R  V  G  V  G  G  E  W  A  V  M  L  A  Q  A  Y  V
TGACGAGGCAGCGTGTGGGCGTGGGAGGGGAGTGGGCTGTGATGCTTGCACAAGCATATG
             15             30             45             60

Y  M  Y  V  Y  D  W  T  H  H  Q  R  L  E  L  D  C  *  A  S
 T  C  M  Y  M  T  G  H  I  I  R  D  *  N  W  I  A  R  L  L
  H  V  C  I  *  L  D  T  S  S  E  T  R  I  G  L  L  G  F  L
TACATGTATGTATATGACTGGACACATCATCAGAGACTAGAATTGGATTGCTAGGCTTCT
             75             90            105            120

TAG
*
```

BACK OF CLONE

```
A  G  T  E  T  H  S  N  S  G  D  R  D  T  Q  I  L  R  A  H
 Q  A  R  R  P  I  A  I  Q  G  T  E  I  R  R  S  *  G  L  T
  R  H  G  D  P  *  Q  F  R  G  Q  R  Y  A  D  P  E  G  S  L
GCAGGCACGGAGACCCATAGCAATTCAGGGGACAGAGATACGCAGATCCTGAGGGCTCAC
             15             30             45             60

W  L  T  A  *  P  E  T  V  S  F  Y  S  S  W  R  P  C  I  K
 G  *  Q  P  S  Q  K  Q  *  V  S  I  P  V  G  D  P  V  S  R
  A  D  S  L  A  R  N  S  E  F  L  F  Q  L  E  T  L  Y  Q  G
TGGCTGACAGCCTAGCCAGAAACAGTGAGTTTCTATTCCAGTTGGAGACCCTGTATCAAG
             75             90            105            120

A  K
 Q  K
  K
GCAAAAG
```

RACE gene product.

SEQUENCE FROM REGION SENSE OLIGONUCLEOTIDE.

```
                    G N R A I E I G F G * N T
     OLIGONUCLEOTIDE G I G Q * K S A S G K I P
  A V E R A R R E * G N R N R L R V K Y P
CTGCAGTTGAAAGAGCTCGAAGGGAATAGGGCAATAGAAATCGGCTTCGGGTAAAATACC
             15              30              45              60

R A R Q Q R D I * L F T C V I A V K N D
 V R D N N V I F S F S L A L S R * K T T
  C A T T T * Y L A F H L R Y R G E K R R
CGTGCGCGACAACAACGTGATATTTAGCTTTTCACTTGCGTTATCGCGGTGAAAAACGAC
             75              90             105             120

V L F I I F N E R T M L N S H C H C R I
 C C L S S S M N E P C * I A T A I A A S
  V V Y H L Q * T N H A E * P L P L P H P
GTGTTGTTTATCATCTTCAATGAACGAACCATGCTGAATAGCCACTGCCATTGCCGCATC
            135             150             165             180

Q S A Q G C S H N T H V T R I D L C F H
 S P L K D V P I I P T L H A S T F A S I
  V R S R M F P * Y P R Y T H R P L L P S
CAGTCCGCTCAAGGATGTTCCCATAATACCCACGTTACACGCATCGACCTTTGCTTCCAT
            195             210             225             240

Q A * P R A G K I R S G C F F F Y R P D
 R P D H G L G K Y V R V A S S S T G Q T
  G L T T G W E N T F G L L L L L Q A R R
CAGGCCTGACCACGGGCTGGGAAAATACGTTCGGGTTGCTTCTTCTTCTACAGGCCAGAC
            255             270             285             300

V T R G V H R Q I K R S A G * I L I C A
 * P V A Y T V K S N A L L G K S * F V Q
  D P W R T P S N Q T L C W V N P D L C R
GTGACCCGTGGCGTACACCGTCAAATCAAACGCTCTGCTGGGTAAATCCTGATTTGTGCA
            315             330             345             360

G M T P A P V I C D T E T W H D S * T A
 A * R Q H P * F A I R K P G M I H K Q P
  H D A S T R N L R Y G N L A * F I N S H
GGCATGACGCCAGCACCCGTAATTTGCGATACGGAAACCTGGCATGATTCATAAACAGCC
            375             390             405             420

T A N F C C R A W S T S R K N * S R
 L Q I F A V V P G L L V V K I D R
  C K F L L S C L V Y * S * K L I A
ACTGCAAATTTTTGCTGTCGTGCCTGGTCTACTAGTCGTAAAAATTGATCGCGG
            435             450             465
```

Sequence of Product L.

FRONT OF PRODUCT

```
F  F  E  L  F  Q  L  E  Y  Y  L  G  P  N  I  I  I  A  C  L
 S  S  S  S  F  N  *  N  I  I  *  G  Q  I  L  S  *  L  V  *
  L  R  A  L  S  T  R  I  L  S  R  A  K  Y  Y  H  S  L  S  E
TTCTTCGAGCTCTTTCAACTAGAATATTATCTAGGGCCAAATATTATCATAGCTTGTCTG
             15            30            45            60

K  K  C  T  F  P  S  S  M  Q  *  N  K  L  L  G  T  G  T  G
 R  S  V  P  F  L  V  V  C  S  E  I  S  C  *  A  L  G  Q  D
  E  V  Y  L  S  *  *  Y  A  V  K  *  A  A  R  H  W  D  R  M
AAGAAGTGTACCTTTCCTAGTAGTATGCAGTGAAATAAGCTGCTAGGCACTGGGACAGGA
             75            90           105           120

C  L  E  L  T  S  E
 A  *  N  *  L  V  N
  P  R  T  D  *  *  I
TGCCTAGAACTGACTAGTGAATT
             135
```

BACK OF GENE

```
Q  E  H  T  R  I  D  S  S  T  L  G  C  H  H  Q  H  A  I  S
 K  S  I  Q  E  L  T  A  A  L  W  V  V  I  I  S  T  P  Y  R
  R  A  Y  K  N  *  Q  Q  H  F  G  L  S  S  S  A  R  H  I  V
CAAGAGCATACAAGAATTGACAGCAGCACTTTGGGTTGTCATCATCAGCACGCCATATCG
             15            30            45            60

*  L  S  N  C  K  W  S  S        OLIGONUCLEOTIDE
 S  *  V  T  V  S  G  H  Q  V  E  R  A  R  R  T  T
  A  K  *  L  *  V  V  I  R
TAGCTAAGTAACTGTAAGTGGTCATCAGGTAGAAAGAGCTCGAAGAACAACA
             75            90           105
```

Sequence of Product P2

FRONT OF GENE

```
 F  Y  I  D  Q  S  S  M  A  V  I  *  N  E  S  I  C  N  G  I
  S  T  L  T  R  V  P  W  Q  S  F  E  M  S  Q  Y  V  M  E  S
   L  H  *  P  E  F  H  G  S  H  L  K  *  V  N  M  *  W  N  Q
TTCTACATTGACCAGAGTTCCATGGCAGTCATTTGAAATGAGTCAATATGTAATGGAATC
        10        20        30        40        50        60

 S  S  S  *  K  -  -  E  V  I  Q  L  Q  A  *  F  K  T  R  N
  V  L  P  E  K  -  -  K  *  F  N  C  K  P  N  L  R  L  E  I
   F  F  L  K  K  -  *  S  N  S  T  A  S  L  I  *  D  *  K  S
AGTTCTTCCTGAAAAAGNNNTGAAGTAATTCAACTGCAAGCCTAATTTAAGACTAGAAAT
        70        80        90       100       110       120

 R  I  R  K  T  R  C  K  G  H  Q  *  *  K  -  L
  A  L  G  K  Q  D  A  R  V  I  S  N  K  -  -  F
   H  *  E  N  K  M  Q  G  S  S  V  I  K  -  T  S
CGCATTAGGAAAACAAGATGCAAGGGTCATCAGTAATAAAAANNACTTCA
       130       140       150       160
```

BACK OF GENE

```
 F  R  E  N  S  L  S  Q  R  Q  N  G  E  T  E  H  N  E  F  R
  S  E  K  I  A  *  V  K  D  R  M  V  R  Q  S  I  M  N  L  G
   Q  R  K  *  P  E  S  K  T  E  W  *  D  R  A  *  *  I  *  G
TTCAGAGAAAATAGCCTGAGTCAAAGACAGAATGGTGAGACAGAGCATAATGAATTTAGG
        10        20        30        40        50        60

 D  R  N  F  R  Y  R  W  H  C  V  E  R  A  R  R  T  T
  T  G  I  L  G  I  G  G  I  V      OLIGONUCLEOTIDE
   P  E  F  *  V  *  V  A  L  C
GACCGGAATTTTAGGTATAGGTGGCATTGTGTTGAAAGAGCACGTAGAACAACA
        70        80        90       100       110
```

Sequence of clone P4

FRONT OF CLONE

```
 P  Q  -  L  T  -  -  L  L  D  S  N  L  C  F  *  F  L  P  F
  H  N  -  *  -  P  -  Y  *  I  L  T  C  A  S  S  S  Y  P  F
   T  M  -  D  -  L  -  T  R  F  *  L  V  L  L  V  L  T  L  S
CCACAATGNCTGACANCCTTNCTACTAGATTCTAACTTGTGCTTCTAGTTCTTACCCTTT
        10        20        30        40        50        60

 H  *  R  Y  V  H  L  L  *  Q  S  L  S  K  K  T  D  -  -  R
  I  K  D  T  S  I  C  S  D  S  H  C  L  R  R  L  I  -  -  V
   L  K  I  R  P  F  A  L  T  V  I  V  *  E  D  *  *  -  T  Y
CATTAAAGATACGTCCATTTGCTCTGACAGTCATTGTCTAAGAAGACTGATAGNNNACGT
        70        80        90       100       110       120

 M
  *
   D
ATG

TAC
 H
  S
   I
```

BACK OF CLONE

```
F  L  A  V  H  A  Q  N  *  P  K  A  P  L  V  P  R  Q  L  Y
 F  L  L  F  M  L  R  T  D  L  K  H  L  *  F  H  G  N  F  I
  S  C  C  S  C  S  E  L  T  *  S  T  S  S  S  T  A  T  L  *
TTTCTTGCTGTTCATGCTCAGAACTGACCTAAAGCACCTCTAGTTCCACGGCAACTTTAT
                                                           72

K  H  L  P  Q  T  P  H  R  S  L  F  V  L  A  C  S  I  S  R
 N  T  C  P  K  P  L  I  G  V  Y  L  C  L  P  A  V  *  V  E
  T  P  A  P  N  P  S  *  E  F  I  C  A  C  L  Q  Y  K  *  K
AAACACCTGCCCCAAACCCCTCATAGGAGTTTATTTGTGCTTGCCTGCAGTATAAGTAGA
                                                           12


K  S  S
 R  A  R
  E  L  E
AAGAGCTCGAA
```

SEQUENCE OF CLONE Q

FRONT OF GENE

```
 OLIGO T  P  F  F  S  S  R  L  N  I  K  Q  E  R  K  R  N  H
  R  G  P  L  F  F  P  P  D  *  I  *  N  K  R  G  K  E  T  T
        P  F  F  F  L  P  I  K  Y  K  T  R  E  E  K  K  P  Q
TAGAGGACCCCTTTTTTTTCCTCCCGATTAAATATAAAACAAGAGAGGAAAAGAAACCAC
        10        20        30        40        50        60

 K
  R
   E
AAGAA
```

BACK OF GENE

```
 K  I  *  K  K  Y  R  I  K  *  K  M  L  S  K  I  V  V  P  N
  K  Y  K  R  N  I  G  *  N  K  K  C  Y  R  K  *  W  F  Q  T
   N  I  K  E  I  S  D  K  I  K  N  V  I  E  N  S  G  S  K  P
AAAATATAAAAGAAATATCGGATAAAATAAAAAATGTTATCGAAAATAGTGGTTCCAAAC
        10        20        30        40        50        60

 L  V  T  K  N  V  K  N  G  G  M  K  I  E  N  I  S  G  M  E
  *  *  R  K  T  *  R  M  V  G  *  K  S  K  I  Y  L  E  W  N
   S  D  E  K  R  K  E  W  W  D  E  N  R  K  Y  I  W  N  G  M
CTAGTGACGAAAAACGTAAAGAATGGTGGGATGAAAATCGAAAATATATCTGGAATGGAA
        70        80        90       100       110       120

 *  Y  V  H  *  H  I  K  K  V  V  R  P  L
  D  M  C  T  D  I  *  R  K  W  S  G  R  S
   I  C  A  L  T  Y  K  E  S  G  Q  A  A  H
TGATATGTGCACTGACATATAAAGAAAGTGGTCAGGCCGCTCA
       130       140       150       160
```

Sequence of Clone R2

FRONT OF CLONE

```
 L  R  *  K  V  N  I  F  P  *  N  E  T  E  G  F  *  E  T  S
  Y  G  E  K  *  I  S  S  H  K  T  R  Q  K  D  S  E  K  Q  V
   T  V  K  S  K  Y  L  P  I  K  R  D  R  R  I  L  R  N  K  F
CTACGGTGAAAAGTAAATATCTTCCCATAAAACGAGACAGAAGGATTCTGAGAAACAAGT
        10        20        30        40        50        60

 L  *  C  V  Y  S  A  N  R  V  E  P  L  F  *  C
  C  D  V  C  T  Q  L  T  E  W  N  L  S  F  D  A OLIGONUCLEOTIDE
   V  M  C  V  L  S  *  Q  S  G  T  S  L  L  M  Q  Q  F  S  G
TTGTGATGTGTGTACTCAGCTAACAGAGTGGAACCTCTCTTTTGATGCAACAATTTTCCG
        70        80        90       100       110       120


 G
  G
   E
GGG
```

Sequence of clone R6

FRONT OF GENE

```
 OLIGONUCLEOTIDE  R  M  T  E  V  *  L  *  F  L  Y  T  K  C
                     G  *  L  K  C  N  Y  N  S  F  I  P  N  V
   P  M  Y  V  G  E  D  D  *  S  V  I  I  I  P  L  Y  Q  M  C
TTCCTATGTATGTTGGAGAGGATGACTGAAGTGTAATTATAATTCCTTTATACCAAATGT
                                                            60

V  C  I  L  Q  H  L  M  G  F  L  Q  N  F  H  L  K  I  S  P
 Y  A  F  S  S  I  *  W  D  S  S  K  I  S  I  S  K  S  A  H
   M  H  S  P  A  F  D  G  I  P  P  K  F  P  S  Q  N  Q  P  I
GTATGCATTCTCCAGCATTTGATGGGATTCCTCCAAAATTTCCATCTCAAAATCAGCCCA
                                                           120

S  V  E  Q  S  H  A  A  S  *  L  Y  G  M  *  W  F  V  A  V
 Q  *  N  S  H  M  L  P  V  N  Y  M  E  C  D  G  L  W  L  Y
   S  R  T  V  T  C  C  Q  L  I  I  W  N  V  M  V  C  G  C  I
TCAGTAGAACAGTCACATGCTGCCAGTTAATTATATGGAATGTGATGGTTTGTGGCTGTA
                                                           180

F  Q  Q  S  T  M  T
 S  N  R  A  P  *  H
   P  T  E  H  H  D
TTCCAACAGAGCACCATGACAC
```

Sequence of Clone R7 - Putative *P. falciparum* enolase.

```
                                                                    60
TTCCTATGTATGTAGGTGATGAAGGTGGATTTGCTCCAAATATATTAAACGCTAATGAAG
                      K  V  D  L  L  Q  I  Y  *  T  L  M  K
  OLIGONUCLEOTIDE      R  W  I  C  S  K  Y  I  K  R  *  *  S
  P  M  Y  V  G  D  E  G  G  F  A  P  N  I  L  N  A  N  E  A

                                                                   120
CTCTTGATTTATTAGTAACTGCCATTAAATCAGCTGGTTATGAAGGAAAGGTTAAAATTG
L  L  I  Y  *  *  L  P  L  N  Q  L  V  M  K  E  R  L  K  L
 S  *  F  I  S  N  C  H  *  I  S  W  L  *  R  K  G  *  N  C
  L  D  L  L  V  T  A  I  K  S  A  G  Y  E  G  K  V  K  I  A

                                                                   180
CTATGGATGTTGCCAGCTCTGAATTTCGACAAGTGAACAAAACATACGATTTAGATTTCA
L  W  M  L  P  A  L  N  F  D  K  *  T  K  H  T  I  *  I  S
 Y  G  C  C  Q  L  *  I  S  T  S  E  Q  N  I  R  F  R  F  Q
  M  D  V  A  S  S  E  F  R  Q  V  N  K  T  Y  D  L  D  F  K

                                                                   240
AAACTCCAAATAATGACAAATCATTAGTTAAGACTGGAGCTCAATTAGTTGACTTATACA
K  L  Q  I  M  T  N  H  *  L  R  L  E  L  N  *  L  T  Y  T
 N  S  K  *  *  Q  I  I  S  *  D  W  S  S  I  S  *  L  I  H
  T  P  N  N  D  K  S  L  V  K  T  G  A  Q  L  V  D  L  Y  I


TTGATTTAGTAAAGAAATATCCAATTGTTTCTATT
L  I  *  *  R  N  I  Q  L  F  L
 *  F  S  K  E  I  S  N  C  F  Y
  D  L  V  K  K  Y  P  I  V  S  I
```

Sequence of clone R10 - Rat GLUT2

FRONT OF GENE

```
            R  L  L  Q  P  H  S  G  V  P  W  A  L  F  T  N
     OLIGO  D  C  S  N  H  T  Q  G  C  P  G  H  S  S  P  T
  Y  V  G  E  I  A  P  T  T  L  R  G  A  L  G  T  L  H  Q  L
TGTACGTTGGAGAGATTGCTCCAACCACACTCAGGGGTGCCCTGGGCACTCTTCACCAAC
                                                            60

W  L  L  Y  R  H  S  Y  *  S  D  C  W  L  S  S  F  W  A  I
 G  S  C  T  G  I  P  I  S  Q  I  A  G  S  V  H  S  G  Q  S
  A  L  V  Q  A  F  L  L  V  R  L  L  A  Q  F  I  L  G  N  Q
TGGCTCTTGTACAGGCATTCCTATTAGTCAGATTGCTGGCTCAGTTCATTCTGGGCAATC
                                                           120
```

BACK OF CLONE

```
 V  G  K  K  A  *  R  L  G  P  E  T  S  P  K  I  L  M  S  E
  S  A  R  K  L  E  D  *  G  L  R  R  H  Q  R  Y  *  *  V  R
   R  Q  E  S  L  K  T  R  A  *  D  V  T  K  D  I  N  E  *  G
GTCGGCAAGAAAGCTTGAAGACTAGGGCCTGAGACGTCACCAAAGATATTAATGAGTGAG
        10        20        30        40        50        60

 E  R  K  G  R  G  V  D  *  A  E  G  L  R  D  P  A  L
  K  E  K  E  E  A  S  T  E  Q  K  V  S  V  I  Q  L  L
   K  K  R  K  R  R  R  L  S  R  R  S  P  *  S  S  S  *
GAAAGAAAAGGAAGAGGCGTCGACTGAGCAGAAGGTCTCCGTGATCCAGCTCTT
        70        80        90       100       110
```

Sequence of Clone R14

FRONT OF CLONE

```
R  P  R  R  *  G  E  M  K  W  P  T  Y  V  T  K  N  Y  I  T
 D  R  E  D  E  G  K  *  N  G  L  H  M  *  Q  K  T  T  *  P
  T  E  K  M  R  G  N  E  M  A  Y  I  C  N  K  K  L  H  N  Q
AGACCGAGAAGATGAGGGGAAATGAAATGGCCTACATATGTAACAAAAAACTACATAACC
                                                            60

N  I  F  C  *  S  I  F  G  I  F  T  *  G  E  K  Q  T  N  K
 T  F  F  V  S  P  S  L  A  Y  L  H  K  E  K  N  K  Q  I  K
  H  F  L  L  V  H  L  W  H  I  Y  I  R  R  K  T  N  K  *
AACATTTTTTGTTAGTCCATCTTTGGCATATTTACATAAGGAGAAAAACAAACAAATAAA
                                                           120
```

BACK OF CLONE

```
 P  N  V  Y  A  F  S  S  I  *  W  D  S  S  K  I  -  -  S  K
  Q  M  C  M  H  S  P  A  F  D  G  I  P  P  K  -  -  S  Q  -
   K  C  V  C  I  L  Q  H  L  M  G  F  L  Q  N  -  -  L  K  -
CCAAATGTGTATGCATTCTCCAGCATTTGATGGGATTCCTCCAAAATTXXCXTCTCAAAA
        10        20        30        40        50        60
GGTTTACACATACGTAAGAGGTCGTAAACTACCCTAAGGAGGTTTTAA--G-AGAGTTTT
 W  I  H  I  C  E  G  A  N  S  P  I  G  G  F  N  -  -  *  F
  L  H  T  H  M  R  W  C  K  I  P  N  R  W  F  -  -  R  L  -
   F  T  Y  A  N  E  L  M  Q  H  S  E  E  L  I  -  -  E  F  -
```

Sequence of Clone S1

```
V  P  M  Y  V  G  E  K  E  M  I  S  L  F  Y  I  A  E  L  *
  OLIGONUCLEOTIDE  K  K  K  *  Y  H  Y  F  I  *  L  N  Y  S
                      K  R  N  D  I  I  I  L  Y  S  *  I  I  V
GTACCTATGTATGTTGGAGAAAAAGAAATGATATCATTATTTTATATAGCTGAATTATAG
                                                            60

S  I  V  Y  N  T  T  F  L  L  S  F  H  P  L  M  D  T  *  V
 P  L  Y  I  I  P  H  F  Y  Y  L  F  I  L  *  W  T  L  R  L
  H  C  I  *  Y  H  I  F  I  I  F  S  S  F  D  G  H  L  G  *
TCCATTGTATATAATACCACATTTTTATTATCTTTTCATCCTTTGATGGACACTTAGGTT
                                                           120

D  Y  M  S  L  L  L  *  I  L  L  *  *  T  Y  N  A  S  I  F
 I  T  C  L  C  Y  C  E  Y  C  C  D  K  H  T  M  Q  V  S  F
  L  H  V  F  V  I  V  N  T  A  V  I  N  I  Q  C  K  Y  L  F
GATTACATGTCTTTGTTATTGTGAATACTGCTGTGATAAACATACAATGCAAGTATCTTT
                                                           180

L  L  Q  *  L  F  P  W  *  M  H  S  S  G  I  D  G  W  N  V
 Y  Y  S  D  Y  F  L  G  R  C  I  V  V  G  L  M  D  G  M  L
  I  T  V  I  I  S  L  V  D  A  *  *  W  D  *  W  M  E  C  *
TTATTACAGTGATTATTTCCTTGGTAGATGCATAGTAGTGGGATTGATGGATGGAATGTT
                                                           240

S  S  V  F  S  P  *  E  I  S  I  L  F  P  *  R  L  Y  *  F
 V  L  F  L  V  L  E  K  S  P  Y  C  F  H  R  G  C  I  N  L
  F  C  F  *  S  L  R  N  L  H  I  V  S  I  E  V  V  L  I  *
AGTTCTGTTTTTAGTCCTTGAGAAATCTCCATATTGTTTCCATAGAGGTTGTATTAATTT
                                                           300

N  F  T  P  T  V  Y  K  H  F  H  F  S  V  S  S  P
 T  S  H  Q  Q  Y  I  S  I  S  I  S  L  Y  P  H  L
  L  H  T  N  S  I  *  A  F  P  F  L  C  I  L  T
AACTTCACACCAACAGTATATAAGCATTTCCATTTCTCTGTATCCTCACCTA
```

Sequence of Clone S3

FRONT OF GENE

```
       OLIGONUCLEOTIDE   P  R  R  *  G  E  M  K  C  L  H  M
  F  V  P  M  Y  V  G  D  R  E  D  E  G  K  *  N  A  Y  I  C
                        T  E  K  M  R  G  N  E  M  P  T  Y  V
TTTTGTTCCTATGTATGTTGGAGACCGAGAAGATGAGGGGAAATGAAATGCCTACATATG
        10        20        30        40        50        60

 *  Q  K  L  H  N  Q  H  F  C  *  S  I  F
  N  K  N  Y  I  T  N  I  F  V  S  P  S  L
   T  K  T  T  *  P  T  F  L  L  V  H  L  Y
TAACAAAAACTACATAACCAACATTTTTGTTAGTCCATCTTT
        70        80        90       100
```

BACK OF GENE

```
 K  P  S  H  S  I  I  I  N  W  A  A  C  D  C  S  T  D  -  -
  N  H  H  I  P  L  *  L  T  G  Q  H  V  T  V  L  L  -  -  -
   T  I  T  F  H  Y  N  *  L  G  S  M  *  L  F  Y  *  -  -  L
AAACCATCACATTCCATTATAATTAACTGGGCAGCATGTGACTGTTCTACTGATKKKKKT
        10        20        30        40        50        60

 *  F  *  D  G  N  F  G  G  I  P  S  N  A  G  E  C  I  H  I
  D  F  E  M  E  I  L  E  E  S  H  Q  M  L  E  N  A  Y  T  F
   I  L  R  W  K  F  W  R  N  P  I  K  C  W  R  M  H  T  H  L
TGATTTTGAGATGGAAATTTTGGAGGAATCCCATCAAATGCTGGAGAATGCATACACATT
        70        80        90       100       110       120

 W  Y  K  G  T  Y  N  L  H  F  S
  G  I  K  E  L  I  T  Y  T  S  V
   V  *  R  N  L  *  L  T  L  Q  S
TGGTATAAAGGAACTTATAACTTACACTTCAGTCA
       130       140       150
```

Sequence of Clone S6

FRONT OF CLONE

```
                        M  R  Y  I  R  A  K  R  H  S  Y  F  F
     OLIGONUCLEOTIDE    *  G  T  S  E  L  K  D  T  V  I  S  S
   V  P  M  Y  V  G  D  E  V  H  Q  S  *  K  T  Q  L  F  L  Q
TTGTACCTATGTACGTTGGAGATGAGGTACATCAGAGCTAAAAGACACAGTTATTTCTTC
        10         20         30         40         50         60

 K  R  V  C  E  L  V  V  *  R  L  T  *  T  V  L  W  L  T  C
  K  E  F  V  N  L  W  Y  K  D  *  H  R  Q  C  Y  G  L  H  V
   K  S  L  *  T  C  G  I  K  I  D  I  D  S  V  M  A  Y  M  *
AAAAGAGTTTGTGAACTTGTGGTATAAAGATTGACATAGACAGTGTTATGGCTTACATGT
        70         80         90        100        110        120

 E  L  Y  V  H  V  H  *
  N  Y  M  Y  M  Y  I  S
   I  I  C  T  C  T  L  A
GAATTATATGTACATGTACATTAG
       130        140
```

BACK OF CLONE

```
 V  L  Y  T  *  F  G  D  G  N  *  K  Y  Q  T  V  *  *  T  V
  F  Y  T  R  N  L  G  T  V  I  K  N  T  K  Q  Y  N  E  Q  S
   S  I  H  V  I  W  G  R  *  L  K  I  P  N  S  I  M  N  S  H
GTTCTATACACGTAATTTGGGGACGGTAATTAAAAATACCAAACAGTATAATGAACAGTC
        10         20         30         40         50         60

 I  Y  S  R  H  K  Q  M  C  I  I  I  *  F  C  P  C  K  N  I
  Y  I  P  D  T  S  R  C  A  L  L  S  N  F  V  L  A  K  I  L
   I  F  Q  T  Q  A  D  V  H  Y  Y  L  I  L  S  L  Q  K  Y  Y
ATATATTCCAGACACAAGCAGATGTGCATTATTATCTAATTTTGTCCTTGCAAAAATATT
        70         80         90        100        110        120

 T  I  E  V  L  C  P  F  Y  K  *  E  N  G  G  S
  R  *  K  C  Y  A  L  S  I  N  E  K  M  E  A  H
   D  R  S  V  M  P  F  L  *  M  R  K  W  R  L  T
ACGATAGAAGTGTTATGCCCTTTCTATAAATGAGAAAATGGAGGCTCAC
       130        140        150        160
```

Sequence of Clone S8

FORWARD SEQUENCE

```
 G  P  M  Y  V  G  E  Y  I  Y  I  Y  I  A  F  F  W  H  C  S
   OLIGONUCLEOTIDE  N  I  Y  I  F  I  *  H  F  S  G  T  V  H
                       I  Y  I  Y  L  Y  S  I  F  L  A  L  F  T
GGTCCTATGTATGTTGGAGAATATATATATATTTATATAGCATTTTTCTGGCACTGTTCA
        10        20        30        40        50        60

 L  F  I  H  I  Y  V  C  V  Y  I  Y  I  T  L  I  -  -  I  Y
  S  L  Y  I  Y  M  C  V  C  I  Y  I  *  L  *  -  -  -  Y  I
   L  Y  T  Y  I  C  V  C  V  Y  I  Y  N  F  D  -  -  -  I  F
CTCTTTATACATATATATGTGTGTGTGTATATATATATAACTTTGATTNNNNNNNATATAT
        70        80        90       100       110       120

 F  H  R  S
  S  I  G  H
   P  S  V  I
TTCCATCGGTCATT
       130
```

REVERSE SEQUENCE

```
              R  E  D  F  I  S  H  L  E  D  M  *  N  S  K  T
      OLIGO   G  R  T  L  F  L  I  W  K  T  C  E  T  A  K  P
   Y  V  G  E  G  G  L  Y  F  S  S  G  R  H  V  K  Q  Q  N  P
TGTATGTTGGAGAGGGAGGACTTTATTTCTCATCTGGAAGACATGTGAAACAGCAAAACC
        10        20        30        40        50        60

 L  L  V  L  Y  M  G  R  R  *  E  A  H  T  V  S  W  S  F  S
  F  W  F  C  I  W  A  D  V  E  K  P  I  R  S  H  G  P  F  P
   S  G  F  V  Y  G  Q  T  L  R  S  P  Y  G  L  M  V  L  F  L
CTTCTGGTTTTGTATATGGGCAGACGTTGAGAAGCCCATACGGTCTCATGGTCCTTTTCC
        70        80        90       100       110       120
GAAGACCAAAACATATACCCGTCTGCAACTCTTCGGGTATGCCAGAGTACCAGGAAAAGG

 L  Y  A  G  S  *  G  D  D  S  *  G  I  Q  E  I  K  S  S  N
  C  M  L  A  P  E  G  M  I  P  K  E  F  K  R  *  N  Q  A  I
   V  C  W  L  L  R  G  *  F  L  R  N  S  R  D  K  I  K  Q  *
TTGTATGCTGGCTCCTGAGGGGATGATTCCTAAGGAATTCAAGAGATAAAATCAAGCAAT
       130       140       150       160       170
```

Sequence of clone S11

FRONT OF CLONE

```
 P  M  Y  V  G  E  G  G  L  Y  F  S  S  G  R  H  V  K  Q  Q
 OLIGONUCLEOTIDE R  E  D  F  I  S  H  L  E  D  M  *  N  S  K
                   G  R  T  L  F  L  I  W  K  T  C  E  T  A  K
CCTATGTACGTTGGAGAGGGAGGACTTTATTTCTCATCTGGAAGACATGTGAAACAGCAA
        10        20        30        40        50        60

 N  P  S  G  K  V  Y  G  Q  T  L  R  S  P  Y  C  L  M  V  L
  T  L  L  E  K  Y  M  G  R  R  *  E  A  H  T  V  S  W  S  F
   P  F  W  K  S  I  W  A  D  V  E  K  P  I  L  S  H  G  P  F
AACCCTTCTGGAAAAGTATATGGGCAGACGTTGAGAAGCCCATACTGTCTCATGGTCCTT
        70        80        90       100       110       120

 F  L  V  C  W  L  L  R  G  S  Y  P  R  K  F  K  R  *  K  S
  S  L  Y  A  G  S  *  G  V  V  I  Q  G  N  S  R  D  K  N  Q
   P  C  M  L  A  P  E  G  *  L  S  K  E  I  Q  E  I  K  I  K
TTCCTTGTATGCTGGCTCCTGAGGGGTAGTTATCCAAGGAAATTCAAGAGATAAAAATCA
       130       140       150       160       170       180

 S  K  *  L
  A  N  N  Y
   Q  I  I  I
AGCAAATAATTA
       190
```

BACK OF CLONE

```
 K  T  L  L  E  K  Y  M  G  S  V  E  K  P  S  V  S  R  S  F
  K  P  F  W  K  S  I  W  A  A  L  R  S  H  R  S  L  G  P  F
   N  P  S  G  K  V  Y  G  Q  R  *  E  A  I  G  L  *  V  L  F
AAAACCCTTCTGGAAAAGTATATGGGCAGCGTTGAGAAGCCATCGGTCTCTAGGTCCTTT
        10        20        30        40        50        60

 S  L  Y  A  G  L  L  R  G  *  F  L  R  K  F  K  R  *  K  S
  P  C  M  L  A  S  *  G  D  D  S  *  G  N  S  R  D  K  N  Q
   L  V  C  W  P  P  E  G  M  I  P  K  E  I  Q  E  I  K  I  K
TCCTTGTATGCTGGCCTCCTGAGGGGATGATTCCTAAGGAAATTCAAGAGATAAAAATCA
        70        80        90       100       110       120

 S  K  *  Y  *  E  *  P  S  A  V  Q
  A  N  N  I  K  N  D  H  Q  Q  Y  K
   Q  I  I  L  R  M  T  I  S  S  T  K
AGCAAATAATATTAAGAATGACCATCAGCAGTACAAAA
       130       140       150
```

Sequence of Clone T3

FORWARD SEQUENCE

```
                    N  I  Y  I  F  I  *  H  F  S  G  T  V  H
   OLIGONUCLEOTIDE  I  Y  I  Y  L  Y  S  I  F  L  A  L  F  T
  P  M  Y  V  G  E  Y  I  Y  I  Y  I  A  F  F  W  H  C  S  L
TTCCTATGTACGTTGGAGAATATATATATATTTATATAGCATTTTTCTGGCACTGTTCAC
                                                            60

S  L  Y  I  Y  M  C  V  C  I  Y  I  *  L  D  *
 L  Y  T  Y  I  C  V  C  V  Y  I  Y  N  L  I
  F  I  H  I  Y  V  C  V  Y  I  Y  I  T  *  L
TCTTTATACATATATATGTGTGTGTGTATATATATATAACTTGATTAA
```

Sequence of Clone T8

FRONT OF CLONE

```
 P  L  V  L  S  T  S  W  S  R  W  Y  S  E  S  N  L  W  S  I
  L  *  C  S  Q  P  A  G  V  V  G  I  L  N  P  I  F  G  L  F
   F  S  A  L  N  Q  L  E  S  L  V  F  *  I  Q  S  L  V  Y  S
CCTTTAGTGCTCTCAACCAGCTGGAGTCGTTGGTATTCTGAATCCAATCTTTGGTCTATT

 H  L  G  S  K  S  Y  T  Q  L  I  H  C  S  Q  S  F
  I  L  G  L  R  A  I  P  N  L  Y  I  V  A  S  P  L
   S  W  V  *  E  L  Y  P  T  Y  T  L  *  P  V  L  Y
CATCTTGGGTCTAAGAGCTATACCCAACTTATACATTGTAGCCAGTCCTTT
        70        80        90       100       110
```

BACK OF CLONE

```
 G  -  R  L  E  Y  P  A  V  L  T  T  H  C  H  F  H  H  A  P
  -  -  V  *  S  T  Q  L  Y  *  Q  P  T  V  I  S  I  M  L  Q
   -  A  F  R  V  P  S  C  I  N  N  P  L  S  F  P  S  C  S  S
GGXGXGCGTTTAGAGTACCCAGCTGTATTAACAACCCACTGTCATTTCCATCATGCTCCA

 A  L  S  V  A  L  W  I  N  V  V  F  Y  Y  S  M  R  I  V  Q
  L  S  Q  *  L  S  G  S  M  L  S  S  I  T  Q  *  E  S  F  K
   S  L  S  S  S  L  D  Q  C  C  L  L  L  L  N  E  N  R  S  R
GCTCTCTCAGTAGCTCTCTGGATCAATGTTGTCTTCTATTACTCAATGAGAATCGTTCAA
        70        80        90       100       110       120

 G  C  R  W  L  E  K  -  -  I  L  *  I  S  A  G  -  -  G  *
  D  A  G  G  L  R  -  -  -  S  Y  R  L  V  Q  -  -  -  V  N
   M  Q  V  A  *  E  -  -  N  P  I  D  *  C  R  -  -  -  L  I
GGATGCAGGTGGCTTGAGAAGKKKKKAATCCTATAGATTAGTGCAGGAKKKKKKGGTTAA
       130       140       150       160       170       180

 Y  Y  L  L  D  C  S  L  S  V  C  G    OLIGONUCLEOTIDE
  I  I  F  L  T  V  V  S  L  F  V  V  E  R  A  R  R  R  T
   L  S  S  *  L  *  S  L  C  L  W  *
TATTATCTTCTTGACTGTAGTCTCTCTGTTTGTGGTAGAAAGAGCTCGAAGAAGAACA
       190       200       210       220       230
```