Characterisation of genes identified during a RADES-PCR screen of concanavalin A-treated procyclic *Trypanosoma brucei rhodesiense*

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by

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Abstract

Previous studies demonstrated that procyclic *Trypanosoma brucei* could be induced to die by culturing cells in the presence of the lectin Concanavilin A, and that throughout the three day death process *de novo* gene expression could be detected. The current study utilised a **d**ifferential **d**isplay **r**everse **t**ranscription **p**olymerase **c**hain **r**eaction (DDRT-PCR) technique known as **r**andomly **a**mplified **d**ifferentially **e**xpressed **s**equences (RADES) PCR to identify cDNAs whose expression levels fluctuated during the time-course of death. 64 PCR products were detected, of which 27 were re-amplified and sequenced. Database searches were conducted using the BLAST algorithm, identifying 17 significant matches with known genes in the database. 8 of these encoded novel *T. brucei* genes. Northern blot analysis was attempted in order to confirm expression patterns indicated by RADES-PCR. However, the data obtained was inconclusive due to lack of a marker constitutively expressed during cell death. Two genes identified during this study, *QM* and *MOB1*, were characterised further.

The human *QM* gene was first identified as a transcript upregulated in a non-tumourigenic Wilms' tumour microcell hybrid relative to the parental cell line, and subsequent experiments suggested that the *QM* gene encoded a transcription factor. More recent evidence indicates that QM is actually a ribosomal protein associated peripherally with the 60S ribosomal subunit. During the current study Southern blot analysis was conducted, indicating the presence of 2 copies of the *T. brucei QM* gene, one of which was isolated from a genomic λ library. Sequence analysis revealed 60% amino acid identity between the *T. brucei* QM and QM homologues from diverse eukaryotes. A recombinant epitope-tagged QM was inducibly expressed in procyclic *T. brucei*. Indirect immunofluorescence microscopy revealed nuclear exclusion and co-localisation with GPI8, a component of the transamidase complex responsible for glycosylphosphatidylinositol (GPI) anchor attachment which is hypothesised to localise to the endoplasmic reticulum. Tagged QM in cellular extracts was demonstrated to be insoluble following lysis in a 1% Triton X-100 buffer, suggesting an association with a large protein complex. Taken together these results suggest that the *T. brucei* QM is a ribosomal protein.

MOB1 is an essential yeast gene required for completion of mitosis and maintenance of ploidy. While a number of interacting partners have been identified for MOB1, the function of this protein remains unknown. During the current study Southern blot analysis showed the presence of 2 copies of the *T. brucei MOB1* gene within 12 kb of each other. Both of these genes were isolated from a single genomic λ clone and were named *MOB1-1* and *MOB1-2*. Sequence analysis revealed that while >96% of the amino acid sequence encoded by *MOB1-1* was conserved in the *MOB1-2* gene, the latter had a predicted N-terminal extension of 82 residues. Inducible expression of an antisense *MOB1-1* mRNA in procyclic *T. brucei* resulted in a significant reduction in proliferation, indicating a role for MOB1 in cell cycle progression. A recombinant epitope-tagged MOB1-1 was inducibly expressed in procyclic *T. brucei*. Indirect immunofluorescence microscopic analysis revealed a homogenous distribution throughout the cell, allowing no insight into function. A recombinant MOB1-1 polypeptide was produced in *E. coli* and used to inoculate rabbits. Resultant antiserum detected a *T. brucei* protein of the predicted size of MOB1-1. This antiserum will prove invaluable for future studies, allowing the subcellular location of native MOB1-1 to be established, and purification of interacting partners through co-immunoprecipitation to be carried out.

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The research reported in this thesis is my own original work, except where otherwise stated, and has not been submitted for any other degree.

Simon G. Lillico

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CHAPTER ONE

Introduction

"Biological phenomena are in part frozen remnants of complex evolutionary histories and cannot be completely explained teleologically merely in terms of energy efficiency and the most parsimonious mechanisms" (Simpson and Theimann, 1995)

1.1.1 Evolution of the kinetoplastids

Together with their sister group the euglenoids, the kinetoplastid protozoa represent the earliest extant group of eukaryotic organisms containing a mitochondrion (Sogin, 1991). Based on morphological differences, kinetoplastids are subdivided into two suborders: the biflagellate Bodonina containing the families Bodonidae and Cryptobiidae, and the uniflagellate Trypanosomatina with a single family Trypanosomatidae. While bodonids/cryptobiids include free-living and commensal organisms, ecto- and endoparasites of fish and some invertebrates as well as some fish blood parasites, trypanosomatids are all obligate parasites. Several genera of trypanosomatid are composed largely or wholly of monogenetic parasites of a diverse group of invertebrates, but are found predominantly in insects. Digenetic lifecycles occur in species from the genera *Trypanosoma*, *Leishmania* and *Endotrypanum* and involve cyclical transmission through an insect vector and a vertebrate host (Vickerman, 1994; Maslov and Simpson, 1995; Lukes *et al.*, 1997).

There are two major hypotheses for the origin and evolution of parasitism in kinetoplastids, and the debate has now been going for almost 100 years. Both theories agree that free-living bodo-like flagellates gave rise to the parasitic trypanosomatids, but disagree on the nature of the primary host. The 'invertebrate first' hypothesis was proposed by Leger in 1904 (cited in Maslov and Simpson, 1995), and assumes that parasitism by this group was first established in the digestive tract of Precambrian invertebrates. These primitive organisms went on to become the ancestors of modern insects and leeches, explaining the wide distribution of kinetoplastids among invertebrates today. Under this hypothesis digenetic lifecycles arose with the advent of haematophagy, parasites being inoculated into the vertebrate host during invertebrate

feeding. If the invertebrate first hypothesis holds true then phylogenetic analysis should place monogenetic parasites of invertebrates on the earliest diverging branches of a tree, with digenetic parasites distributed amongst more recently evolving lineages (Maslov and Simpson, 1995).

The 'vertebrate first' hypothesis proposed by Minchin in 1908 (cited in Maslov and Simpson, 1995) states that parasitism first arose in the guts of vertebrates, and that parasitism of blood and tissues arose subsequently (Wallace, 1966). With the advent of haematophagous arthropods it is thought that blood-blood transmission was adopted by parasites, and gut infections became increasingly rare. If this hypothesis holds true then phylogenetic analysis should reveal that the monogenetic parasites of invertebrates arose from digenetic ancestors, explaining why most invertebrates that harbour trypanosomatids belong to haematophagous groups (Maslov and Simpson, 1995).

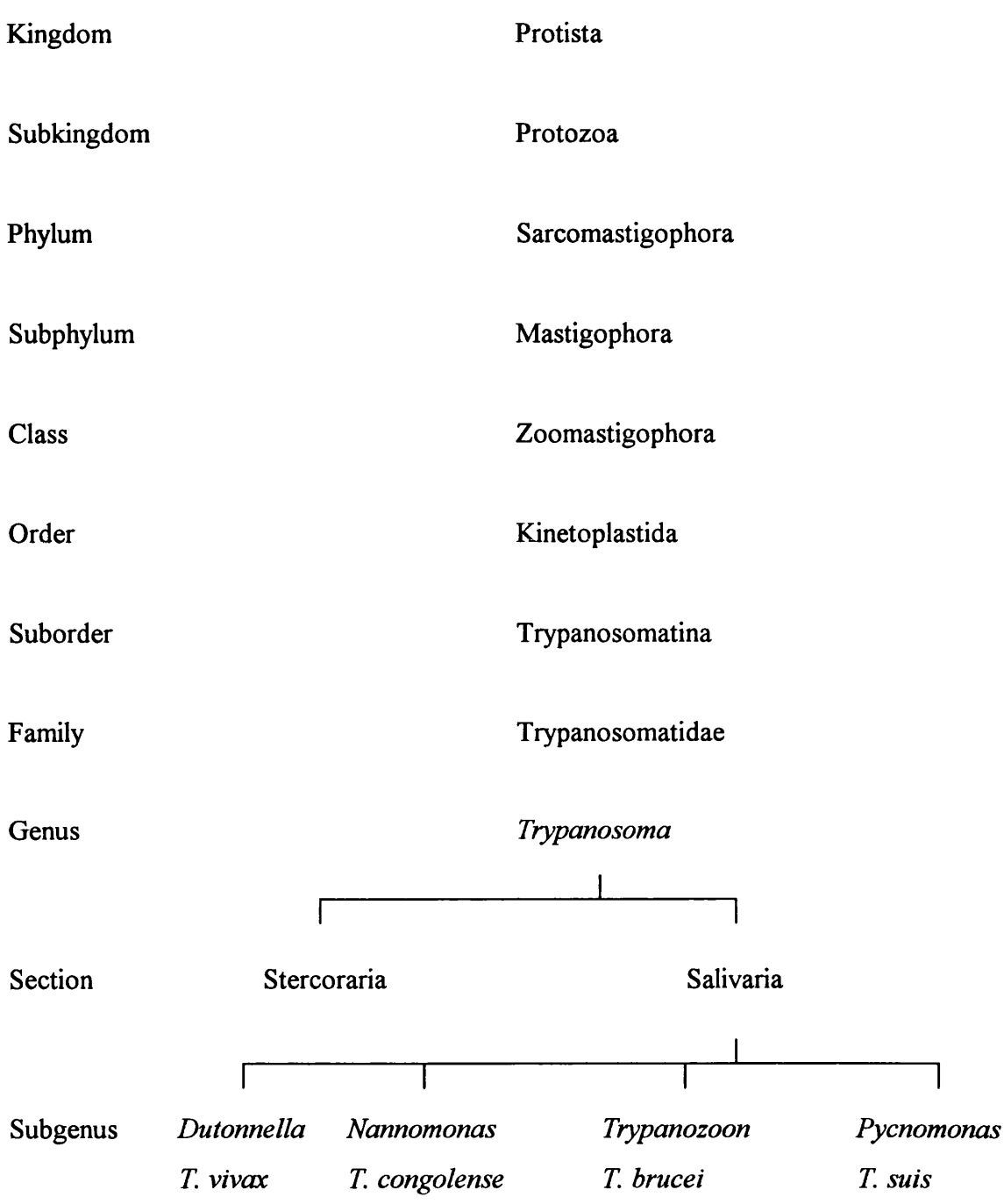
Phylogenetic trees based on nuclear small- and large-subunit rRNAs provide support for both hypotheses, with digenetic organisms interspersed with monogenetic ones. This led to the conclusion that digeneity evolved independently several times from monogenetic invertebrate parasites (Fernandes *et al.*, 1993), further supported by the dissimilar immune evasion strategies employed by digenetic kinetoplastids within their vertebrate hosts. However, as the 'vertebrate first' hypothesis also relies on digeneity being a derived character this debate seems set to continue (Maslov and Simpson, 1995).

1.1.2 Trypanosomiasis

The trypanosomiases are a group of diseases which affect both man (sleeping sickness and Chagas' disease) and animals (nagana, surra, mal de caderas, murina, derrengadera and dourine) and are of both medical and economic importance throughout large areas of Africa, Asia and South America.

All members of the genus *Trypanosoma* (except *T. equiperdum*) are heteroxenous, or are at least transmitted by an animal vector. They are haemoflagellate parasites of all classes of vertebrates, living in the blood and tissue fluids although some, most notably *T. cruzi*, may occupy intracellular habitats as well. The majority are transmitted by blood-feeding arthropods, although exceptions to this rule do exist, as exemplified by *T. equiperdum* which can only be transmitted sexually, *T. equinum* which is primarily transmitted mechanically by tabanid flies but can also be transmitted by vampire bats, and *T. cruzi* which can be passed from donor to host during blood transfusions (Schmidt and Roberts, 1989). Tsetse flies (genus *Glossina*) are the vectors of *Trypanosoma brucei* s.l. (subgenus Trypanozoon) which cause disease in both man and livestock in sub-Saharan Africa. Sleeping sickness, a disease thought to be well under control in the 1960's, has re-emerged in the last decade as a threat to the lives of the rural poor in several African countries. WHO estimate there are 55 million people at risk of sleeping sickness in Africa (25,000 cases were reported in 1995 but only 4 million people were under surveillance - if surveillance were adequate, WHO estimate there would be between 300,000 and 500,000 cases per annum) (WHO web site at http://www.who.int/emc/diseases/tryp/trypanoepidat.html).

Figure 1.1 Phylogeny of salivarian trypanosomes



There are three subspecies of *T. brucei*: *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense*. All three subspecies are cyclically transmitted by blood-sucking tsetse flies, parasitise the blood and body fluids of their mammalian hosts and are morphologically,

biochemically and serologically indistinguishable (Hajduk *et al.*, 1992). Only *T. b. rhodesiense* and *T. b. gambiense* are able to infect man, and the distribution of these two subspecies is determined for the main part by the ecological and geographical distribution of the tsetse species that transmit them. *T. b. rhodesiense* is found mainly in East Africa, from Ethiopia and eastern Uganda south to Zambia and Zimbabwe. It is transmitted by woodland, savannah and riverine tsetse species including *G. morsitans*, *G. pallidipes* and *G. fuscipes* and is a zoonosis, having a reservoir in wild game animals as well as domestic livestock. It causes an acute disease, with the patient usually dying within a few months (Cheesbrough, 1987; Schmidt and Roberts, 1989). In contrast *T. b. gambiense* is found in West, western Central and Northern regions, from Senegal across to Sudan and down to Angola. It is transmitted by riverine and lakeside tsetse species including *G. palpalis*, *G. fuscipes* and *G. tachinoides*. Gambian sleeping sickness is predominantly an anthroponosis, although a reservoir in domestic pigs does exist (Cheesbrough, 1987; Schmidt and Roberts, 1989). The disease course is chronic with the initial phase involving the blood and lymphatic tissues lasting for several years. Death is often from secondary causes such as malnutrition, pneumonia, other parasitic diseases or a severe fall (Cheesbrough, 1987; Schmidt and Roberts, 1989; Hajduk *et al.*, 1992). The clinical differences between Gambian and Rhodesian sleeping sickness are not clear-cut and are mainly a question of time scale. Early in the disease there is high fever with persistent headache and joint pains. The lymph glands close to the site of inoculation become swollen; in *T. b. gambiense* infection the posterior cervical lymph nodes at the back of the neck are involved (Winterbottom's sign), while in *T. b. rhodesiense* infections it is usually the glands under the jaw, in the arm-pit, at the base of the elbow or in the groin. As the disease progresses the spleen becomes enlarged and anaemia due to immune-complex-induced haemolysis is common. In the late stages the

CNS may be invaded, inducing trembling, inability to speak properly, progressive mental dullness, apathy, excessive sleeping and incontinence. In the absence of chemotherapy, coma develops then death in almost all cases. These symptoms are more common in *T. b. gambiense* infections, as with *T. b. rhodesiense* the patient often dies from toxaemia or heart failure before symptoms can become fully developed (Poltera, 1985; Van Meirvenne and Le Ray, 1985).

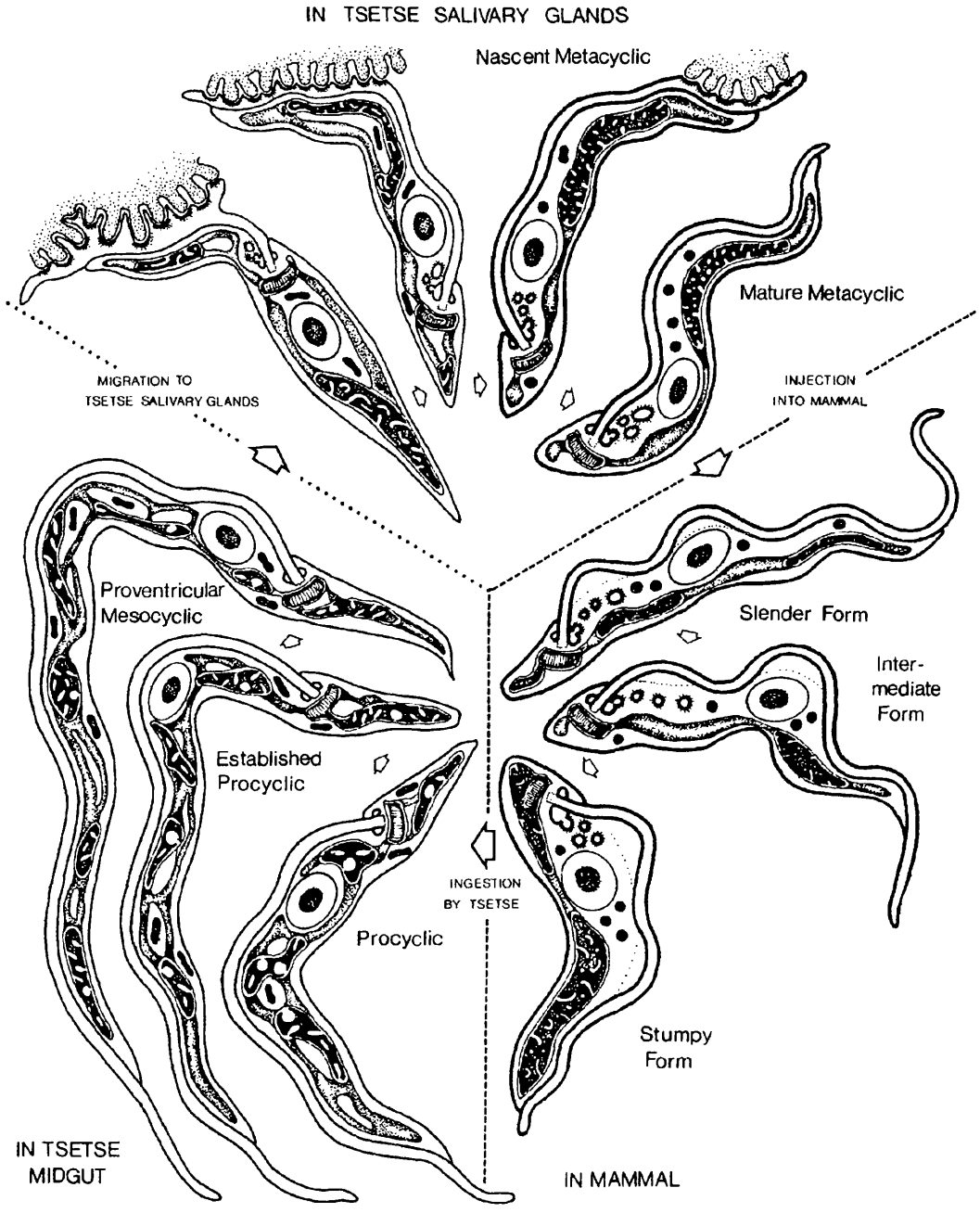
The greatest limitation currently faced in the chemotherapy of African trypanosomiasis is the lack of a drug with activity against all stages of the disease and all species of the parasite. Chemotherapy for *T. brucei* spp relies heavily on the drugs pentamidine, suramin and melarsoprol, all of which have been in use for almost 60 years. Side effects of these drugs can be severe, with death occurring in approximately 5% of patients treated with the arsenical melarsoprol (Pepin and Milord, 1994; Gutteridge, 1985). The reasons behind the lack of more suitable drugs for the prophylaxis and treatment of this disease are both scientific and commercial. On the scientific front the problem relates mainly to getting any drug not only into the trypanosome itself, but also into the parasites' microhabitat within the host. CNS involvement imposes a significant pharmacological barrier to many drugs, and is a problem yet to be solved. On the commercial front the costs involved in developing, testing and marketing a new drug are likely to be higher than the potential return (Gutteridge, 1985).

1.1.3 The lifecycle of *T. brucei* spp.

Trypanosoma brucei s.l. infections are transmitted by the infected bite of tsetse flies. On biting a host an infected tsetse injects metacyclic trypomastigotes in the dermal connective tissue, causing a local inflammatory reaction called a chancre to develop. This lesion develops for a period of about two weeks, and after multiplying within it for several days the trypanosomes access the draining lymphatics and then the blood, where they multiply by binary fission as long slender trypomastigotes with a doubling time of about 6 hours.

Host parasitaemias are characterised by a gradual increase in the numbers of trypanosomes followed by antibody mediated decimation. Evasion of the host's immune response is achieved by means of antigenic variation on the part of the parasites (Cross, 1975; Vickerman, 1978). Long slender dividing trypomastigotes predominate during the ascending parasitaemia with the majority of the population belonging to a single antigenic type (the 'homotype'). The IgM response mounted by the host against the homotype results in the parasite population going into remission as individuals of that variable antigenic type (VAT) are killed (Seed, 1977), with non-dividing short stumpy forms seen at the peak and during the declining parasitaemia (Brown *et al.*, 1973). Serological studies have shown that as well as the homotype several other VATs, termed 'heterotypes', coexist within the population. These continue to multiply during remission until one of them outgrows the others and becomes the new homotype, causing a recrudescence of the parasitaemia (Vickerman, 1985; Van Meirvenne and Le Ray, 1985).

Figure 1.2 Schematic representation of the *T. brucei* life cycle



Lifecycle taken from Vickerman (1985)

Bloodstream form trypomastigotes possess a cell surface coat 12-15nm thick (Vickerman, 1969) consisting of more than 10^7 densely packed variable surface glycoprotein (VSG) molecules (Cross, 1975). Each VAT arises from the expression of a single VSG gene from a repertoire of approximately 1000, the majority of which are situated in large arrays in the interior of the larger chromosomes (Van der Ploeg *et al.*, 1982a). However, a subset of VSG genes are located at the telomeres of chromosomes, most notably the approximately 100 minichromosomes (Van der Ploeg *et al.*, 1982b; Agur *et al.*, 1989; Graham and Barry, 1996; Horn and Cross, 1997; Turner, 1997). VSG genes are expressed in one of many polycistronic telomeric expression sites along with at least eight expression site-associated genes (ESAGs) (Borst *et al.*, 1997; Cross *et al.*, 1998), all under the control of a polymerase I-like promoter situated 40-60 kb upstream of the VSG gene (Pays *et al.*, 1989; Zomerdijk *et al.*, 1990). The surface coat may be switched either by a DNA rearrangement (the creation of an expression-linked copy of a transcriptionally silent VSG gene and insertion of this into the currently active expression site, with concomitant destruction of the original VSG gene occupying that site), or by transcriptional activation of a second expression site already containing a different VSG, with concomitant deactivation of the originally active site (Graham and Barry, 1996; Rudenko *et al.*, 1996; Borst *et al.*, 1997; Cross *et al.*, 1998). Repression of transcription from all but one of the bloodstream-form expression sites is critical for mutually exclusive VSG expression and parasite survival in the mammalian host. Rate of switching between different VSG genes has been a matter of some debate, with syringe passaged (monomorphic) lines showing per capita rates of 10^7-10^5 switches/cell/generation (Lamont *et al.*, 1986), while lines cyclically transmitted through tsetse can show rates of greater than 10^2 switches/cell/ generation (Turner, 1997). Switching appears to be

spontaneous, requiring neither the host's immune system or other factors in the blood for induction (Hajduk *et al.*, 1992).

1.1.4 Parasite development in the insect vector

When infected blood is ingested by a feeding tsetse fly, trypanosomes are taken up first into the crop and then the lumen of the midgut. Parasites face radical changes in their environmental conditions such as a drop in temperature from 37°C to about 25°C, tsetse digestive enzymes and a non-specific immune system. Furthermore glucose, the principal energy source of bloodstream form trypanosomes, disappears from the tsetse bloodmeal within about 15 minutes of ingestion, so a switch from utilisation of glucose to utilisation of proline is adopted (Evans and Brown, 1972). Under these conditions the long slender bloodstream form either dies or transforms into the short stumpy form in the anterior midgut. Short stumpy trypomastigotes are thought to be preadapted to these conditions and subsequently differentiate into the procyclic form, involving an increase in body length, expansion of the mitochondrion (Hecker *et al.*, 1972), transformation of the glycosome from a branched to bacilliform structure and loss of the VSG coat (Overath *et al.*, 1983). These changes all take place within 48-72 hours of ingestion (Vickerman, 1985). The VSG coat is shed within about 4 hours of ingestion and is simultaneously replaced with an invariant coat made of procyclin (also called procyclic acidic repetitive protein or PARP) (Mowatt and Clayton, 1987; Richardson *et al.*, 1988) attached to the plasma membrane by a C-terminal glycosylphosphatidyl-inositol (GPI) anchor (Field *et al.*, 1991). There are approximately 5 x 10^6 PARP molecules coating each cell (comparable to VSG molecules in blood-stream forms), forming a protective glycocalyx around the parasite (Ferguson, 1997).

From day four onwards the procyclic (insect form) parasites begin to invade the ectoperitrophic space (Evans and Ellis, 1983) - the space between the peritrophic membrane and the midgut epithelium. In the days following this invasion the ectoperitrophic space becomes packed with dividing trypanosomes, the substrate utilisation of which remains a mystery. Once an infection is established in a susceptible fly, procyclic trypanosomes will reside in the ectoperitrophic space for the life of the fly, which may be several months in a female tsetse. During this period the parasite population density remains remarkably constant (Welburn and Maudlin, 1997). Some parasites migrate towards the proventriculus growing in length as they do so and ceasing to divide. At this stage they are referred to as mesocyclic forms, and it is thought that it is these trypanosomes which go on to reinvade the endoperitrophic space, migrate via the oesophagus to the mouthparts and from there through the salivary ducts to the salivary glands (Vickerman, 1985). The main proliferative stage within the salivary gland is the epimastigote, which, like procyclic forms is coated by procyclin (Hehl *et al.*, 1994; Roditi *et al.*, 1998). Epimastigotes attach to the microvilli of the epithelial cells lining the gland lumen by means of outgrowths from the flagellum, termed 'flagellipodia', that wrap themselves around the microvilli, forming junctional complexes at focal points of contact. Epimastigotes differentiate into 'premetacyclics', retaining the branched mitochondrion and bacilliform glycosomes of the epimastigote together with the ability to proliferate, but with reduced flagellipodia. These in turn become 'nascent metacyclics' through cessation of cell division and acquisition of a VSG coat (Tetley and Vickerman, 1985). While still attached by means of the flagellum they now have the unbranched mitochondrion and spherical glycosome, preadapting the parasite for life in the mammalian bloodstream. Mature metacyclics inhabit the lumen of the salivary gland and

are heterogeneous with respect to VAT (Vickerman, 1985; Tetley *et al.*, 1987). Metacyclic trypomastigotes express a distinct subset of VSG genes numbering no more than 27 (Turner *et al.*, 1988), located at telomeric metacyclic-specific expression sites (Cornelissen *et al.*, 1985; Barry *et al.*, 1990). These metacyclic VSGs are subject to life cycle stage-specific control of transcription initiation (Graham and Barry, 1995; Graham *et al.*, 1998), a situation unique in Kinetoplastida, where all other genes are regulated, at least partly, post-transcriptionally (Graham, 1995).

1.1.5 The kinetoplast and RNA editing

Members of the Order Kinetoplastida all possess a kinetoplast, a differentiated region of the single mitochondrion, containing the mitochondrial genome (kDNA). This structure is unique in nature, and contains thousands of interlocked circular DNA molecules (see Borst and Hoeijmakers, 1979 for electron micrographs) that represent 5-30% of the total cellular DNA in an array that has been likened to chain mail (Stuart, 1983; Shapiro and Englund, 1995). These DNA circles are of two types: 25-50 maxicircles, each 20-40kb (depending on species) and 5000-27000 minicircles, each 0.46-2.5kb. Maxicircles all have the same DNA sequence and are the functional homologues of mitochondrial DNA of other eukaryotes, carrying genes for mitochondrial ribosomal RNAs and subunits of some proteins involved in electron transport and ATP synthesis, although it appears that they do not code for tRNAs. In contrast, minicircles are heterogenous in sequence and evolve rapidly within a given trypanosome stock. They make up 90-95% of the mass of the kDNA network and encode small (70 nucleotides) guide RNAs (gRNAs) involved in mRNA editing (Sturm and Simpson, 1990). The degree of heterogeneity of minicircles within species of trypanosomatid reflects the number of gRNAs required for editing (Borst, 1991; Shapiro and Englund, 1995).

Many mRNA transcripts within the kinetoplast of *T. brucei* require editing in order to form mature functional mRNAs (Arts and Benne, 1996), a process involving the insertion or deletion of uridine residues (Blum *et al.*, 1990; Cruz-Reyes *et al*, 1998). In some cases more than half of the nucleotides within the open reading frame of a mitochondrially encoded mRNA require to be introduced during editing (Feagin *et al.*, 1988). gRNAs are antisense to portions of the edited mRNAs, and can form anchor duplexes with pre-edited mRNA just downstream of the sequence that is to be edited (Maslov and Simpson, 1992). It is generally agreed that mismatches between a gRNA and mRNA identify specific sites to be edited by a putative editosome, but the precise mechanism by which this occurs is not clear. After a stretch of mRNA has been edited the bound gRNA is removed, allowing subsequent gRNAs to bind and continue editing the upstream fragment of the transcript (Maslov and Simpson, 1992; Simpson and Theimann, 1995; Shapiro and Englund, 1995).

1.1.6 The nucleus and trans-splicing

The chromosomes of *T. brucei,* as visualised by pulsed field gradient gel electrophoresis, have been divided into three classes: minichromosomes number between 50 and 100 copies, and are of 50-150 kb. Over 90% of the length of these chromosomes consists of tandem arrays of 177 bp repeats (Weiden *et al.*, 1991), with non-transcribed basic copy VSG genes located at the telomeres. Intermediate length chromosomes range from 200-900 kb and are thought to be composed primarily of repeat units, with VSG expression sites located at their telomeres. The ploidy of mini- and intermediate chromosomes is currently unknown. Megabase chromosomes range in size from around one Mb to more than 5 Mb, and encode most (possibly all) of the housekeeping genes, as well as many basic copy VSG genes. Interestingly, while megabase chromosomes are diploid,

homologues of a pair may be of significantly different sizes (Melville *et al.*, 1998; Melville *et al.*, 1999). The trypanosomes genome contains large numbers of repeated and transposable elements, and together with high levels of homologous recombination evolves rapidly (Van der Ploeg *et al.*, 1984; Gull *et al.*, 1998; Melville *et al.*, 1999).

The chromosomes of trypanosomes do not condense during mitosis, possibly as a result of differences in the amino acid composition of their histones from those of higher eukaryotes (Burri *et al.*, 1994). Electron microscopic analysis revealed that the mitotic spindle of *T. brucei* contained insufficient microtubules to provide a conventional centromere-microtubule interaction for each of the minichromosomes (Vickerman, 1994). A model has now been proposed whereby the minichromosomes congregate in the centre of the nucleus at the start of M-phase where they associate with the emerging central spindle. The mass of minichromosomes then divides into 2 clusters and separates, one moving to each spindle pole. This model is strongly supported by microscopic observations. However, it is still unknown whether this mechanism results in faithful segregation of minichromosomes (Ersfeld and Gull, 1997).

Protein encoding genes in higher eukaryotes are comprised of a number of introns (non-coding) and exons (protein encoding). Transcription is directed by a promoter situated in the 5' flank of the gene, begins at an initiation site 5' of the coding region and continues until a transcription termination site is reached in the 3' flank of the gene (Alberts *et al.*, 1994). Processing of primary transcripts involves the addition of an mGppp cap to the 5' residue of the RNA, polyadenylation at a pre-determined site within the 3' UTR and cis-splicing to remove introns, simultaneously joining adjacent exons. Genes are generally arranged as monocistronic units, that is, each gene is under the

control of its own promoter. Consequently, one of the major determinants of the level of gene product within a cell is the activity of the promoter controlling transcription of that gene (transcriptional regulation). Manipulation of downstream events such as mRNA stability (post-transcriptional regulation), rate of translation (translational regulation) and protein turnover (post-translational regulation) may also be utilised in order to influence the final level of both mRNAs and proteins within the cell (Graham, 1995).

In contrast to the genetic organisation described above, trypanosomatid genes tend to be arranged in polycistronic transcriptional units, that is, several genes separated by short intergenic sequences under the control of a single 5' promoter (Imboden *et al.*, 1987; Muhich and Boothroyd, 1988). Transcription of such units yields polycistronic precursor RNA encoding several genes that may or may not have related functions (Imboden et al., 1987; Berberof *et al.*, 1991; Paindavoine *et al.*, 1992). Analysis of the entire 300 kb sequence of *L. major* chromosome 1 revealed 79 putative protein encoding genes distributed between 2 coding units of 29 and 50 genes, arranged in head-to-head orientation on opposing strands. Genes were categorised by function and were determined not to display the operon-like clustering of prokaryotes. Whether the 2 coding units observed represent large polycistronic units or contain promoter sites within them is currently unknown (Myler *et al.*, 1999).

As a promoter drives transcription of all genes within a polycistron at the same rate, organisation of a genome into polycistronic units results in the inability to transcriptionally regulate individual genes. However, markedly different levels of gene expression have been reported for various members of the same array (Vanhamme *et al.*, 1999), indicating that control of gene expression in trypanosomes operates primarily at

post-transcriptional levels in order to generate appropriate cellular concentrations of gene products (Graham, 1995; Vanhamme and Pays, 1995).

All known trypanosomatid genes lack introns (Graham, 1995; Ismaili *et al.*, 1999; Myler *et al.*, 1999), and thus generation of mature transcripts does not require a cis-splicing mechanism (Graham, 1995). Instead, individual mRNAs are produced from polycistronic transcripts by trans-splicing of a common cap-bearing sequence termed the spliced leader (or mini-exon) to the 5' end of primary transcripts (Sather and Agabian, 1985; Murphy *et al.*, 1986), and polyadenylation of their 3' terminus (Huang and van der Ploeg, 1991) (for schematic representation see Graham, 1995). It is thought that trans-splicing and polyadenylation may be functionally linked, explaining the lack of a consensus signal for polyadenylation in trypanosomatids (Graham, 1995; Vanhamme and Pays, 1995).

The spliced leader (SL) sequence of mature trypanosome mRNAs was first discovered during the characterisation of cDNA clones encoding VSGs. It was identified as a 35 nucleotide long stretch of DNA common to all VSG cDNA clones but absent in the basic copy genes (Boothroyd and Cross, 1982; Van der Ploeg *et al.*, 1982b). It is now known that the SL sequence is in fact 39 nucleotides in length, is trans-spliced onto the pre-mRNA transcript 10-200 nucleotides upstream of the genes initiator methionine codon and is present in all known mature trypanosome mRNAs (Donelson and Zeng, 1990). A 1.35kb repeat unit, present in at least 200 copies within the genome, encodes the SL sequence. The primary transcript of this unit, termed spliced-leader derived RNA (SL-RNA), is 141 nucleotides long and contains the 39 nucleotide SL sequence at it's 5' terminus (Parsons *et al.*, 1984; Bonen, 1993; Pellé and Murphy, 1993a; Vanhamme and

Pays, 1995). At the 5' end of the SL-RNA is a 'cap 4' structure consisting of a 7-monomethylguanosine linked via a 5'-5'-triphosphate to four highly modified nucleosides (Perry *et al.*, 1987). The presence of this cap structure on SL-RNA appears to be necessary for trans-splicing and may act as a recognition signal for components of the trans-splicing machinery (Bonen, 1993).

Many features of trans-splicing in trypanosomatids resemble the molecular mechanisms of cis-splicing in higher eukaryotes. Trans-splicing adds a SL from a SL-RNA to a pre-mRNA at a 5' consensus splice acceptor site by means of a normal 3'-5' phosphodiester bond. The 3' end of the SL-RNA and the portion of the pre-mRNA upstream of the SL acceptor site are thus analogous to a fragmented intron in a cis-splicing system, and form a Y-branched structure analogous to the cis-splicing lariat. Trans-splicing in trypanosomes has been demonstrated to occur within large ribonucleoprotein complexes (as with cis-splicing in higher eukaryotes) which contain homologues of spliceosomal U2, U4, U5 and U6 small nuclear RNAs (snRNAs), primary cofactors of cis-splicing. A homologue for the U1 snRNA has also recently been discovered in trypanosomatids (Mottram *et al.*, 1989; Agabian, 1990; Bonen, 1993; Dungan *et al.*, 1996; Metzenberg and Agabian, 1996; Goncharov *et al.*, 1998; Schnare and Gray, 1999).

1.2.1 Discovery of apoptosis in mammals

The term apoptosis was adopted to define an active process of physiological cell death, and is derived from a Greek word meaning "a falling away" as of leaves from trees (Kerr *et al.*, 1972). The earliest reports of apoptotic bodies (remnants of cells that have died by apoptosis) described them as being widely distributed in histological sections of many healthy tissues. This led to the proposition that apoptosis was an important mode of cell death, and that it may in fact be the only mode of controlled cell death contributing to the regulation of cell populations in a variety of tissues under physiological conditions (Kerr *et al.*, 1972).

It is now generally accepted that the death of individual cells within a multicellular organism is, more often than not, essential for the continued life and development of that organism (Hardy, 1999; Abrams, 1999). Cell death forms an essential counterpoint to mitosis, and in embryological development, morphogenesis and metamorphosis is used to help shape and structure tissues, organs and organisms. Such processes as the transformation of caterpillars to butterflies (reviewed by Ellis *et al.*, 1991) or the development of the pentadactyl limb through interdigital cell death (Garcia-Martinez *et al.*, 1993; Zakeri *et al.*, 1994) appear to be under genetic control.

Apoptosis as defined by Kerr *et al.* (1972) is a form of programmed cell death (PCD) with its own characteristic morphology. As a result apoptosis is commonly referred to as PCD, but the two terms should not be used synonymously as there are many cases when the death of cells by apoptosis is not predetermined (as is the case with PCD), but a response to changing environmental stimuli.

1.2.2 Necrosis, apoptosis and mitosis

The word necrosis, like apoptosis, is derived from Greek and means "to make dead". Necrosis is commonly caused by significant deviations from physiological conditions such as hypotoxia, major changes in temperature, exposure to toxins or disruption of cell membranes. It is characterised by huge increases in permeability of both plasma and organellar membranes with resultant osmotic swelling and eventual rupture of the cell. In solid tissue it is typical for a number of contiguous cells to be involved simultaneously, and the debris produced following their rupture can lead to tissue inflammation and secondary necrosis of bystander cells. Debris is eventually removed by professional phagocytes (Wyllie *et al.*, 1980; Martin, 1993; Hetts, 1998). The timeframe of cellular necrosis is typically short, and can occur within seconds (Willingham, 1999).

In contrast to necrosis, apoptosis within a tissue tends to affect single cells, which separate from their neighbours. Cytoplasmic condensation results in vacuolisation of the cell, while protuberances form on the cell membrane (often referred to as membrane blebbing). Characteristic changes can also be seen in the nucleus where the chromatin collapses into electron-dense masses that become marginated around the periphery of the organelle. Nuclear pores move away from the marginalised chromatin facilitating the dissolution of the nucleus into discrete fragments (Earnshaw, 1995). Cellular disassembly follows with the entirety of the contents being packaged into membrane-bound vesicles termed apoptotic bodies which are derived from the protuberances on the cell surface. Apoptotic bodies vary greatly in size and may even contain intact organelles. Changes in the makeup of surface proteins and lipids allows apoptotic bodies to be readily identified and engulfed by neighbouring cells, as well as professional phagocytes. Formation and removal of apoptotic bodies can take as little as 24 hours.

As cellular constituents are not released directly into the intercellular matrix an inflammatory response does not occur, making this process suitable for tissue homeostasis. Throughout the entire process both the cell as a whole, and the resultant apoptotic bodies retain their membrane integrity (c.f. necrosis) (Wyllie *et al.*, 1980; Earnshaw, 1995; Hetts, 1998). Apoptotic cell death is a slower process than necrosis, and can take from a few hours to several days (Willingham, 1999).

Apoptosis is particularly common in cells derived from highly mitotic cell lines, raising the possibility that apoptosis may be a type of abnormal or mis-timed mitosis (King and Cidlowski, 1998). Supporting this theory is the fact that apoptosis is frequently associated with disrupted growth factor production/reception (Liu and Zhu, 1999; Napier *et al.*, 1999), activation of oncogenes (Zornig and Evan, 1996) and involvement of cell-cycle regulatory genes (Choi *et al.*, 1999) - all important in mitosis. Furthermore, in both apoptosis and mitosis cells become rounded, the plasma membrane becomes "blebbed", the nuclear lamina disassembles and the chromatin condenses (King and Cidlowski, 1998). However, while chromatin condensation during mitosis is an active process requiring a number of cell-cycle-related kinases, this is not thought to be the case in apoptosis, where condensation appears to be the result of loss of structural integrity of several nuclear components (Hendzel *et al.*, 1998).

Apoptotic cell death occurs in two distinct phases, widely termed the commitment and execution phases. The commitment phase is of extremely variable length and consists of the time between which the cell receives an apoptotic stimulus (which may be either intrinsic or extrinsic) and the onset of apoptotic morphology. Transition to the execution phase follows, during which the morphological features of apoptosis are observed over a

predetermined period of time (Earnshaw, 1995). Apoptotic signals are diverse, including DNA damage (Uberti *et al.*, 1999), withdrawal of trophic factors (Mesner *et al.*, 1992; Syroid *et al.*, 1999) and parasitic (Seydel and Stanley, 1998; Leguizamon *et al.*, 1999) and viral infection (Allsopp *et al.*, 1998).

For several years after its discovery apoptosis was thought of as a form of cellular "suicide". This view derived from the fact that early studies on rodent thymocytes and T-cell hybridomas under diverse apoptotic stimuli demonstrated that inhibitors of RNA and/or protein synthesis could abrogate death. Continuing macromolecular synthesis therefore seemed to be an essential part of the apoptotic process, indicating that these cells had control over their own demise (Wyllie *et al.*, 1981; Wyllie *et al.*, 1984). The validity of this observation has however been called into question as there are now numerous reports of systems in which apoptosis is either not blocked, is enhanced, or may be triggered directly as a result of RNA or protein synthesis inhibitors (reviewed in Martin, 1993). Consequently, mechanisms leading to apoptotic cell death may be divided into three distinct groups: induction mechanisms (those that required protein synthesis), transduction mechanisms (those that did not require protein synthesis), and release mechanisms (those in which the proteins required for apoptosis were constantly held in check and interruption of protein synthesis resulted in cell death) (Cohen *et al.*, 1992).

1.2.3 Methods of detecting apoptosis

There are several problems involved in the detection and accurate quantitation of apoptosis. These derive partly from the fact that the majority of cell populations studied are asynchronous, and many of the processes operating in apoptotic cells are transient. As a result cells that have been dead for a period of time are unlikely to share the same biochemical or morphological characteristics as cells currently undergoing apoptosis or those in the commitment phase. Furthermore, apoptotic bodies are phagocytosed by neighbouring cells, and in populations where apoptosis and proliferation occur simultaneously, determining the number of cells that have died can be problematic. Consequently, while multiple factors that influence apoptosis in cell populations have been discovered and studied, very little is known about how an individual cell decides to activate its cell death machinery or the biochemical sequence of events that follows.

Methods for the detection and measurement of apoptosis fall into three classes: biochemical (DNA fragmentation, activation of caspases and cell viability assays), altered cellular characteristics (including observations on cell morphology, chromatin status within the nucleus, DNA content, *in situ* end labelling, and the surface expression of phosphatidylserine), and following the fate of an individual cell over time (Table 1.2).

Table 1.1 Summary of the various techniques available for detection of apoptosis

Technique	Advantages	Disadvantages
Biochemical	Usually simple	Asynchronous populations
- **DNA fragmentation**	Journals usually require demonstration of this hallmark for publication	Identity of nuclease(s) involved uncertain. Oligonucleosomal fragmentation may be non-specific and/or nonessential. Difficult to generate from asynchronous cells. Prone to artefacts.
- **Proteolytic assays**	Caspases strongly implicated in apoptosis. Many kits commercially available to detect cleaved substrates.	No clear proof that many substrates of caspases involved directly in apoptosis. Caspase activation does not necessarily imply apoptosis. Only practical with cell cultures.
- **Viability assays**	Easy and rapid. Allows informed choice to be made of time points to be used for further experiments.	Loss of membrane permeability is a late event. Apoptotic bodies exclude vital dyes. Viability assays do not differentiate between apoptosis and necrosis.
Cellular		
- **Chromatin status**	Can be observed with either light or electron microscope. Variety of dyes/stains give good results.	Apoptotic and necrotic nuclei look very similar if inexperienced.
- **DNA content**	Apoptotic cells contain sub-G1 content, and this can be determined with flow cytometer.	
- ***in situ* end labelling**	Easy to use on fixed sections. Reveals degradation status of individual nuclei.	False positives and negatives have been reported.
- **Phosphatidylserine**	In mammalian systems this molecule is "flipped" to the outer membrane during apoptosis where it is detected by macrophages. Annexin V binds specifically and can be detected by flow cytometry.	Only applicable to living cells, not fixed tissue sections. Occurs in less than 1/3 of cells within a population. Detects necrotic cells when membranes rupture.
Time lapse photography	Allows systematic morphologic analysis of individual cells over time. Differences between apoptosis and necrosis obvious.	Requires specialised equipment. Slow acquisition of data. Limited sample size. Quantitation difficulties. Only applicable to adherent cultured cells.

Based on McCarthy and Evan (1998) and Willingham (1999)

1.2.4 DNA fragmentation

Degradation of DNA is the most characteristic feature of apoptosis and occurs at 3 levels. In some cell systems large DNA fragments have been observed (50-300kb), possibly corresponding to chromatin loops on the nuclear scaffold (Filipski *et al.*, 1990). Cleavage of DNA between linker regions on histones resulting in a characteristic regular ladder of fragments of 180-200bp is the most frequent marker while the third type of

fragmentation comprises single-strand breaks, although its relevance in respect to apoptosis remains unknown (Wyllie, 1980; Bortner *et al.*, 1995). Several endogenous endonucleases have been implicated in DNA cleavage in different cell death systems; DNase I (Pietsch *et al.*, 1993), DNase II (Barry and Eastman, 1993), NUC18 (Gaido and Cidlowski, 1991), various cyclophilins (Montague *et al.*, 1997), NUC70 (Urbano *et al.*, 1998) and caspase-activated deoxyribonuclease (Enari *et al.*, 1998; Liu *et al.*, 1999). It is possible that different cell types utilise different nucleases (Earnshaw, 1995).

The significance of DNA fragmentation in the apoptotic process remains uncertain and in some cases fragmentation has not been detected. The time at which DNA fragmentation becomes evident varies with both cell type and apoptotic stimulus, in some cases appearing early after receipt of the appropriate stimulus and in other cases manifesting as a late phenomenon. DNA fragmentation appears to mark a point of no return, as cells which have initiated cleavage cannot be recovered by removal of the apoptotic stimulus (Bortner *et al.*, 1995).

1.2.5 *Caenorhabditis elegans* death genes and their mammalian homologues

Cell death during the developmental process of the nematode *Caenorhabditis elegans* organism is extremely precise, involving elimination of 131 of the original 1090 somatic cells (Ellis *et al.*, 1991) though the action of specific genes which are conserved throughout animal evolution (Cohen, 1997). *ced-3* and *ced-4* have been shown to be essential for execution of the apoptotic pathway, while expression of a third gene, *ced-9*, has shown an inhibitory effect on the process (Hengartner and Horvitz, 1994b). When CED-4 receives an appropriate signal it binds to and activates pro-CED-3 to mature CED-3, which in turn cleaves a number of cellular proteins, 'death substrates', which

include DNA repair enzymes, components of the nuclear membrane and endonucleases. CED-9 acts upstream of this, binding CED-4 and thus preventing activation of pro-CED-3 (Earnshaw, 1995; Hetts, 1998). These three *C. elegans* death genes have homologues in mammals which display both sequence conservation and conserved function. The mammalian homologue of *ced-3,* interleukin-1β converting enzyme (ICE), induced apoptosis in fibroblasts if ectopically expressed (Miura *et al.*, 1993). ICE has since been shown to be a member of a large group of genes called caspases (cysteine proteases). There are now 10 known members of this family in man, and all have homology to *ced-3,* the only member of this family to have been found in the nematode. There is also a mammalian homologue of the *C. elegans* gene *ced-4* called apoptotic protease activating factor-1 (*Apaf-1*) and which seems to serve a very similar function to its *C. elegans* counterpart (Zou *et al.*, 1997). When cytochrome c is released from the mitochondrion it binds to the C-terminus of Apaf-1, enabling the caspase recruitment domain of Apaf-1 to bind pro-caspase 9 (a CED-3 homologue). Apaf-1 also has the ability to self-associate, allowing aggregation of pro-caspase-9, facilitating autoactivation (Green, 1998; Srinivasula *et al.*, 1998). An ATP binding domain has recently been discovered in Apaf-1, offering an explanation as to why the energy status of a cell plays a critical role in its mode of death (Zou *et al.*, 1999). In mammals members of the *Bcl-2* gene family are homologues of the *C. elegans* anti-apoptotic gene *ced-9* (Hengartner and Horvitz, 1994a). The function of some of the Bcl-2 family members parallels that of their nematode counterpart, binding Apaf-1 (CED-4) and in so doing preventing activation of ICE (CED-3) (Hu *et al.*, 1998a; Pan *et al.*, 1998). However, other family members fulfil a pro-apoptotic role in mammals (Korsmeyer *et al.*, 1993).

1.2.6 Apoptosis and yeast

Database searches using the BLAST algorithm have demonstrated categorically that the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* do not possess proteins with significant sequence homology to either caspases, members of the Bcl-2 family or Apaf-1 (Goffeau *et al.*, 1996; Madeo *et al.*, 1999). Furthermore, apoptosis has not been reported in yeast. Despite this apparent lack of endogenous apoptotic effectors, or possibly because of it, recent years have seen a significant body of work compiled assessing overexpression of several Bcl-2 family members in yeast. Pro-apoptotic members, namely Bax and Bak, result in killing of both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.* Anti-apoptotic members, namely Bcl-2 and Bcl-X_L, are capable of abrogating the action of their pro-apoptotic counterparts, allowing cell survival (Zha *et al.*, 1996; Manon *et al.*, 1997; Tao *et al.*, 1997; Ligr *et al*, 1998; Xu *et al.*, 1999). The phenotype associated with Bax overexpression in yeast is currently the source of much contention, with authors debating its apoptotic nature. Phosphatidylserine externalisation, chromatin condensation, DNA fragmentation, a degree of plasma membrane blebbing, and cytoplasmic vacuolisation have been reported by various authors (Zha *et al.*, 1996; Madeo et al., 1997; Ligr *et al.*, 1998; Xu *et al.*, 1999). Oligonucleosomal DNA fragmentation has not been observed in yeast cells overexpressing Bax, but this phenotype is not universal in mammalian apoptosis (Collins *et al.*, 1991; Oberhammer *et al.*, 1994) and has been observed during cases of necrosis (Dong *et al.*, 1997). Contradictory data have not helped to resolve this issue, with some groups reporting margination of chromatin within the nucleus (Ligr *et al.*, 1998), others describing foci of condensed chromatin distributed throughout this organelle (Madeo *et al.*, 1999), while still others report no detectable chromatin condensation (Zha *et al.*, 1996). Interestingly, both *Tetrahymena thermophila* (Davis *et al.*, 1992) and

Dictyostelium discoideum (Cornillon *et al.*, 1994) demonstrate chromatin condensation during macronuclear and stalk cell death respectively, morphology integral to the programmed events occurring within the cells. Furthermore, while cytoplasmic vacuolisation is not a characteristic normally associated with apoptosis, it has been reported during both *D. discoideum* programmed cell death (Cornillon *et al.*, 1994) and Bax-induced death of mammalian cells in the presence of caspase inhibitors (Xiang *et al.*, 1996).

Whether the morphology described in yeast expressing Bcl-2 family members is apoptotic in nature remains an open question, although it is thought that these proteins are likely to be acting in a manner similar to that documented in mammalian systems (Madeo *et al.*, 1999). This is supported by the fact that Bax-expressing cells release cytochrome c from their mitochondria (Manon *et al.*, 1997). Because yeast are known to be devoid of endogenous homologues of the principal effectors of mammalian apoptosis (caspases, Bcl-2 family members, Apaf-1), they could make an ideal model for dissection of the interactions between these proteins. By over-expressing a mutant form of Bcl-X_L in this system it has already been demonstrated that Bcl-X_L is capable of counteracting the effects of Bax through some mechanism other than physical interaction. This finding suggests that the theory of a direct stoichiometric relationship between Bcl-2 family members determining cell fate may be overly simplistic (Tao *et al.*, 1997). Another study has shown that overexpression of Ced-4 in *S. cerevisiae* results in the death of cells, with morphology reminiscent of apoptosis, despite the absence of Ced-3/caspase homologues with which to interact. Furthermore, Ced-4 mutants that were no longer capable of dimerisation failed to induce cell death (Tao *et al.*, 1999). This then

raises the possibility that a cell death role may exist for Ced-4/Apaf-1 in addition to its documented activation of Ced-3/caspase-8 in *C. elegans* and mammals.

1.2.7 Programmed cell death in single celled eukaryotes

In recent years it has become evident that mammalian cells undergo PCD not only as a physiological control mechanism or in response to irreparable damage, but also as a result of encounters with both parasites and viruses. Upon infection with a virus many mammalian cells respond by undergoing apoptosis, thereby preventing proliferation of the virus and protecting neighbouring cells from infection (Miller, 1997; Seshagiri and Miller, 1997; Fleck *et al.*, 1999; reviewed in Edwards *et al.*, 1999). To counter this strategy many viruses have developed the ability to produce molecules that mimic components of the host apoptotic machinery, influencing the cellular response in favour of maximising viral proliferative capacity (Miura *et al.*, 1993). Similarly, a variety of mammalian cell types are prevented from undergoing apoptosis when parasitised by the coccidian *Toxoplasma gondii* (Nash *et al.*, 1998; Goebel *et al.*, 1999), as are host cell macrophages infected by the trypanosomatid *L. donovani* (Moore and Matlashewski, 1994). By contrast, CD4+ (but not CD8+) T-cells are induced to undergo apoptosis by *Trypanosoma cruzi*, resulting in immunosuppression of the host (Lopes *et al.*, 1995; Lopes and DosReis, 1996).

An active cell death process has been shown to be crucial to the development and homeostasis of multicellular organisms, but while the importance of programmed cell death in metazoans is now universally recognised, the evolutionary origin of the process remains unclear 27 years after its initial discovery. The need to develop an altruistic cell suicide pathway has been associated with selective pressures that apply specifically to

multicellular organisms, and was thus assumed to have arisen in phylogenies after the onset of multicellularity. The discovery of a PCD pathway in the slime mould *Dictyostelium discoideum* (Cornillon *et al.*, 1994) has resulted in the strict association between multicellularity and PCD being called into question, as this organism displays both single- and multi-cellular stages, dependent upon environmental conditions. Furthermore, macronuclear death in the protozoan *Tetrahymena thermophila* has been reported as having similarity to the nuclear changes observed during apoptotic death in mammals (Davis *et al.*, 1992; Christensen *et al.*, 1995; Christensen *et al.*, 1998). These discoveries have led to the inevitable suggestion that a common PCD mechanism may have arisen prior to the advent of multicellularity (Cornillon *et al.*, 1994). This begs the question "why?". In what way could a single celled organism possibly benefit from its own suicide? The answer lies in the relationship between individual cells. The evolutionary constraints which favour altruistic death at the level of the multicellular organism could also be applied to unicellular organisms at the level of the multicellular colony (Ameisen *et al.*, 1995). Therefore, whenever unicellular organisms constitute a multicellular colony, the altruistic death of an individual cell within that colony can be genetically advantageous if it ensures the survival of closely related individuals. This scenario could certainly be applied to protozoa belonging to the genera *Trypanosoma* and *Leishmania* where parasitic populations in both the insect vector and mammalian host are largely clonal (Tibayrenc *et al.*, 1990).

PCD in metazoa serves to control cell populations. Due to the fact that digenetic kinetoplastids of the genera *Trypanosoma* and *Leishmania* must establish and maintain a proliferative cell population within their insect vectors, the idea of a system of social control of cell numbers is appealing. Studies on the dynamics of trypanosome infections

in tsetse flies have revealed that the majority of parasites ingested with the bloodmeal die, despite having successfully differentiated to procyclic form cells (Maudlin and Welburn, 1987; Welburn *et al.*, 1989; Dale *et al.*, 1995). Dying procyclic cells display characteristics of apoptosis in metazoans, including condensation of nuclear DNA and vesiculation of the plasma membrane (Welburn et al., 1989). This cell death occurs within the midgut of the fly, prior to establishment of an infection within the ectoperitrophic space, and can be prevented by addition of glucosamine to tsetse feeds (Maudlin and Welburn, 1987). This led to the hypothesis that this sugar competitively binds a tsetse midgut lectin, inhibiting the ability of the fly to induce parasite death. Following establishment of a midgut infection a substantial period of time elapses before infective metacyclic trypomastigotes are detected within the salivary gland (if at all), typically 2-3 weeks for *T. brucei* (Dale *et al.*, 1995). During this time the number of trypanosomes occupying the ectoperitrophic space of the tsetse midgut remains remarkably constant despite the fact that proliferation is continuous (Welburn and Maudlin, 1997). Maintenance of a stable population of parasites is of advantage to the trypanosome, as competition with the tsetse for an energy source is ongoing, and too great a parasite burden could result in death of the vector prior to transmission. This led to the suggestion that *T. brucei* could be regulating their own population density, balancing proliferation against altruistic death, in order to maintain a viable population within this environment (Welburn *et al.*, 1997).

An analogy has been drawn between the death of *T. brucei* induced by tsetse midgut lectin *in vivo*, and *in vitro* death induced by culturing cells with a tetrameric lectin, concanavalin A (ConA), derived from the Jack bean *Canavalis ensiformis* (Welburn *et al.*, 1996). Transition electron microscopy revealed that cell death induced by this lectin

presented morphological changes similar to those observed in cells dying within the tsetse midgut - namely membrane vesiculation and condensation of chromatin into electron dense masses that accumulated at the periphery of the nucleus. Further studies on cultured cells revealed that plasma membrane integrity was maintained throughout the 72 hours of the death process, and that an oligonucleosomal DNA fragmentation pattern was generated by 72 hours post-treatment (Welburn et al., 1996).

PCD has recently been described in several members of the parasitic protozoa: *Trypanosoma cruzi* (Ameisen *et al.* 1995); *T. brucei rhodesiense* (Welburn *et al.* 1996); *Leishmania amazonenis* (Moriera *et al.* 1996) and *Plasmodium falciparum* (Picot *et al.* 1997). In metazoa, the distinction between apoptotic cell death and necrosis often relies on experimentally demonstrating the formation of oligonucleosomal DNA fragmentation patterns on an agarose gel (Montague and Cidlowski, 1996). All four of these parasitic protozoans were demonstrated to display this phenomenon, leading to the suggestion that a conserved cell death mechanism may be operating in these parasites (Welburn *et al.*, 1996; Picot *et al.* 1997). Further research in this area is of particular interest as these organisms represent the most primitive eukaryotes in which such a phenomenon has been described. A better understanding of the degree to which the cell death programmes of these organisms correspond to those of their metazoan counterparts has implications for our understanding of the origins of PCD in eukaryotic cell survival.

Apoptosis in metazoans is an active process induced by intra- or extra-cellular signalling and culminating in characteristic morphological and biochemical alterations in the target cell. Changes in gene expression are almost inevitable in cells undergoing such a drastic process, and detection of mRNA transcripts differentially expressed during apoptotic

death has been the focus of significant research (Wang *et al.*, 1997; Baudet *et al.*, 1998; Choi *et al.*, 1998; Backert *et al.*, 1999; Fournier *et al.*, 1999). Differentially expressed genes identified to date include the cell cycle inhibitor p21 (Backert *et al.*, 1999), the transcription factor DIO-1 (Garcia-Domingo *et al.*, 1999), members of the Bcl-2 family (Gillardon *et al.*, 1994; Marx *et al.*, 1997; Wang *et al.*, 1998), the proto-oncogene c-Myc (Gillardon *et al.*, 1994) and the tumour suppressor p53 (Wang *et al.*, 1998; Kaya *et al.*, 1999). It is clear that these genes are not acting in isolation and that the pathways leading to cell death are complex.

It has previously been shown that trypanosome death induced with Con A is associated with *de novo* gene expression (Murphy & Welburn 1997, Welburn & Murphy 1998). The identification of genes involved in the death of these unicellular organisms is essential to address issues such as the social control of cell survival and the degree of conservation between unicellular and multicellular PCD pathways (Ameisen 1996, Welburn *et al*, 1997). Characterisation of genes involved in regulation of PCD in *T. brucei* will therefore be of value both from the above perspective, and as a potential therapeutic target for selective induction of parasite death.

To provide an overview of the genetic changes occurring during the execution of the cell death program in *T. b. rhodesiense*, this project used a differential display method [Randomly Amplified Developmentally Expressed Sequences-PCR (RADES-PCR)] for the identification of genes demonstrating differentially expression in cells which had been induced to die. These differentially expressed genes and their encoded products may be implicated directly or indirectly in the cell death mechanism in *Trypanosoma brucei* s.l.

CHAPTER 2

Materials and methods

2.1 Culturing, harvesting and cryopreservation of trypanosomes

Trypanosoma brucei rhodesiense stock D. Obwang (isolated from a patient during a sleeping sickness epidemic in S. E. Uganda, 1990) were used for all experiments involving treatment with Concanavilin A. Procyclics were cultured at 27°C in Cunninghams medium (Cunningham, 1977) containing 17.5% heat inactivated foetal bovine serum [Gibco BRL] and 10 μg/ml gentamycin [Sigma].

Trypanosoma brucei brucei stock EATRO 795 stably transfected with plasmid construct pHD449 (Biebinger *et al.*, 1997) was used for all expression studies. Procyclics were cultured at 27°C in complete SDM 79 (Brun *et al.*, 1979) prepared by Gibco BRL (10% heat inactivated FBS, 10 μg/ml gentamycin, 20 μg/ml zeocin [Invitrogen]).

Cells were harvested by centrifugation at 1000 x g for 10 minutes at room temperature, following which they were washed in an equal volume of sterile PBS. Subsequent to a second centrifugation cells were ready for downstream applications.

Trypanosomes were harvested as described previously then resuspended in an equal volume of fresh medium containing 5% dimethyl sulfoxide (DMSO) [Sigma]. The culture was aliquoted into 1 ml cryovials and slow-frozen at -70°C for 24 hours. Aliquots were then transferred to liquid nitrogen for long term storage.

2.2 Concanavilin A treatment of trypanosomes

Log phase trypanosomes (1 x 10^7/ml) were harvested as described previously then resuspended in an equal volume of fresh Cunninghams medium containing 17.5% heat inactivated FBS, 10 μg/ml gentamycin and 10 μg/ml Concanavilin A (ConA) type V

[Sigma]. The culture was aliquoted appropriately and maintained at 27°C. At designated time-points cells were harvested and pellets placed at -70°C for storage. Control cultures were harvested at the beginning of each experiment and were not treated with ConA.

2.3 Preparation of total and poly[A]$^+$ RNA

Isolation of total RNA from trypanosomes was based on the method of Chomczynski and Sacchi (1987), with improvements by Puissant and Houdebine (1991). Briefly, cells were harvested as described previously then resuspended at $1x10^9$ cells/ml in RNA denaturation solution by vigorously vortexing. 2M sodium acetate pH 4.0, water saturated phenol and chloroform were added to final concentrations of 87 mM, 43% v/v and 9% v/v respectively. The lysate was vortexed for 20 seconds, placed on ice for 10 minutes with occasional mixing, then centrifuged at 4°C in a microfuge for 10 minutes at 14000 rpm. The aqueous upper layer containing RNA was extracted to a fresh Eppendorf tube, an equal volume of isopropanol was added, and the solution was placed at -70°C for 10 minutes. The solution was centrifuged as previously and the supernatant discarded, following which the pellet was re-dissolved in 400 µl RNA denaturation solution by heating to 50°C and vortexing. 1 ml ice cold ethanol was added and after mixing the tube was placed at -70°C for 10 minutes. Centrifugation was as described previously, the supernatant was discarded and the pellet was washed with ice cold 70% ethanol. The pellet was dried at 50°C for 5 minutes following which it was dissolved in 200µl RNase free water and placed at -70°C for storage.

Poly[A]$^+$-enriched RNA was prepared directly from harvested trypanosomes using the PolyATract System 1000 [Promega] as specified by the manufacturer. This kit purifies

messenger RNA by utilizing the high affinity interaction between a biotinylated oligo(dT) probe and streptavidin-bound magnetic particles. While the manufacturer recommends samples used with this protocol to be as fresh as possible it was found to be more convenient to store samples at -70°C for a period of time prior to RNA extraction.

2.4 RNA gel electrophoresis and Northern blotting

RNA gel electrophoresis was carried out according to the protocol of Pellè and Murphy (1993b). Briefly, gel tanks, gel combs and gel formers were all immersed in 0.5% SDS for 30 minutes followed by a thorough rinse in distilled water. A 1.4% (w/v) agarose gel was made by boiling multi purpose agarose [Boehringer Mannheim GmbH] in running buffer consisting of 10 mM sodium phosphate (pH 6.8) containing 0.1 μg/ml ethidium bromide. 1 - 10 μg RNA in a total volume of 10 μl was rapidly denatured prior to running by addition of 2 μl 6 x RNA loading buffer followed by incubation at 75°C for 5 minutes. Samples were centrifuged then placed on ice prior to loading onto the submarine gel together with size markers (0.363-9.488 kb [Promega]). Gels were electrophoresed at 3 - 7 V/cm with constant recirculation of buffer. Progress of the migrating RNA was monitored by visualisation with medium-wave UV light.

Following electrophoresis RNA was transferred directly to Hybond N^+ membrane [Amersham] by blotting overnight using 20x SSC as the transferrant as outlined in Sambrook *et al.* (1989). RNA was then fixed using a CL-1000 Ultraviolet Crosslinker [Genetic Research Instrumentation Ltd.] set at 120 J/cm^2.

2.5 Preparation and amplification of first strand cDNA

First strand cDNA was synthesised from both total and poly[A]$^+$ RNA using the Reverse Transcription System [Promega] as directed by the manufacturer. Briefly, 1 μg RNA was heat denatured at 70°C for 10 minutes in the presence of 0.5 μg Oligo(dT)$_{15}$ primer in a final volume of 13 μl, then incubated at 42°C for one hour with 20U recombinant RNasin® ribonuclease inhibitor, 15U avian myeloblastosis virus (AMV) reverse transcriptase, 1 mM dNTPs, reverse transcription buffer and 5 mM $MgCl_2$. Heat inactivation of the AMV reverse transcriptase was carried out at 99°C for 5 minutes. Single-stranded cDNA was stored at -20°C then used directly in downstream applications.

First strand cDNA was amplified by the polymerase chain reaction (PCR) using Oligo(dT)$_{15}$ and nucleotides 16 - 39 of the conserved 5' miniexon sequence of trypanosomes as primers (this primer contained a 5' *Not* I site). PCR reactions were carried out in 100 μl final volume containing 100ng cDNA template, 2.5U *Taq* DNA polymerase [Promega], *Taq* DNA polymerase reaction buffer, 2 mM $MgCl_2$, 100 ng of each aforementioned primer, and dNTPs (4 μl from 5mM stock). The reaction mixture was overlaid with mineral oil [Sigma]. Reactions were carried out using 500 μl eppendorf tubes in a PTC-100™ Programmable Thermal Controller [MJ Research, Inc.]. Cycling conditions were 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes for 40 cycles followed by 5 minutes at 72°C for primer extension. Enzyme, buffer, primers and dNTPs were removed from products by ultrafiltration using Centricon 30 columns [Amicon, Inc.] as directed by the manufacturer. Products were diluted to 20 ng/μl in TE buffer, aliquoted and stored at -20°C.

2.6 Differential display PCR

Differential display PCR was carried out according to the method of Murphy and Pellè (1994) using two different template concentrations. The reaction mix was as follows: 1 µl template (20 ng/µl and 2 ng/µl), 20 ng single arbitrary 10-mer primer, 2.5U Taq DNA polymerase [Promega], *Taq* DNA polymerase reaction buffer, 3 mM $MgCl_2$, dNTPs (1 µl from a 5 mM stock) and sterile distilled water to 20 µl. The reactions were carried out in 500 µl eppendorf tubes as described previously with cycling conditions as follows: 94°C for 45 seconds, 40°C for 1 minute, 72°C for 1 minute for 40 cycles followed by 5 minutes at 72°C for primer extension.

A 2% (w/v) agarose gel was made by boiling multi purpose agarose [Boehringer Mannheim GmbH] in TAE buffer containing 0.1 µg/ml ethidium bromide. 2 µl 6 x loading buffer [Promega] was added to 10 µl PCR products which were then loaded onto the submarine gel together with size markers (100 bp PCR Molecular Ruler [Bio Rad]) and electrophoresed at 8 V/cm. Progress of the migrating DNA was monitored by visualisation with medium-wave UV light.

Gel plugs were taken from differentially expressed bands and boiled in 50 µl water for 10 minutes, following which 1 µl was taken as template for re-amplification using the PCR conditions described above. In order to facilitate subsequent T-vector cloning re-amplified PCR products underwent terminal addition of dATP as follows. The PCR reaction mix was heated to 95°C for 20 minutes then cooled to 4°C. 2U Taq DNA polymerase and 5 µl 2 mM dATP were added. The reaction was then heated to 72°C for 20 minutes. Products were electrophoresed as described above and appropriate bands

excised. DNA was extracted from the gel slices using an SL-8520 Nucleon® GX kit [Scotlab Bioscience] as directed by the manufacturer.

2.7 Cloning of PCR products

PCR products generated as described previously were ligated into either pGEM®-T or pGEM®-T Easy vectors [Promega], subject to availability and according to the manufacturers guidelines. Ligations were used to transform heat-shock competent JM109 cells and transformants selected by their ability to grow on LB (Luria-Bertani) agar plates supplemented with 50 μg/ml ampicillin. Presence of PCR products was initially determined by blue/white selection of colonies (0.5 mM isopropylthio-β-D-galactodise [IPTG], 80 μg/ml 5-Bromo-4-chloro-3-indoyl-β-D-galactoside [X-gal]), following which positives were screened either by colony PCR using SP6 and T7 RNA Polymerase Promoter Sequencing Primers [Promega], or by restriction enzyme digestion.

2.8 Procedures involving *Escherischia coli*

2.8.1 Production of heat-shock competent JM109

Heat-shock competent JM109 {*e*14-(McrA-), *rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17(r_k-, m_k^+), *sup*E44, *rel*A1, delta(*lac-pro*AB), [F′, *tra*D36, *pro*AB+, *lac*I^qZdeltaM15]} were used for cloning of all plasmids. Cells were made competent as follows: a single JM109 colony was picked from a streaked plate, inoculated into 10 ml LB medium and grown in an orbital shaker at 37°C overnight. 500 μl of the overnight culture was used to inoculate 50 ml fresh LB medium, then grown to an OD_{600} of 0.7. Cells were incubated on ice for 10 minutes then centrifuged at 1800 x g for 15 minutes at 4°C. The supernatant was discarded and the pellet gently resuspended in 16 ml sterile solution

RF1. Cells were again incubated on ice for 15 minutes then centrifuged at 1600 x g for 15 minutes at 4°C. The supernatant was discarded and the cells gently resuspended in 4 ml sterile solution RF2. Cells were incubated on ice for one hour, aliquoted, snap frozen in liquid nitrogen then stored for up to 2 months at -70°C.

2.8.2 Transformation of heat-shock competent JM109

Competent JM109 cells were removed from storage at -70°C and incubated on ice for 5 minutes. An appropriate amount of plasmid or ligation was added to 50 µl thawed cells and the incubation on ice continued for a further 30 minutes. Cells were heat-shocked in a 42°C waterbath for 45 seconds then returned to ice for 2 minutes. 1 ml LB medium supplemented with 100 µl/ml 1M glucose was added to the cells, which were then transferred to an orbital shaker at 37°C for 1 hour. Cells were plated on appropriately selective LB agar plates and cultured overnight at 37°C.

2.8.3 Colony PCR

Following overnight growth on plates recombinant bacterial colonies were screened for DNA insertion into the multiple cloning sites of plasmids as follows: using a 200 µl pipette tip a small amount of bacterial material was taken from the periphery of colonies and suspended in 18.3 µl distilled water. To this was added 5 pmol of each primer, 1U *Taq* DNA polymerase, 3 µl 10 x *Taq* DNA polymerase reaction buffer, 2 mM $MgCl_2$, and dNTPs (1µl from 5 mM stock). Reactions were carried out in 500 µl eppendorf tubes as described previously with cycling conditions as follows: 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute for 30 cycles followed by 72°C for 5 minutes for primer extension. PCR products were analysed on an agarose gel as described previously.

Clones of interest were used to inoculate 5 ml of appropriately selective LB medium. Cells were cryopreserved by addition of 150 µl glycerol to 850 µl overnight bacterial culture, thorough mixing then slow-freezing to -70°C. Cells were maintained at this temperature for long term storage.

2.8.4 Plasmid preparation

Plasmid preparation methodology was dependent on downstream applications. Plasmids destined for sequencing were purified using the Qiagen Plasmid Mini Kit [Qiagen]. For subsequent transfection of trypanosomes, plasmids were purified using the Wizard® PureFection Plasmid DNA Purification System [Promega]. Plasmids to be used for restriction enzyme digestion purposes were purified using Wizard® Plus Minipreps [Promega], QIAprep Spin Miniprep kit [Qiagen], or by the alkaline lysis method (Sambrook *et al.*, 1989) depending on availability and the number of samples to be processeed.

2.8.5 Y1090

E. coli strain Y1090 were made competent for infection by bacteriophage λ as described by Sambrook *et al.* (1989). Briefly, 50 ml LB medium supplemented with 0.2% maltose, 10 mM $MgCl_2$ and 50 µg/ml ampicillin was inoculated with a single bacterial colony and incubated in an orbital shaker at 37°C overnight. The culture was centrifuged at 4000 x g for 10 minutes at room temperature, resuspended in 20 ml 10 mM $MgCl_2$ and stored at 4°C for up to 2 weeks.

2.8.6 Production and purification of a MOB1-MBP fusion protein

Production of MBP-Mob1 fusion protein in *E. coli* was basically as directed by the manufacturer with minor alterations. Briefly, 1 litre of LB medium supplemented with 2 g glucose and 100 μg/ml ampicillin was inoculated with 10 ml of an overnight culture of pMobMFPMAL and incubated in an orbital shaker at 37°C until an optical density of A_{600} 0.5 was achieved. IPTG was added to a final concentration of 0.1 mM, and the culture incubated in an orbital shaker for a further 6 hours at 30°C. Cells were chilled to 4°C, harvested by centrifugation at 4000 x g for 20 minutes at 4°C, resuspended in 50 ml column buffer and frozen overnight at –20°C. Cells were thawed in cold water then sonicated in an ice water bath for 3 minutes with 5 second pulses using a Vibra Cell™ VC100 [Sonics and Materials Inc., West Kenosia Avenue, Danbury, Conneticut, USA]. Following centrifugation of the crude lysate at 8000 x g for 40 minutes at 4°C, the supernatant was collected and diluted 1:5 with column buffer. Soluble lysate was placed at 4°C for temporary storage.

An affinity column was prepared at 4°C as follows; a 25 ml pipette was employed as the barrel of the column and its tip plugged with sterile glass wool. 10 ml amylose resin [New England Biolabs] was introduced into the column. After being allowed to settle the column was washed with 80 ml de-gased column buffer.

240 ml soluble bacterial lysate was loaded onto the column at 4°C at a rate of approximately 1 ml/minute. The column was then washed with a further 150 ml column buffer. Elution of the MBP-MOB1 fusion protein was by addition of 20 ml column buffer containing 10 mM maltose. Protein from 1 ml fractions collected throughout the

elution was detected using a DC Protein Assay [Bio Rad]. Fractions containing greater than 0.5 mg/ml protein were pooled and placed at 4°C for temporary storage.

2.9 Screening of bacteriophage lambda libraries

10^3 plaque forming units (pfu) from a quantified phage stock were added to 100 µl competent Y1090, vortexed and incubated at 37°C for 15 minutes. 8 ml molten (47°C) top agarose was added to the cells and the tube was inverted 3 times to mix the contents. The cells were immediately poured onto the centre of a 14 cm petri dish containing 80 ml hardened NZCYM bottom agar supplemented with 50 µg/ml ampicillin, being careful to avoid introduction of bubbles. Plates were closed and stored at room temperature for 1 hour before being inverted and incubated at 37°C overnight.

Confluent plates were placed at 4°C for 2 hours to ensure solidity of top agarose. A Protran BA 85 nitrocellulose disc [Schleicher and Schuell] labelled with indelible marker was laid onto each plate, orientation marks added with Indian ink, and the plates returned to 4°C for a further 15 minutes. Filters were removed from plates, placed in DNA denaturation solution for 10 minutes, fixed using a CL-1000 Ultraviolet Crosslinker [Genetic Research Instrumentation Ltd.] set at 120 J/cm^2, placed in DNA neutralisation solution for 10 minutes then fixed as previously. Plates were returned to 4°C for storage.

Filters were prehybridised for 4 hours in hybridisation solution 1 then hybridised overnight at 65°C. Washes with 2 x SSC, 0.1% SDS were carried out at 65°C with intermittent monitoring to determine background. Following autoradiographic detection plates were aligned with film and positive plaques removed as plugs into 500 µl SM

medium with 50 µl chloroform. Subsequent storage was at 4°C. Tenfold serial dilutions were made of the liquid phase of stored plugs, 1 µl of each then being used to infect 100 µl competent Y1090 as described previously. Dilutions giving approximately 100 plaques per plate were used for subsequent screens.

2.10 Preparation of bacteriophage lambda DNA for subcloning

Approximately 10^4 pfu bacteriophage were used to infect 100 µl competent Y1090 cells. The mixture was incubated, plated and cultured overnight as described previously. 10 ml SM medium was added to the confluent plate and incubation at 37°C resumed for a further 1 hour with gentle agitation. The SM medium was decanted from the plate into a corex tube, and 0.5 volumes of DEAE-sephacyl [Pharmacia Biotech] added. The mixture was incubated at room temperature on a roller for 1 hour, following which it was centrifuged at 8000 x g for 15 minutes in a Beckman J-21 with a swing bucket rotor. The supernatant was transferred to a clean corex tube and Tris-HCl (pH 7.5), NaCl and PEG 6000 added to final concentrations of 25 mM, 1 M and 10% respectively. The mixture was incubated on ice for 15 minutes then centrifuged at 8000 rpm as previously, at 4°C. The supernatant was decanted leaving a pellet of phage, which was resuspended in 400 µl SM medium and extracted with an equal volume of chloroform. The solution was treated with 100 µg/ml RNaseA at room temperature for 1 hour, then SDS and EDTA added to final concentrations of 0.05% and 4 mM respectively. Two extractions were performed with phenol/chloroform, following which the DNA was precipitated with 2 volumes of ice cold absolute ethanol. DNA was washed once with 70% ethanol before being resuspended in 100 µl TE buffer.

2.11 Subcloning of *T. b. brucei* genomic DNA from bacteriophage λgt11

Bacteriophage λgt11 DNA prepared as described previously was quantified spectrophotometrically at 260 nm. 20 µl *Eco*R I buffer [Promega] was added to 25 µg DNA and the volume made to 193 µl with TE buffer (pH 8.0). 7 µl *Eco*R I (12 U/µl) [Promega] was added and the digest pipetted briefly to mix, before being incubated at 37°C for 3 hours. DNA fragments resulting from the digest were analysed on a 0.7% agarose gel (w/v) as described previously.

T. b. brucei DNA excised from bacteriophage λgt11 was in the range 2-6 kb. DNA was extracted from agarose gel slices using an SL-8520 Nucleon® GX kit [Scotlab Bioscience] as directed by the manufacturer. Purified fragments were quantified by running on a 1% (w/v) agarose gel with known amounts of standard.

10 µg pBluescript was digested with 20U *Eco*R I in a final volume of 50 µl for 1 hour at 37°C, following which 0.2 U calf intestine alkaline phosphatase [Promega] was added and the incubation continued for a further 15 minutes. The solution was extracted once with phenol/chloroform, precipitated with 3 volumes of ice cold ethanol and resuspended in 20 µl dH_2O. Linearised plasmid was quantified by running on a 1% (w/v) agarose gel with known amounts of standard.

200 ng genomic DNA was ligated into either 100 ng pBluescript (prepared as described above) or 100 ng commercial pUC18 *Eco* RI/BAP [Pharmacia Biotech] using a Rapid DNA Ligation Kit [Boehringer Mannheim] as directed by the manufacturer. Heat-shock competent JM109 were transfected and screened for inserts as described previously.

2.12 ^{32}P random-primed labelling of DNA

DNA was labelled using the Prime-It® II random primer labelling kit (Stratagene) as directed by the manufacturer. This kit utilises random 9-mer primers and exonuclease-deficient Klenow polymerase in association with [^{32}P]dCTP (Dupont) to generate labelled fragments of 500-1000 nucleotides in length. Unincorporated nucleotides were separated from labelled probes using NucTrap® probe purification columns (Stratagene) together with a Push Column Beta Shield Device (Stratagene) as directed by the manufacturer.

2.13 Automated sequencing of DNA

Sequencing of cloned DNA was performed by the University of Glasgow Molecular Biology Support Unit. Sequencing reactions were carried out with a Dye Terminator Cycle Sequencing Ready Reaction kit [Perkin-Elmer Applied Biosystems], and the products analysed on a Perkin-Elmer ABI 373 Stretch DNA Sequencer.

2.14 DNA sequence analysis

Raw sequence data was imported into EditSeq [DNASTAR, Madison, Wisconsin, USA] for editing, following which individual sequence fragments were compiled into contigs using SeqMan II [DNASTAR]. Homology searching was carried out against nucleotide sequences lodged in non-redundant GenBank, EMBL, DDBJ, PDB and EST databases using the Basic Local Alignment Search Tool (BLAST) programme through the National Centre for Biotechnology Information (NCBI) [http://www.ncbi.nlm.nih.gov]. Homology searches were also carried out against kinetoplastid databases through the Parasite Genome BLAST Server [http://mercury.ebi.ac.uk/parasites/parasite_blast_server.html].

2.15 Transfection of *T. brucei*

10 ml procyclic cells in mid-log phase (6 x 10^6 / ml) were pelleted at 1000 x g for 10 minutes at room temperature then resuspended in 1 ml Zimmerman post-fusion medium (ZPFM). Cells were pelleted as previously and again resuspended in 1 ml ZPFM. 5 – 100 μg DNA resuspended in sterile dH_2O was added to 0.5 ml cells in a 0.4 cm pulse cuvette [Bio Rad] and electroporated at 1500 volts / 25 μF twice using a Gene Pulser™ [Bio Rad] with the Pulse Controller disconnected. Cells were immediately transferred to 5 ml pre-warmed complete SDM 79 and allowed to recover overnight at 27°C. Following recovery transfected cells were selected by addition of hygromycin B [Boehringer Mannheim GmbH] to 50 μg / ml and cloned immediately by limiting dilution.

2.16 Induction of construct expression

Stably transfected clonal cell lines were grown to mid-log phase, pelleted at 1000 x g then resuspended in an appropriate volume of complete SDM 79 medium containing 50 μg/ml hygromycin B and 20 ng/ml tetracycline [Sigma]. Cells were cultured overnight at 27°C then harvested as described previously.

2.17 Analysis of proteins

2.17.1 Electrophoresis of proteins

Glycine and tricene SDS-PAGE gels were made as indicated in Appendix III using a Mini-PROTEAN II cell with 0.75 mm spacers [Bio Rad]. Cell pellets were resuspended in 75 μl 0.25% Triton®-X 100 by vortexing and repeated passage through a 21 gauge needle. 25 μl 4 x Laemmli buffer [Sigma] was added, following which samples were

heated at 100°C for 5 minutes. 20 µl samples were loaded onto gels with appropriate standards and electrophoresed as indicated in Appendix III until the dye front reached the bottom of the gel.

2.17.2 Coomassie staining of gels

Protein gels were simultaneously fixed and stained by immersion in 50% methanol, 10% acetic acid, 0.125% Coomassie Brilliant Blue G 250 for 1 hour at room temperature. Gels were destained with several changes of 50% methanol, 10% acetic acid until optimum staining / background was observed, following which they were washed in several changes of distilled water and vacuum dried.

2.17.3 Western blotting of gels and immuno-detection of proteins

Protein gels were equilibrated in Western transfer buffer for 10 minutes, then blotted onto Hybond-C Super [Amersham] using a Mini Trans-Blot cell [Bio Rad] set at either 200 mA for 2 hours or 50 mA overnight at 4°C. After transfer of proteins the membrane was incubated with 5% Marvel dried skimmed milk powder [Premier Beverages] in TBST for 1 hour at room temperature or overnight at 4°C. Membranes were incubated with primary antibody in 0.5% Marvel / TBST on a roller for 1 hour at room temperature, washed 3 x with 5 ml 0.5% Marvel / TBST for 5 minutes each then incubated with secondary antibody / HRP conjugate [Promega] in 0.5% Marvel / TBST as previously. Membranes were washed 3 x with 5 ml TBST for 2 minutes each then incubated with 2 ml TBST, 300 µl SuperSignal® Chemiluminescent Substrate Stable Peroxide Solution [Pierce, Rockford, Illinois, USA] and 300 µl SuperSignal® Chemiluminescent Substrate Luminol/Enhancer [Pierce] for 5 minutes on a roller at room temperature. Membranes were encased in cling-film and exposed to X-Ray film

for 5 minutes. Subsequent exposure times were based on the initial autoradiograph, light emission from membranes remaining stable for up to 1 hour.

2.18 Immunofluorescence microscopy

Log-phase procyclic cells were harvested and washed as described previously then resuspended in PBS. Cells were spread on slides and allowed to settle until an appropriate cell density was achieved, following which slides were sequentially fixed in methanol and acetone for 3 minutes each at -20°C. After drying, wells were marked on slides using a rolling ball-point paint marker, and subsequent hybridisation performed in individual wells. Wells were blocked for 30 minutes with PBS / 0.5 % Marvel dried skimmed milk powder, then treated for 30 minutes with PBS / 0.5 % Marvel / primary antibody. Wells were washed thoroughly with PBS / 0.5 % Marvel then treated for 30 minutes with PBS / 0.5 % Marvel / secondary antibody / 2 μg/ml 4, 6-diamidino-2-phenylindole (DAPI) [Sigma] in the dark. Following thorough washing with PBS slides were mounted with PBS / 50 % glycerol containing 2.5 % 1, 4-diazabicyclo[2.2.2]octane (DABCO) [Sigma] as antifade. Microscopy was carried out using a Zeiss Axioplan [Carl Zeiss Inc., Thornwood, NY, USA] at 1000 x magnification. Images were captured using a Hamamatsu C4742-95 cooled digital CCD camera and processed using Openlab 2.02 (Improvision, University of Warwick Science Park, Coventry).

2.19 Preparation of *T. brucei* genomic DNA

1 x 10^9 mid-log cells were harvested as described previously, resuspended in 5 ml ice cold DNA digestion buffer and pelleted at 1500 x g for 10 minutes at 4°C. Cells were resuspended in 5 ml digestion buffer then SDS and RNAse A [Sigma] added to 1% and

100 μg/ml respectively. The solution was incubated at 50°C for 1 hour, following which proteinase K [Sigma] was added to 1 mg/ml and incubation resumed at 55°C for a further 2 hours with periodic gentle inversions. The solution was extracted twice with 50:50 phenol/chloroform and once with chloroform as described in Sambrook *et al.* (1989). 1/20 volume 3 M sodium acetate and 2 volumes absolute ethanol were added sequentially with mixing, and precipitated DNA extracted with a sealed pasteur pipette hook. DNA was repeatedly washed in 70% ethanol, air-dried briefly then dissolved in an appropriate volume of TE buffer. DNA was quantified spectrophotometrically at 260 nm and stored at 4°C.

2.20 Southern blotting

Southern blotting (Southern, 1975) was carried out as described by Sambrook *et al.* (1989). Briefly, following agarose gel electrophoresis of restriction digested DNA a photograph was taken of the gel with a ruler laid alongside. The gel was immersed in 0.25 M HCl for 15 minutes, following which it was immersed in 0.5 M NaOH, 1.5 M NaCl twice for 15 minutes each. The gel was washed thoroughly in dH_2O then immersed in 1 M Tris-HCl pH 8.0, 1.5 M NaCl twice for 15 minutes each. The DNA was transferred onto Hybond N^+ membrane [Amersham] by blotting overnight using 20x SSC as the transferrant. DNA was then fixed using a CL-1000 Ultraviolet Crosslinker [Genetic Research Instrumentation Ltd.] set at 120 J/cm^2.

2.21 Culture media and solutions

Ampicillin

Stock solution 100mg/ml in dH_2O. Storage at $-20^{\circ}C$

BBL Bottom agar

BBL Trypticase peptone	1% (w/v)
NaCl	100mM
Agarose	1% (w/v)

Autoclave and store at room temperature

BBL Top agarose

BBL Trypticase peptone	1% (w/v)
NaCl	100mM
$MgCl_2$	12mM
Agarose	0.7% (w/v)

Autoclave and store at room temperature

Column buffer

Tris-HCl (pH 7.4)	20 mM
NaCl	200 mM
EDTA	1 mM
Sodium azide	0.05% (w/v)

Cunninghams medium (Cunningham, 1977)

	mg/100 ml
NaH_2PO_4	53
$MgCl_2.6H_2O$	304
$MgSO_4.7H_2O$	370
KCl	298
$CaCl_2$	15
Glucose	70

Fructose	40
Sucrose	40
L-Malic acid	67
α-Ketoglutaric acid	37
Fumaric acid	5.5
Succinic acid	6
β-Alanine	200
DL-Alanine	109
L-Arginine	44
L-Asparagine	24
L-Aspartic acid	11
L-Cysteine HCl	8
L-Cystine	3
L-Glutamic acid	25
L-Glutamine	164
Glycine	12
L-Histidine	15
L-Isoleucine	9
L-Lysine	9
DL-Methionine	20
L-Phenylalanine	20
L-Proline	690
DL-Serine	20
L-Taurine	27
DL-Threonine	10
L-Tryptophan	10
L-Tyrosine	20
L-Valine	21

BME Vitamin mixture (100x - Gibco BRL) 0.2 ml

The above components were combined, a small amount of phenol red indicator was added, and the pH was adjusted to 7.4 with 2 M NaOH. The medium was filter sterilised through 0.22 μm filters, aliquoted and frozen.

Denhardts (50x)

Ficol	5 g
Polyvinylpyrrolidone	5 g
BSA	5 g

Sterile distilled water to 500 ml, then filter sterilise

DNA denaturation solution

NaCl	1.5 M
NaOH	0.5 M

DNA digestion buffer

Tris-HCl (pH 7.5)	10 mM
EDTA	1 mM
NaCl	100 mM

DNA loading buffer (6x)

Ficoll	15 % (w/v)
Orange G	0.2 % (w/v)
Xylene cyanol	0.1 % (w/v)

DNA neutralisation solution

NaCl	1.5 M
Tris (pH 7.2)	0.5 M

Ethidium bromide

10mg/ml stock solution in dH_2O

Hybridisation solution (Church Gilberts)

Na_2HPO_4	0.34 mM
$NaH_2PO_4.H_2O$	0.16 mM
SDS	7 % (w/v)
EDTA (pH 8.0)	1 mM

Laemmli sample buffer (4x)

SDS	4 % (w/v)
Glycerol	10 % (v/v)
2-mercaptoethanol	10 % (v/v)
Trizma base (pH 6.8)	0.125 M
Bromophenol blue to colour	

Luria-Bertani (LB) medium

	g/litre
Bacto-tryptone	10
Bacto-yeast extract	5
NaCl	10

Solutes were dissolved in 950 ml deionized water and the pH adjusted to 7.0 with 5 M NaOH. The volume was adjusted to 1 litre prior to autoclaving.

LB agar

LB medium as described above with addition of 15 g/litre bacto-agar.

LSG

MOPS (pH 7.2)	50 mM
NaCl	100 mM
EDTA	0.1 mM
EGTA	0.1 mM
NaOVanadate	1 mM
NaF	10 mM
Triton X-100	1 % (v/v)
Glycerol	10 % (v/v)

LSGI

LSG	10 ml
Phenanthroline	20 µl from 500 mM stock
Pepstatin	50 µl from 1 mg/ml stock
Leupeptin	100 µl from 10 mg/ml stock

PMSF	50 µl from 10 mg/ml stock
Pefabloc SC	50 µl from 100 mg/ml stock

Phosphate buffered saline (PBS)

KH_2PO_4/K_2HPO_4 (pH 7.4)	10 mM
NaCl	137 mM

PBST

PBS	1 x
Tween-20	1% (v/v)

***Pfu* DNA polymerase reaction buffer**

KCl	50 mM
Tris-HCl (pH 9.0)	10 mM
Triton®X-100	0.1% (v/v)

Reverse transcription buffer

Tris-HCl (pH 9.0)	10 mM
KCl	50 mM
Triton®X-100	0.1% (v/v)

RF1

RbCl	100 mM
$MnCl_2.4H_2O$	50 mM
KAc	30 mM
$CaCl_2.2H_2O$	10 mM
Glycerol	15% (v/v)

Solution was adjusted to pH 5.8 with 0.2 M acetic acid and filter sterilised.

RF2

MOPS (pH 6.8)	10 mM
RbCl	10 mM
$CaCl_2.2H_2O$	75 mM

Glycerol	15% (v/v)

Solution was filter sterilised.

RNA denaturation solution

Guanidinium thiocyanate	4M
Sodium citrate pH 7.0	25 mM
Sarkosyl	0.5% (v/v)
β-mercaptoethanol	0.1 M

RNA loading buffer (6x)

Bromophenol blue	0.25% (w/v)
Xylene cyanol	0.25% (w/v)
Glycerol	30% (v/v)
SDS	1.2% (w/v)
Sodium phosphate (pH 6.8)	60 mM

SM medium

NaCl	5.8 g
$MgSO_4.7H_2O$	2 g
1 M Tris (pH 7.5)	50 ml
2% Gelatin	5 ml

Sterile distilled water to 1 litre

TAE (1 x)

Tris-acetate	40mM
EDTA	1mM

***Taq* DNA polymerase reaction buffer**

KCl	50 mM
Tris-HCl (pH 9.0)	10 mM
Triton®X-100	0.1%

TBST

Tris-HCl (pH 8.0)	10 mM
NaCl	0.15 M
Tween 20	0.05% (v/v)

Western transfer buffer

Tris	5 mM
Glycine	2 mM
Methanol	20% (v/v)

ZPFM medium

NaCl	132 mM
KCl	8 mM
Na_2HPO_4	8 mM
KH_2PO_4	1.5 mM
$(C_2H_3O_2)_2Mg{\cdot}4H_2O$	1.5 mM
$Ca(C_2H_3O_2)_2{\cdot}H_20$	90 μM

2.22 Abbreviations

bp	Base pairs
ConA	Concanavilin A type V
DABCO	1, 4-diazabicyclo[2.2.2]octane
DAPI	4,6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FBS	Foetal bovine serum
HRP	Horse radish peroxidase
IPTG	Isopropylthio-β-D-galactodise
kb	Kilobase pairs
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SSC	Salt, sodium citrate
TEMED	N,N,N',N'-tetramethylethylenediamine
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactoside
ZPFM	Zimmerman post-fusion medium

Chapter 3

Identification, cloning and sequence analysis of *Trypanosoma brucei rhodesiense* mRNA transcripts differentially expressed during ConA induced cell death

3.1 Introduction

Alteration in gene expression is the core regulatory mechanism that controls cell biology. The repertoire of genes expressed by a population of cells at any given time influences processes such as development and differentiation, homeostasis, cell cycle regulation, ageing and programmed cell death (Laing and Pardee, 1992). Consequently, isolation and characterisation of genes that are differentially expressed between cell populations in response to varying stimuli can provide insights into the biological processes occurring within those cells.

Analysis of gene expression can be conducted at either the mRNA or protein level. As protein is one of the "final products" of the cell, analysis of fluctuation in cellular protein constituents would seem to be the ideal method for monitoring changes in gene expression. While proteome analysis using two dimensional gel electrophoresis has become more widespread over recent years (Byrjalsen *et al.*, 1999; Costa *et al.*, 1999; Jungblut *et al*, 1999), most studies monitoring differential gene expression focus on variations in the level of mRNA. Several techniques have been developed for the comparison of mRNA expression between cell populations, including subtractive hybridisation (Sargent, 1987; Diatchenko *et al.*, 1996; Konietzko and Kuhl, 1998), differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) (Liang and Pardee, 1992; Murphy and Pellè, 1994; Dimopoulos *et al.*, 1996; Feghali and Wright, 1999), the use of cDNA microarrays (Schena *et al.*, 1995; Schena *et al.*, 1996; Amundson *et al.*, 1999), and serial analysis of gene expression (Velculescu *et al.*, 1997).

Subtractive hybridisation was one of the earliest techniques developed for identification of differentially expressed genes (Sargent, 1987). Annealing of control cDNA from one

cell population with experimental mRNA from another is followed by separation of unhybridised mRNA from the cDNA/mRNA hybrids. After several rounds of such hybridisation, cloning and sequencing of the surviving mRNA is carried out. Drawbacks to subtractive hybridisation include technical difficulty, requirement for a relatively large amount of template mRNA, inability to detect transcripts that are downregulated with respect to the control and the restriction of comparing only two samples during each screen.

The use of cDNA microarrays provides a sensitive and semi-quantitative method for screening thousands of transcripts in a single hybridisation, identifying those that are both up- and downregulated with respect to the control. This is a very involved technique requiring amplification of cDNA clones from a control population by PCR, construction of a microarray and screening with labelled mRNA from the experimental population (Schena *et al.*, 1995; Amundson *et al.*, 1999). The inherent limitation of this technique is of course that the mRNA expression levels of experimental populations may only be compared with the control, and not directly with each other.

Serial analysis of gene expression (SAGE) allows identification and quantitation of all mRNA being expressed within a cell population. With this technique short ESTs (9-11 bp) are generated from double stranded cDNA, annealed together to form concatemers then sequenced. Identity and frequency of ESTs can then be determined (Velculescu *et al.*, 1997). The major drawback of this technique is its *a priori* requirement for complete genome sequence, thus limiting the number of species to which it can be applied currently.

Differential display PCR is the most commonly used method for identifying mRNAs that are differentially expressed between different populations of cells. Variations of this technique abound (Welsh *et al.*, 1992; Murphy and Pellè, 1994; Mathieu-Daude *et al.*, 1998), but the general principle underlying them remains the same. mRNA is converted to first strand cDNA, and is then amplified by PCR using one or more primers to produce a variety of products that are visualised by electrophoresis. Differential display PCR is a relatively uninvolved and sensitive technique that allows comparison of multiple samples, identifying transcripts that are both up- and downregulated. However, drawbacks include a bias towards more abundant transcripts (Bertioli *et al.*, 1995), a requirement for high quality template mRNA, and an abundance of false positive results (Zhang *et al.*, 1996; Poirier *et al.*, 1997).

Randomly amplified differentially expressed sequences PCR (RADES-PCR) was designed specifically for the identification of differentially expressed transcripts from trypanosomes (Murphy and Pellè, 1994). Like other differential display PCR techniques, the first steps of the RADES-PCR protocol involve isolation of RNA from cells, followed by reverse transcription to produce first strand cDNA. cDNA is then amplified by PCR using primers specific to the fixed 5' spliced leader and 3' poly[A]$^+$ tail of all trypanosome mRNAs studied to date (Parsons *et al.*, 1984). This trypanosome-specific amplification step facilitates analysis of samples contaminated with material from either host or vector, and allows utilisation of very small amounts of template in numerous differential screens. Differential display PCR is carried out with a single 10-mer primer, producing a fingerprint that is resolved by agarose gel electrophoresis (Murphy and Pellè, 1994).

In recent years a number of studies have been undertaken with the aim of identifying transcripts that were differentially expressed within a particular apoptotic system. The goal of such research has been a better understanding of the control of gene expression, and by association cellular events, occurring throughout this process, presumably with a view to manipulating elucidated pathways in the future. While 2 groups have identified novel Bcl-2 family members (Thomson *et al.*, 1997; Fournier *et al.*, 1999), the majority of genes identified thus far (such as a ribosomal protein, α-tubulin, monocyte chemotactic protein-1, Sm protein G or aldolase C) had not had previous links to apoptosis (Chen *et al.*, 1998; Choi *et al*, 1998).

In the current work the RADES-PCR technique was utilised to identify differential gene expression in procyclic *T. brucei* undergoing ConA-induced cell death. 27 putatively differentially expressed transcripts were identified and sequenced. Three of these were subjected to Northern blot analysis. Phase microscopy and DAPI staining were conducted on ConA-treated cells, revealing a phenotype dissimilar to apoptosis in metazoans.

3.2 Results

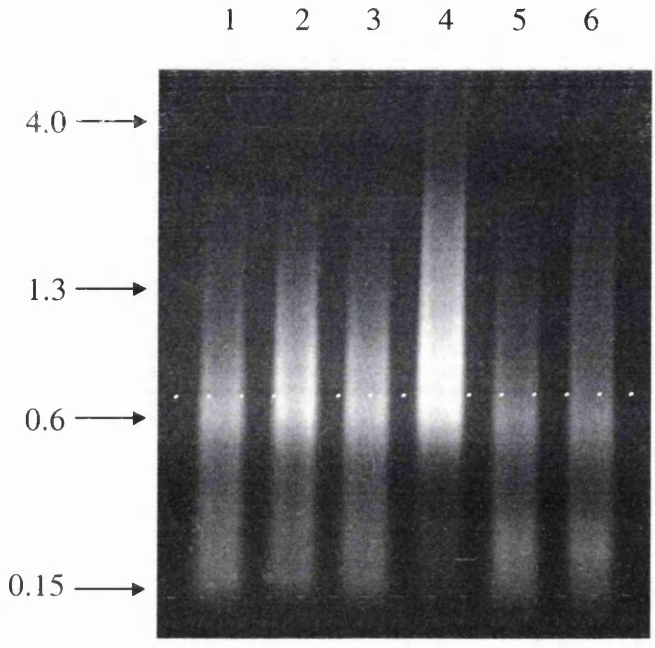
3.2.1 Identification of transcripts displaying differential expression during ConA-induced cell death of *T. brucei*

5 x 10^8 trypanosomes in mid-log phase were harvested, then resuspended at 2 x 10^6/ml in fresh Cunninghams medium containing 10 μg/ml ConA. Cells were equally distributed between five culture flasks and incubated at 27°C for 4, 8, 24, 48 or 72 hours, following which trypanosomes were harvested and placed at –70°C for storage. 1 x 10^8 control cells were not subjected to ConA treatment.

Poly[A]$^+$-enriched RNA was prepared directly from trypanosome cell pellets and resuspended in 50 μl RNase free dH_2O. First strand cDNA was synthesised and amplified as described previously (Section 2.5). Successful amplification of cDNA was verified by electrophoresis of 10 μl of each sample (from 100 μl) on a 1.5% agarose gel (Figure 3.1). Enzymes, buffer, primers and dNTPs were removed from samples by ultrafiltration as described previously (Section 2.5). The concentration of cDNA was determined spectrophotometrically, following which samples were diluted to 20 ng/μl with TE buffer. Aliquoted cDNA was stored frozen at –20°C.

RADES-PCR was carried out on samples as described in Section 2.6 using a total of 36 arbitrary primers (kindly supplied by N. Murphy, ILRI, Nairobi). Sequences of RADES primers can be found in Table 3.1. PCR reactions on each sample were, where possible, carried out at two template concentrations in order to detect more accurately those products displaying a reproducible pattern, thus reducing the probability of selecting artefactual bands. PCR products were electrophoresed on 2% agarose gels and progress of the migrating bands monitored by medium-wave UV transillumination. RADES gels are displayed in Figure 3.2.

Figure 3.1 Amplified cDNA electrophoresed on a 1.5 % agarose gel



cDNA derived from ConA treated trypanosomes was subjected to agarose gel electrophoresis to verify successful reverse transcription and PCR amplification. Lanes 1-6 represent samples derived from cells cultured in the presence of ConA for 0, 4, 8, 24, 48, and 72 hours respectively. Numbers on the left denote molecular weight in kb.

Table 3.1 Sequences of arbitrary RADES primers

Primer number	Primer sequence	Primer number	Primer sequence
101	CGAGCACAATG	1205	ACGCCGGGGC
505	CGGACGTCGC	1206	GCGGTCGGCG
508	CGGCCCCTGT	1209	GGCCCCCGCT
509	TGGTCAGTGA	1213	CGCGCGGGGA
524	CGCGCCCGCT	1216	ATGGCTCGGC
526	GCCGTCCGAG	1281	CCGGGCCGTG
527	GCGCGCAGCG	1285	TCGCGCGCCG
539	CGGCGGAGCT	1297	CCGTCGGCCC
540	GAGGGGGCGT	1298	CGAGACGGAG
541	GCGGCTGCCA	1300	CAGTCGGGTC
542	GGGTGCGCGG	1365	CGAGCACAAT
543	GTGTCCGGCG	1384	GAAGGCTGCG
867	CGTTCCCCGC	1387	GCTAGGGCGG
873	CATGTGCAGG	1499	AAGCGAGCCG
875	GTCCGTGAGC	1501	CGGCCGGTCA
921	CGGCTACGGG	1504	GGAGACGCCC
1197	GCTCTGGGTC	1505	GCCTGTGAGG
1201	CCCAGCCCCA	1506	ACGGTGCGCC
1204	GACGGCGCAA	1508	CCACATACCC

Significant changes in banding pattern were sought, including bands which either appeared/disappeared or were judged to demonstrate greater than 3-fold increase/decrease across the time course. Where two template concentrations were employed products were required to be present in both lanes in order to be selected. On occasions where PCR was carried out at only one template concentration such a safeguard against artefacts was not possible. Failure of the PCR on one or more samples was not uncommon (Figure 3.2: primer 527, lane 5; primer 1501, lane 3) and was taken into account when seeking differentially expressed bands. Each primer produced a unique fingerprint, and significant variation in the number of amplified products was observed. PCR products ranged in size from 100 bp to 2 kb, but were generally in the range of 300 bp to 1 kb.

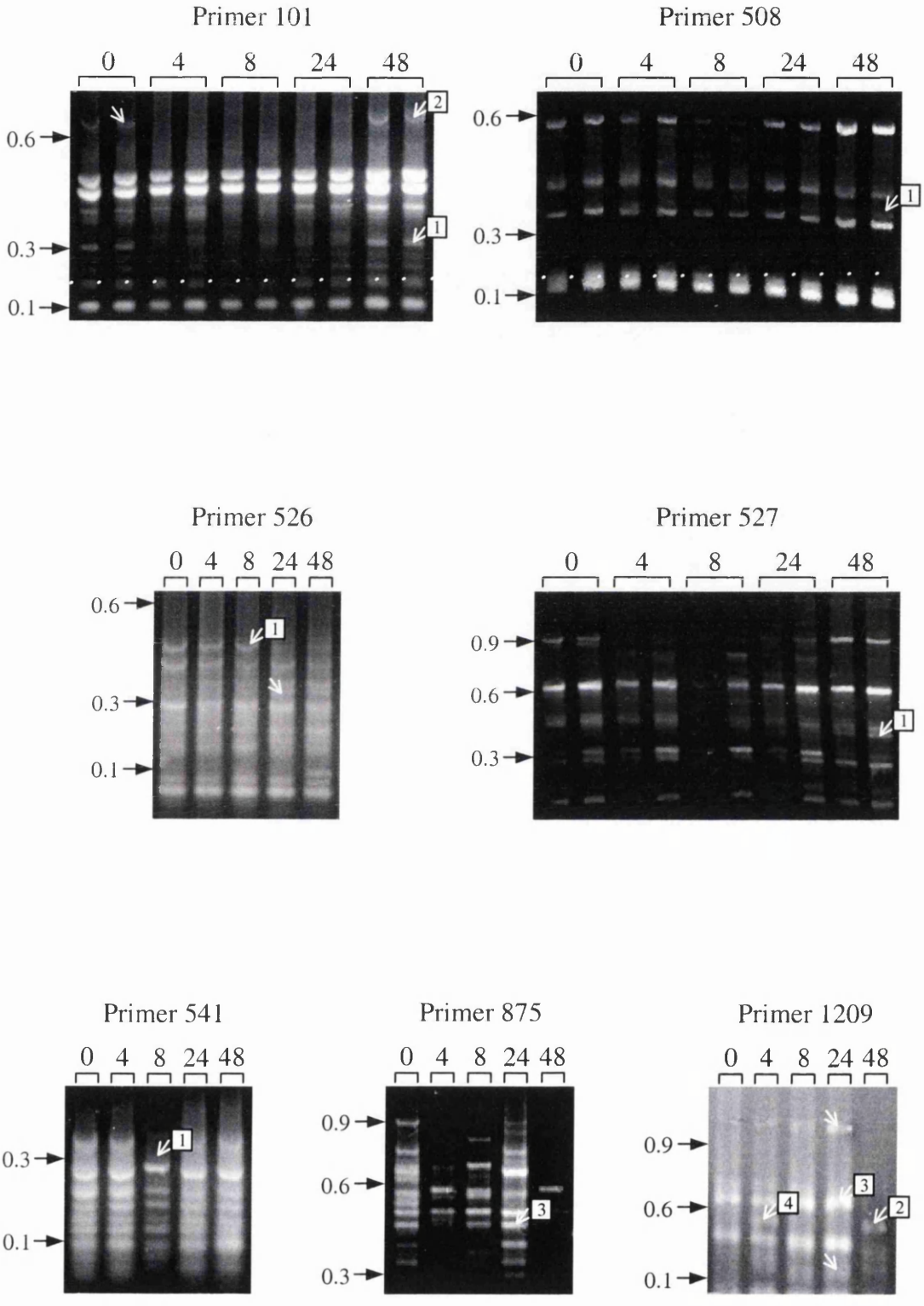
Plugs were taken from selected bands using a 200 µl pipette tip with the terminal 5 mm removed, resulting in a bore of approximately 1 mm. Gel plugs were expelled into 50 µl dH_2O, boiled for 10 minutes in a water bath, then used as template for re-amplification as described in Section 2.6. The resulting PCR products were electrophoresed on a 2% agarose gel as previously and appropriate bands excised. Examples of re-amplified products are presented in Figure 3.3. Multiple bands were often produced during re-amplification, indicating that the template DNA had consisted of several PCR products. Co-migrating bands have previously been reported as one of the major causes of false positive results when using differential display PCR (Laing *et al.*, 1993; Murphy and Pellé, 1994; Zhang *et al.*, 1998), so this phenomenon was not unexpected. Of the 64 bands identified during the course of this study, 49 (77%) were successfully re-amplified. There was a tendency for small RADES products (300 – 800 bp) to re-amplify more successfully than larger ones (> 1 kb) under the conditions employed. In theory it would have been possible to optimise reamplification conditions for each

amplicon, but due to the number of products detected during this study such an approach was not deemed to be necessary.

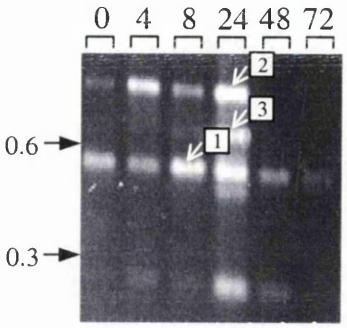
Re-amplified RADES-PCR products were T-vector cloned as described in Section 2.7. The presence of inserts in bacterial colonies was determined by colony PCR using commercial SP6 and T7 RNA polymerase promoter sequencing primers [Promega]. Of the 49 products which re-amplified, 27 (55%) were successfully cloned. The size of cloned products was confirmed prior to sequencing by restriction enzyme digestion of plasmids (*Not* I/*Nco* I for pGEM-T or *Eco* RI for pGEM-T easy), followed by agarose gel electrophoresis. On several occasions fragments of disparate size were cloned from the same re-amplified product. On all but two occasions it was possible to determine by size alone which were the products of interest, and which re-amplification artefacts. However, both 1499(3) and 1504(4) produced 2 clones, each of which contained an insert approximating the predicted amplicon size, but of noticeable different sizes to each other. As it was not possible to determine which clones represented the original amplicon, both clones, henceforth referred to as p1499(3)/p1499(3a) and p1504(4)/p1504(4a) respectively, were sequenced in each case. Sequencing was with commercial SP6 and T7 RNA polymerase promoter sequencing primers. Sequence data was edited using the EditSeq programme [DNASTAR] then used to homology search databases using the BLAST algorithm. Sequence data is lodged in Appendix I.

Figure 3.2 RADES-PCR products from ConA treated trypanosomes

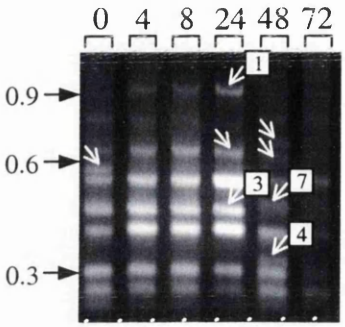
RADES-PCR was carried out on ConA treated procyclic trypanosomes, using the primers indicated above each image. Numbers above each photograph denote time in hours post-treatment of cells with ConA. Where two lanes are indicated as corresponding to a single time-point, the first represents a template concentration of 20 ng/μl and the second 2 ng/μl. Numbers in the left margin denote size in kb. Numbered arrows (↙) denote products that were successfully cloned and sequenced. Arrows without numbers (↘) denote bands that were selected as being differentially expressed but which failed at either the re-PCR or cloning step.


Primer 101
0 4 8 24 48
0.6
0.3
0.1
2
1
Primer 508
0 4 8 24 48
0.6
0.3
0.1
1
Primer 526
0 4 8 24 48
0.6
0.3
0.1
1
Primer 527
0 4 8 24 48
0.9
0.6
0.3
1
Primer 541
0 4 8 24 48
0.3
0.1
1
Primer 875
0 4 8 24 48
0.9
0.6
0.3
3
Primer 1209
0 4 8 24 48
0.9
0.6
0.1
4
3
2

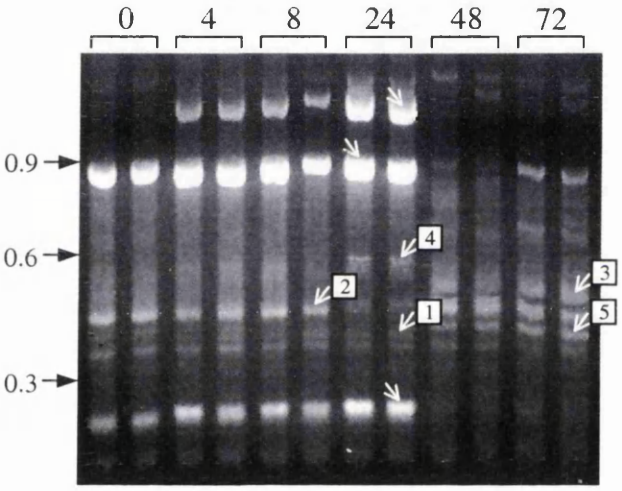
Primer 1365



Primer 1504



Primer 1499



Primer 1501

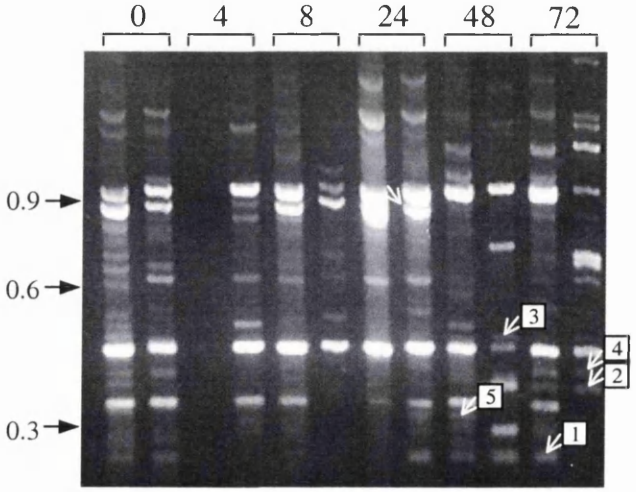
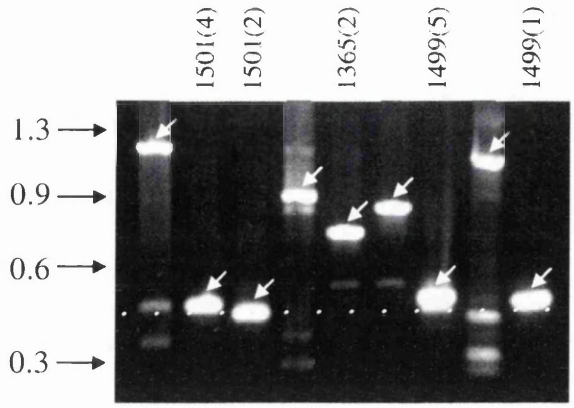


Figure 3.3 Re-amplified RADES-PCR products



Re-amplified RADES-PCR products were electrophoresed on a 2% agarose gel. Appropriate bands were identified (indicated by white arrows) and excised for T-vector cloning. Bands that were successfully cloned and sequenced are labelled above, corresponding to amplicons indicated in Figure 3.2. Numbers on the left denote size in kb.

BLAST searches were carried out using the programmes pre-set parameters. A Score (in bits) and a statistical significance for the match (e-value) accompanied each BLAST alignment. The Score indicated the search space needed to be examined in order to find a match as good as the one presented by chance alone. Larger bit scores indicated more significant alignments, and were independent of the size of the database in which a matching sequence was found. The "Expect" value gave an estimate of the statistical significance of the Score and was based on both the size of the query sequence and the size of the database. A Score of 30 and an Expect value of 2e-05 would therefore indicate that a search space of 1 billion bits (2^{30}) would have to be examined in order to find, by chance alone, a match as good as the one presented, and that on a database of the size searched 2×10^{-5} matches with a Score of 30 would be expected by chance alone.

Comparison of sequence data derived from RADES-PCR products with that held on internet databases was carried out primarily using the BLAST-X programme. This translated the experimental nucleotide sequence in all six reading frames then compared these with the 6-frame translation of sequences on the database. The output from BLAST-X searches using the T7 sequence of amplicon 3, Primer 875 [875(3)T7], and the SP6 sequence of amplicon 5, Primer 1499 [1499(5)SP6] are presented in Figures 3.4 and 3.5 respectively as examples. At the top of each results page was a list of definition lines that referred to alignments arranged sequentially further down the page. The alignments in both Figures 3.4 and 3.5 were from the first sequence listed. Alignments were preceded by a definition line containing information such as the database on which the target sequence was lodged (sp, dbj, emb etc.), the sequence accession number, a bit Score, and an Expect value. The sequence identity (Identities), similarity (Positives) and nucleotide reading frame were also provided. On the alignment itself the query line referred to the sequence being compared against database entries, and the subject line gave the sequence of the database match. The intervening sequence denoted residues that were identical (letters) or conserved (+).

A bit Score greater than 50 and an Expect value lower than 1×10^{-5} were required in order for a match to be deemed significant. Sequence 875(3)T7 (Figure 3.4) returned a high Score of 31 and a corresponding Expect of 3.1. In accordance with the aforementioned parameters this match was therefore not classed as significant. By contrast, sequence 1499(5)SP6 (Figure 3.5) returned a high Score of 179 and a corresponding Expect of 6×10^{-45}. This match was therefore highly significant. Figure 3.7 contains BLAST results from all sequences derived from RADES-PCR of ConA

treated cells. Of the 29 clones analysed, 17 returned significant matches from one or both sequences.

BLAST-N searches, comparing nucleotide query sequences directly with sequences on the database, were carried out on sequences that did not produce significant BLAST-X matches. No additional significant matches were revealed by employing the BLAST-N programme.

Figure 3.4 BLAST-X alignments derived from sequence 875(3)T7

Sequence 875(3)T7 was used to homology search the nr database using the BLAST-X algorithm. This sequence returned matches to a variety of envelope glycoprotein sequences from the human immunodeficiency virus. Values for Score and Expect were low, indicating that these were not significant matches. The alignment denoted by the first of the definition lines is presented. This match was of reasonable quality, but was short.

Sequences producing significant alignments:	Score (bits)	E Value
gi | 1336634 (U57788) envelop glycoprotein 120 [Human immuno...	31	3.1
pir || A40218 envelop glycoprotein gp120 - human immunodefici...	31	3.1
gi | 2853990 (AF041129) envelope glycoprotein [Human immunode...	31	4.0
gi | 1495956 (U27401) envelope glycoprotein [Human immunodefi...	30	9.0
gi | 818222 (U23487) env glycoprotein [Human immunodeficiency...	30	9.0
gi | 3114552 (AF005494) envelope polyprotein [Human immunodef...	30	9.0

gi|1336634 (U57788) envelope glycoprotein 120 [Human immunodeficiency virus type 1] Length = 536

Score = 31.3 bits (69), Expect = 3.1

Identities = 13/30 (43%), Positives = 19/30 (63%)

Frame = -3

```
QUERY:  270 I I R A A E K I W I S F F Y L H P F F L P F S P V Y F C A S 181
            I   R A A E K + W + +   + Y     P   +       +       F C A S
SBJCT:   27 I C R A A E K L W V T V Y Y G V P V W K E A T T T L F C A S 56
```

Figure 3.5 BLAST-X alignments derived from sequence 1499(5)SP6

Sequence 1499(5)SP6 was used to homology search the nr database using the BLAST-X algorithm. This sequence returned matches to the 60S ribosomal protein L10 of *Drosophila melanogaster*, *Oryza sativa*, *Solanum melongena* and *Homo sapiens*. The same region of the sequence also matched a QM family protein from *S. melongena* and *O. sativa*, and a putative protein from *Arabidopsis thaliana*. Values for Score and Expect were high, indicating that these were significant matches. The alignment denoted by the first of the definition lines is presented. This match was of high quality, and was relatively long.

Sequences producing significant alignments:	Score (bits)	E Value
emb|CAB39087.1| (AL034358) predicted using hexExon; L4830.9...	179	6e-45
sp|P45635|R101_ORYSA 60S RIBOSOMAL PROTEIN L10-1 (PUTATIVE ...	143	3e-34
dbj|BAA19414| (AB001582) QM family protein [Solanum melongena]	143	5e-34
sp|P93847|RL10_SOLME 60S RIBOSOMAL PROTEIN L10 (EQM) >gi|19...	143	5e-34
gi|1305525 (U55212) Wilms' tumor-related protein QM [Oryza ...	143	5e-34
gb|AAD14497| (AC005508) 29621 [Arabidopsis thaliana]	142	6e-34
sp|P45633|RL10_MAIZE 60S RIBOSOMAL PROTEIN L10 (QM PROTEIN ...	141	1e-33

emb|CAB39087.1| (AL034358) predicted using hexExon; L4830.9, 60S Ribosomal protein L10, len: 214 aa; Similarity to 60S ribosomal protein L10. D.melanogaster 60S ribosomal protein (SW:RL10_DROME) BLAST Score: 740, sum P(1) = 1.7e-73; 66% iden...Length = 213

Score = 179 bits (449), Expect = 6e-45

Identities = 84/97 (86%), Positives = 90/97 (92%)

Frame = +3

```
QUERY:  3   K E C F H M R I R A H P F H V L R I N K M L S C A G   80
            K +   F H M R   R A H P F H V L R I N K M L S C A G
SBJCT:  82  K D V F H M R T R A H P F H V L R I N K M L S C A G   107

QUERY:  81  A D R L Q T G M R Q S Y G K P N G N C A R V R I G Q   158
            A D R L Q T G M R   + + G K P N G   C A R V R I G Q
SBJCT:  108 A D R L Q T G M R G A F G K P N G V C A R V R I G Q   133

QUERY:  159 I L L S M R T K D T Y V P Q A L E S L R R A K M K F   236
            I L L S M R T K +   Y V P Q A   E + L R R A K M K F
SBJCT:  134 I L L S M R T K E A Y V P Q A F E A L R R A K M K F   159

QUERY:  237 P G R Q I I V I S K Y W G F T N L I R       293
            P G R Q I I V + S K Y W G F T N L I R
SBJCT:  160 P G R Q I I V M S K Y W G F T N L I R       178
```

Table 3.2 BLAST data from RADES clones

Sequence	Seq length	Amplicon length (bp)	BLAST match	Organism	Accession N°	Score	Expect	Detected at hours
101(1)T7	313	313	cDNA 5' similar to 60S ribosomal protein L30	*Trypanosoma brucei*	P49153	175	1e-43	4, 8, 24, 48
101(1)SP6	313		cDNA 5' similar to 60S ribosomal protein L30	*Trypanosoma brucei*	P49153	175	1e-43	
101(2)T7	606	643	cDNA 5' similar to putative mitochondrial carrier protein	*Trypanosoma brucei*	AA186249	115	2e-32	48
101(2)SP6	335		cDNA 5' similar to mitochondrial FAD carrier protein	*Trypanosoma cruzi*	AI664716	73	1e-14	
508(1)T7	377	392	cDNA 5' similar to 40S ribosomal protein S15	*Trypanosoma brucei*	AI215241	257	1e-67	24, 48
508(1)SP6	370		cDNA 5' similar to 40S ribosomal protein S15	*Trypanosoma brucei*	AI215241	252	2e-67	
526(1)T7	264	450	cDNA clone similar to peptide chain release factor 2	*Homo sapiens*	AI621052	59	3e-08	0, 4, 8
526(1)SP6	Sequence failed							
527(1)T7	367	465	cDNA 5' similar to 60S ribosomal protein L13A	*Trypanosoma brucei*	AA063735	201	2e-50	48
527(1)SP6	371		cDNA 5' similar to 60S ribosomal protein L13A	*Trypanosoma brucei*	AA063735	201	8e-51	
541(1)T7	296	296	cDNA clone (protein function unknown)	*Mus musculus*	AI663727	68	2e-12	8
541(1)SP6	296		cDNA clone (protein function unknown)	*Mus musculus*	AI663727	68	2e-12	
875(3)T7	364	470	envelope polyprotein	HIV	U37270	30	8.9	8, 24
875(3)SP6	300		envelop glycoprotein gp120	HIV	A40218	31	4.1	

Sequence	Seq length	Amplicon length (bp)	BLAST match	Organism	Accession N°	Score	Expect	Detected at hours
1209(2)T7	345	500	cDNA clone (protein function unknown)	*Homo sapiens*	AI038033	35	0.008	48, 72
1209(2)SP6	Sequence failed							
1209(3)T7	330	600	predicted using Genefinder	*Caenorhabditis elegans*	Z75553	32	1.9	0, 4, 8, 24
1209(3)SP6	Sequence failed							
1209(4)T7	233	470	cDNA 5' similar to arginine kinase	*Trypanosoma brucei*	AA003463	146	3e-35	4, 8, 24
1209(4)SP6	332		No hit found					
1365(1)T7	578	578	No hit found					All
1365(1)SP6	578		No hit found					
1365(2)T7	435	693	No hit found					
1365(2)SP6	525		cDNA clone similar to MOB1	*Dictyostelium discoideum*	C90606	110	3e-23	0, 4, 8, 24
1365(3)T7	280	630	T-cell receptor alpha chain precursor V	*Mus musculus*	A42242	33	0.69	4, 8, 24
1365(3)SP6	Sequence failed							
1499(1)T7	280	385	factor H homologue	*Homo sapiens*	I72653	31	1.9	4, 8, 24
1499(1)SP6	245		endonuclease PAER7I	*Pseudomonas aeruginosa*	P05104	31	1.8	
1499(2)T7	440	440	cyclophilin 2	*Oryza sativa*	L29470	76	2e-13	0, 4, 8
1499(2)SP6	440		cyclophilin 2	*Oryza sativa*	L29470	76	2e-13	

Sequence	Seq length	Amplicon length (bp)	BLAST match	Organism	Accession N°	Score	Expect	Detected at hours
1499(3)T7	548	548	conserved hypothetical protein	*Thermotoga maritima*	AE001759	49	2e-05	48, 72
1499(3)SP6	548		conserved hypothetical protein	*Thermotoga maritima*	AE001759	49	2e-05	
1499(3a)T7	571	571	ATP-dependent Clp protease regulatory subunit	*Aquifex pyrophilus*	AF027500	35	0.52	48, 72
1499(3a)SP6	571		ATP-dependent Clp protease regulatory subunit	*Aquifex pyrophilus*	AF027500	35	0.52	
1499(4)T7	598	650	Hypothetical protein similar to pteridine transporter	*Trypanosoma brucei*	S33475	70	9e-19	24
1499(4)SP6	Sequence failed							
1499(5)T7	271	411	60S Ribosomal protein L10	*Leishmania major*	AL034358	149	5e-36	48, 72
1499(5)SP6	298		60S Ribosomal protein L10	*Leishmania major*	AL034358	179	6e-45	
1501(1)T7	224	224	invariant surface glycoprotein 65	*Trypanosoma brucei*	A38145	28	8.0	24, 48, 72
1501(1)SP6	224		invariant surface glycoprotein 65	*Trypanosoma brucei*	A38145	28	8.0	
1501(2)T7	205	426	cDNA 5' similar to ribosomal protein S13	*Trypanosoma brucei*	AA052874	116	2e-31	72
1501(2)SP6	303		cDNA 5' similar to ribosomal protein S13	*Trypanosoma brucei*	AA052874	126	2e-28	
1501(3)T7	357	479	olfactory receptor 17-93	*Homo sapiens*	U76377	32	3.7	48
1501(3)SP6	479		ORF2 bases 1807-2850	*Trypanosoma brucei*	M14820	31	8.0	
1501(4)T7	271	453	activated protein kinase c receptor homolog	*Trypanosoma brucei*	AJ234154	130	5e-36	72
1501(4)SP6	453		activated protein kinase C receptor homolog	*Trypanosoma brucei*	AJ234154	166	2e-40	

Sequence	Seq length	Amplicon length (bp)	BLAST match	Organism	Accession N°	Score	Expect	Detected at hours
1501(5)T7	304	370	ALK-2 receptor	*Xenopus laevis*	AF012245	33	0.75	24, 48
1501(5)SP6	285		hypothetical 47.9 kd protein	*Bacillus subtilis*	P14203	39	0.012	
1504(1)T7	347	950	apocytochrome c1	*Trypanosoma brucei*	AF102980	157	2e-38	4, 8, 24
1504(1)SP6	379		cDNA clone (protein function unknown)	*Trypanosoma brucei*	AA701839	158	6e-62	
1504(3)T7	361	450	cDNA clone (protein function unknown)	*Trypanosoma cruzi*	AI664670	82	2e-14	0, 4, 8, 24
1504(3)SP6	347		cDNA clone (protein function unknown)	*Trypanosoma cruzi*	AI664670	148	7e-34	
1504(4)T7	318	318	Similar to brain specific protein CGI-38 (*H. sapiens*)	*Trypanosoma brucei*	W84076	216	1e-56	48
1504(4)SP6	318		Similar to brain specific protein CGI-38 (*H. sapiens*)	*Trypanosoma brucei*	W84076	216	1e-56	
1504(4a)T7	308	308	cDNA 5' similar to 40S ribosomal protein S11	*Trypanosoma brucei*	W69043	408	1e-112	48
1504(4a)SP6	308		cDNA 5' similar to 40S ribosomal protein S11	*Trypanosoma brucei*	W69043	408	1e-112	
1504(7)T7	387	454	cell surface adhesion glycoproteins LFA-1/CR3/P150,95	*Bos taurus*	P32592	35	0.23	48, 72
1504(7)SP6	322		spore protein	*Bacillus subtilis*	AF027868	33	0.63	

RADES clones were sequenced using T7 and SP6 primers. Resultant sequences were compared with database entries using BLAST-X against the nr database (non-redundant protein translations of GenBank + Protein Data Bank + SwissProt + Protein Identification Resource Database + Protein Research Foundation Sequence database), and BLAST-N and TBLAST-X against dbEST (GenBank division containing "single-pass" cDNA sequences, or Expressed Sequence Tags, from a number of organisms). The best match, as determined by Score and Expect, is presented for each sequence. Sequence numbers refer to amplicons indicated in Figure 3.2.

Six of the significant matches returned encoded ribosomal protein homologues, 5 of which had previously been identified as *Trypanosoma brucei* ESTs (p101(1), p508(1), p527(1), p1501(2) and p1504(4a)), and one which represented a novel *T. brucei* gene but which had a *T. cruzi* homologue (p1499(5)). Matches to *T. brucei* sequences never displayed 100% identity. Differences were likely to have resulted either from introduced errors (RADES-PCR reactions were carried out using relatively high $MgCl_2$ concentrations, and were thus of low fidelity) or from strain differences between trypanosome isolates. The RADES products encoding ribosomal proteins were all identified as being upregulated in ConA treated cells with respect to the control (respective bands in Figure 3.2), although variation in the interval between treatment and upregulation was noted. 101(1) was present in all experimental samples but absent in the control. 508(1) became evident at 24 hours, while 527(1), 1499(5) and 1504(4a) were not observed until 48 hours post treatment. 1501(2) did not appear until 72 hours after addition of ConA to trypanosomes.

Analysis of two RADES clones identified sequences encoding proteins associated with the mitochondrion. Amplicon 101(2) was identified as a transcript present at 48 hours in ConA treated trypanosomes but absent in control cells and at previous time points (Figure 3.2). BLAST data revealed a significant match between p101(2) and previously identified ESTs from *T. brucei* and *T. cruzi* that had significant homology to a family of mitochondrial transporter proteins. The major difference between p101(2) and the *T. brucei* EST AA186249 was a temporary loss of alignment due to a frameshift, believed to be the result of sequence errors introduced either during RADES-PCR or during sequencing.

Amplicon 526(1) was identified as a transcript that was present in control cells and ConA-treated 4 and 8 hour timepoints, but that was absent at 24 and 48 hours post treatment (Figure 3.2). BLAST data demonstrated a significant match between the T7 sequence of clone p526(1) and a human cDNA clone (Score 59, Expect 3 x 10^{-8}). The human transcript encoded a homologue for the peptide chain release factor 2 of *Streptomyces coelicolor* (Score 62, Expect 2 x 10^{-9}). Direct comparison between clone p526(1) and *S. coelicolor* revealed a match that was not significant (Score 41, Expect 0.002). The SP6 sequence of clone p526(1) failed.

Amplicon 1209(4) was identified as a transcript present in ConA treated cells at all time points, but absent from the control (Figure 3.2). BLAST data revealed a significant match between the T7 sequence of clone p1209(4) and a *T. brucei* EST encoding the 5' end of arginine kinase. This enzyme is a member of the phosphagen kinase family, responsible for the reversible transfer of a high energy phosphoryl group from ATP to a phosphagen such as creatine or arginine, effectively buffering ATP levels within active cells (Stryer, 1988). The SP6 sequence from p1209(4) did not produce a database match.

Amplicon 1365(2) was identified as a transcript that was present in control lanes and which gradually increased in experimental cells over the first 24 hours following ConA treatment. The transcript was not evident at 48 or 72 hours (Figure 3.2). BLAST analysis of the T7 sequence of clone p1365(2) did not reveal any database matches. However, SP6 sequence data displayed a significant match with a cDNA clone from *Dictyostelium discoideum* with homology to MOB1 sequences from various organisms. Direct comparison between the SP6 sequence of p1365(2) and MOB1 sequences from *Arabidopsis thaliana*, *Homo sapiens*, *Caenorhabditis elegans*, *Schizosaccharomyces*

pombe and *Saccharomyces cerevisiae* revealed significant matches to all, with Score and Expect values ranging from 91 : 5 x 10^{-18} to 69 : 2 x 10^{-11} respectively. To date the MOB1 protein has only been characterised in *S. cerevisiae*, where it is involved in completion of mitosis and maintenance of ploidy. MOB1 is an essential yeast phosphoprotein that has been shown to display two-hybrid interaction with two protein kinases, MPS1 (Luca and Winey, 1998) and DBF2 (Komarnitsky *et al.*, 1998). The function of MOB1 is currently unknown, although a role during late mitosis has been demonstrated (Komarnitsky *et al.*, 1998; Luca and Winey, 1998). A further analysis of the *T. brucei MOB1* is presented in Chapter 5.

Amplicon 1499(2) was identified as a transcript that was present in control cells and at 4, and 8 hours in ConA treated cells, but absent in the 24, 48 and 72 hour timepoints (Figure 3.2). BLAST analysis of both the T7 and SP6 sequence data derived from clone p1499(2) revealed a significant match to cyclophilins from various organisms, the best Score and Expect being against a cDNA encoding cyclophilin 2 of *Oryza sativa.* Cyclophilins are ubiquitous proteins that are highly conserved in bacteria, fungi, plants and vertebrates. Cyclophilins are members of the immunophilin group of peptidyl-prolyl cis-trans isomerases that catalyse the interconversion of peptidyl-prolyl imide bonds in peptide and protein substrates (reviewed in Göthel and Marahiel, 1999).

Amplicon 1499(4) was present in ConA treated cells at the 24 hour timepoint, but was not evident in other samples (Figure 3.2). The T7 sequence derived from clone pGEM1499(4) returned a significant match to a *T. brucei* putative protein (S33475) of unstated function. Significant matches were also made with *Leishmania donovani* putative pteridine transporters FT1 - FT6 (consecutive accession numbers AAD52046-AAD52051 respectively), with Score and Expect values ranging from 64 : 1 x 10^{-13} to

45 : 3 x 10^{-8}. The *T. brucei* putative protein also demonstrated a highly significant match to the *L. donovani* sequences. Trypanosomatid protozoans lack a *de novo* pathway for the biosynthesis of pteridines (pterins and folates)(Scott *et al.*, 1987). Consequently, a sophisticated salvage pathway has arisen within these organisms involved in scavenging pteridines from the host (Nare *et al.*, 1997). The lack of identity between the insert of clone p1499(4) and the *T. brucei* putative protein S33475 suggests that the former represents a novel pteridine transporter family member in this organism.

Amplicon 1501(4) was identified as a transcript present at 72 hours in ConA treated cells, but not evident at earlier time points (Figure 3.2). BLAST analysis revealed a significant match between both T7 and SP6 sequences of clone p1501(4) and the trypanosome homologue of a receptor for activated protein kinase C (TRACK). TRACK had been identified during a previously RADES-PCR screen as a transcript upregulated in *T. brucei* undergoing ConA-induced cell death, and was confirmed as such by Northern blot analysis (Welburn and Murphy, 1998). RACKs are components of protein kinase C (PKC) signalling, binding active PKC via a PKC site distinct from the substrate binding site (Mochly-Rosen *et al.*, 1992). RACK family members have been identified in all eukaryotic species studied to date (Taladriz *et al.*, 1999).

Amplicon 1504(1) was identified as a transcript that was gradually upregulated from 4 to 24 hours post treatment with ConA (Figure 3.2). This transcript was not evident within the control lanes, or at 48 and 72 hours post-treatment. Data derived from the 950 bp insert of clone p1504(1) revealed identity with two different *T. brucei* sequences. The T7 sequence matched AF102980, encoding the full length *T. brucei* apocytochrome c1, the precursor of cytochrome c1. The SP6 sequence matched AA701839, a *T. brucei* EST with no significant homology to other sequences currently

lodged on databases. Alignments revealed that both T7 and SP6 sequences matched their corresponding clones in a 5' → 3' direction, suggesting that the PCR product cloned within p1504(1) was likely to be an artefact. Whether this clone represented the originally identified amplicon is unknown.

BLAST analysis of two RADES clones returned significant matches to database entries that encoded transcripts of as yet unknown function. Amplicon 541(1) was present in experimental trypanosomes at 8 hours post treatment, but was absent at other timepoints. Both T7 and SP6 sequences derived from clone p541(1) demonstrated a significant match to a *Mus musculus* EST isolated from a male Soares mouse mammary gland library. This EST did not return significant matches to any other sequences currently lodged on the databases searched. Amplicon 1504(4) was present in ConA treated trypanosomes at the 48 hour timepoint, but was not evident in other samples. Both T7 and SP6 sequences derived from clone p1504(4) demonstrated identity with a *T. brucei* EST, and significant homology to a novel brain specific protein, CGI-38, from *Homo sapiens* (Score 54, Expect, 2×10^{-7}). The role of this protein is unknown.

Analysis of 12 RADES clones did not return significant BLAST matches, suggesting that either the amplicons they represent derive largely or wholly from the 3' untranslated regions of *T. brucei* mRNAs, or that they represent proteins for which homologues in other organisms do not exist or have yet to be discovered. The former possibility is favoured for the most part, as much of the *T. brucei* sequence data currently available is in the form of ESTs, which by definition represent only the 5' terminus of cDNAs. Furthermore, the non-coding region of *T. brucei* ESTs would not be expected to produce database matches to DNAs from other species. However, bearing in mind that the kinetoplastids are evolutionarily divergent from other

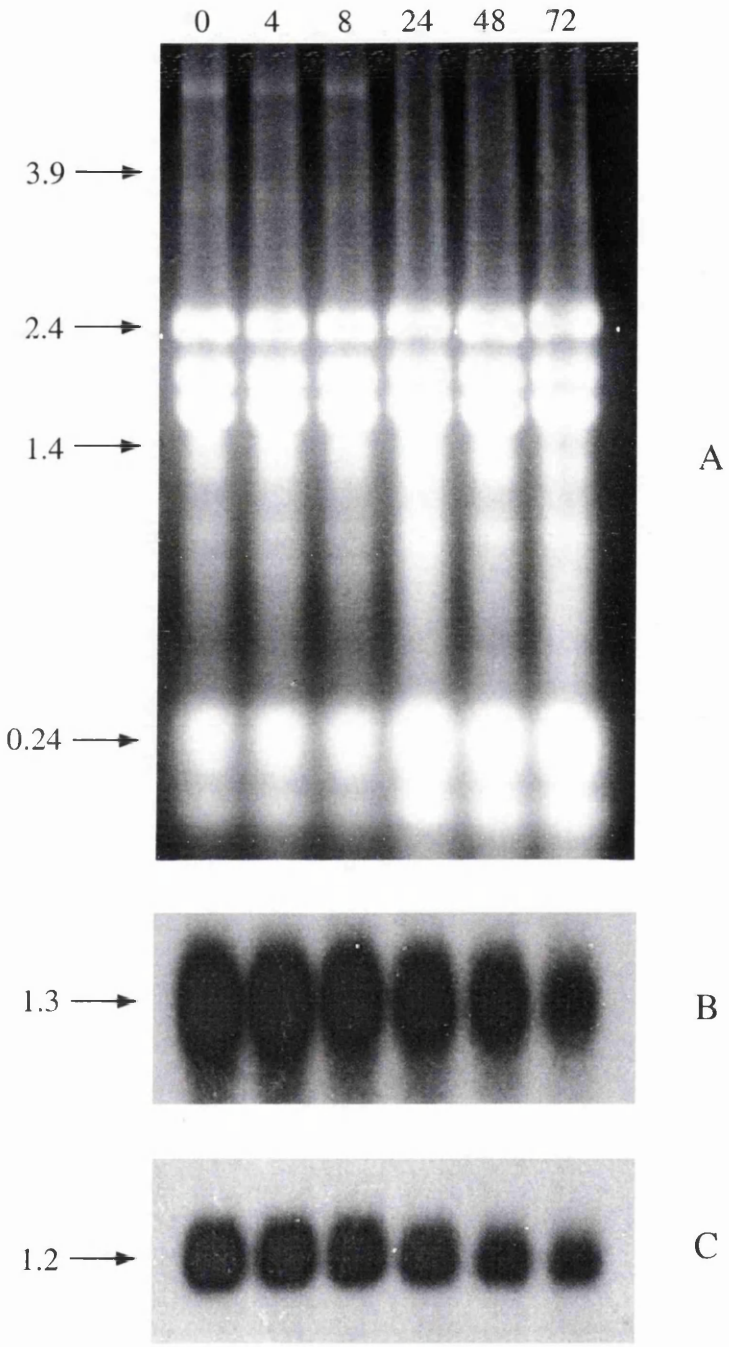
eukaryotic groups (McLaughlin and Dayhoff, 1973), and that many sequencing projects are ongoing (http://parsun1.path.cam.ac.uk), the latter possibility is not without merit.

3.2.2 Northern blot analysis of *T. brucei* transcripts identified during RADES-PCR

In order to confirm differential expression of *T. brucei* transcripts identified during the RADES-PCR screen described in Section 3.2.1, procyclic cells were treated with 10 μg/ml ConA and harvested at 0, 4, 8, 24, 48 and 72 hours post treatment. Total RNA was prepared from cell pellets and relative concentration of samples determined by agarose gel electrophoresis. Approximately 10 μg RNA from each sample was subjected to agarose gel electrophoresis, following which nucleic acid was transferred to Hybond-N+ membrane and fixed by UV cross-linking. The membrane was subjected to Northern blot analysis using the 411 bp *QM* RADES product [1499(5)] as a probe, and signal was detected autoradiographically. The *QM* probe was removed from the membrane by immersion in boiling 0.1% SDS, following which the same membrane was subjected to Northern blot analysis using the 957 bp open reading frame of the *TRACK* gene (Welburn and Murphy, 1998) as a probe. Signal was again detected autoradiographically. Results are displayed in Figure 3.6.

Hybridisation to the *QM* transcript clearly reduced throughout the time-course (Figure 3.6, plate B), contrary to the fact that the *QM* RADES products was identified as being upregulated at 48 and 72 hours in ConA-treated cells. The *TRACK* gene, previously reported to be upregulated during cell death of procyclic *T. brucei* induced by ConA (Welburn and Murphy, 1998) and identified in the present study as upregulated at 72 hours, was used as a positive control on this blot (plate C). This also demonstrated a

Figure 3.6 Northern blot analysis of total RNA from ConA-treated procyclic *T. brucei*



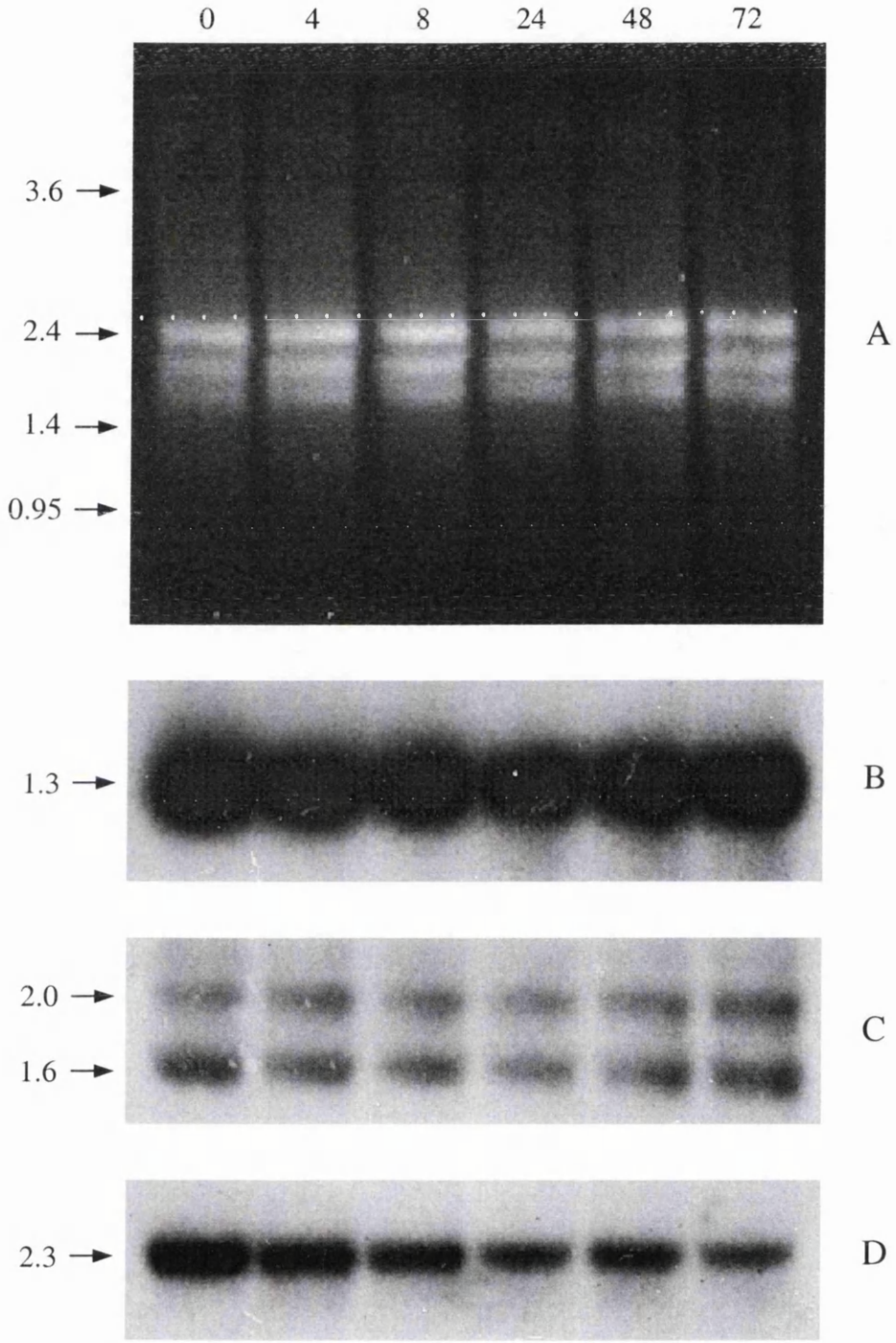
Mid-log phase procyclic cells were treated with 10 μg/ml ConA then harvested at 0, 4, 8, 24, 48 and 72 hours post treatment. Total RNA was prepared from cell pellets. 10 μg RNA from each sample was electrophoresed on a 1.4% agarose gel, photographed (plate A) then transferred to Hybond-N+. Northern blot analysis was carried out using the 411 bp *QM* RADES-PCR product [1499(5)] (plate B) or the 957 bp *TRACK* open reading frame (plate C) as probes. Numbers on the left denote transcript size in kb. Numbers along the top denote post-treatment time in hours.

notable reduction in hybridisation signal over the time-course. This led to two conflicting hypotheses. Firstly, that levels of *QM* and *TRACK* transcripts genuinely reduced during the course of ConA induced cell death (contrary to the results of RADES-PCR with primer 1499 and the published profile of *TRACK*). Secondly, that levels of mRNA in the experimental cells gradually reduced throughout the time-course relative to total RNA, resulting in an artefactual reduction in hybridisation relative to control cells.

In order to address this question cells were treated with ConA as previously, and poly[A]$^+$ RNA prepared from pellets. RNA was quantified spectophotometrically and 1μg from each sample was subjected to agarose gel electrophoresis, following which nucleic acid was transferred to Hybond-N+ membrane and fixed by UV cross-linking. The membrane was sequentially subjected to Northern blot analysis using probes derived from (1) the 411 bp *QM* RADES product [1499(5)], (2) the 693 bp *MOB1* RADES product [1365(2)] and (3) a 600 bp PCR product corresponding to the open reading frame of *T. brucei* β-tubulin gene (kindly supplied by A. Osanya). The membrane was stripped between each hybridisation as described previously, and removal of probe confirmed by autoradiographic analysis. Results are displayed in Figure 3.7.

Neither *QM* (Figure 3.7, plate B) nor *MOB1* (plate C) displayed differential hybridisation throughout the ConA-treatment time-course. β-tubulin, used as a control on this blot, was expected to hybridise equally at all time-points, but demonstrated a significant reduction in the level of detectable transcript (plate D). Phospho-imaging data acquired using a BAS-1500 phosphoimager together with MACBAS v2.02 software (FUJI) confirmed this as a 3-fold reduction in hybridisation signal between 0

Figure 3.7 Northern blot analysis of poly[A]+ RNA from ConA-treated procyclic *T. brucei*



Mid-log phase procyclic cells were treated with 10 μg/ml ConA then harvested at 0, 4, 8, 24, 48 and 72 hours post treatment. Poly[A]+ RNA was prepared from cell pellets. 1 μg RNA from each sample was electrophoresed on a 1.4% agarose gel, photographed (plate A) then transferred to Hybond N+ membrane. Northern blot analysis was carried out using the 411 bp *QM* RADES-PCR product [1499(5)] (plate B), the 693 bp *MOB1* RADES-PCR product [1365(2)] (plate C), or the β-tubulin gene open reading frame (plate D) as probes. Numbers on the left denote size of transcripts in kb. Numbers along the top denote post-treatment time in hours.

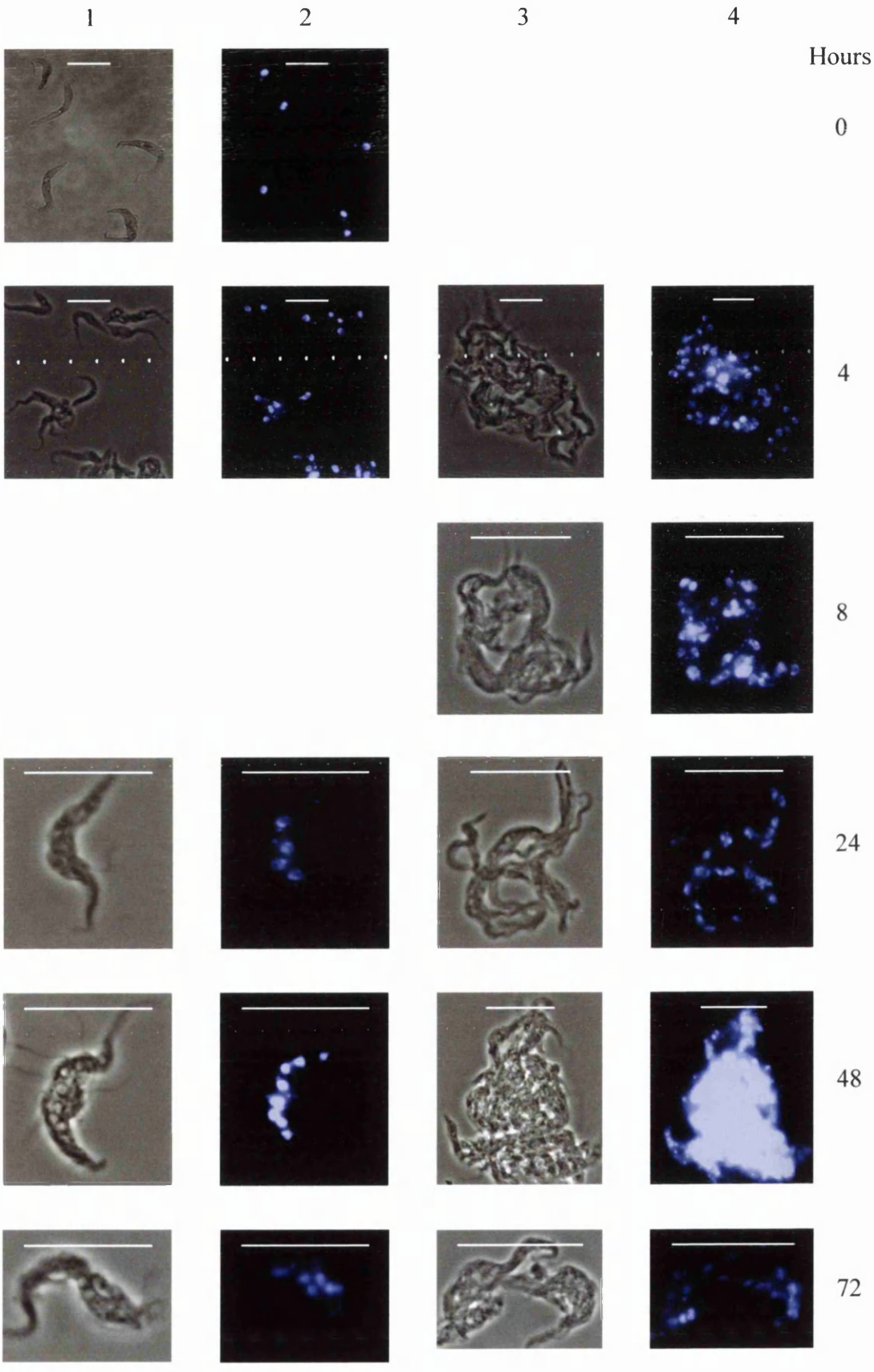
and 72 hours. These results conflicted with both the Northern blot analysis of total RNA and the RADES-PCR data, and led to two mutually exclusive hypotheses. Firstly, that the results of the poly[A]$^+$ Northern blot were directly representative of the mRNA in respective samples. Consequently, neither *QM* nor *MOB1* were differentially expressed during ConA induced death, and β-tubulin was downregulated. Secondly, that mRNA on the gel was not evenly loaded, a distinct possibility considering the high level of rRNA contamination in these preparations (Figure 3.7, plate A). The suggestion would then be that the β-tubulin transcript was evenly expressed at all time-points, and that the *QM* and *MOB1* transcripts were correspondingly up-regulated. As both the *QM* and *TRACK* transcripts demonstrated down-regulation on the total RNA Northern (Figure 3.6, Plates B and C), this would lead to the supposition that *TRACK* was in reality up-regulated, fitting with the published data. The results of the Northern blot analysis were therefore inconclusive and due to time constraints were not pursued further during this study.

3.2.3 Microscopic analysis of ConA-treated procyclic *T. brucei*

5 x 10^8 trypanosomes in mid-log phase were harvested, then resuspended at 2 x 10^6/ml in fresh Cunninghams medium containing 10 μg/ml ConA. Cells were equally distributed between five culture flasks and incubated at 27°C for 4, 8, 24, 48 or 72 hours, following which trypanosomes were harvested and resuspended in an equal volume of PBS. 20 μl cells were immediately spotted onto slides, allowed to air-dry, then fixed sequentially in 70% methanol and 100% acetone for 3 minutes each. Cells were prepared for fluorescence microscopy as described in Section 2.18, incubating slides with DAPI/PBS for 30 minutes in a dark chamber, washing thoroughly, then mounting with PBS/glycerol/DABCO. Results are displayed in Figure 3.8.

Figure 3.8 Microscopic analysis of procyclic *T. brucei* treated with ConA

Procyclic *T. brucei* cells cultured with 10 μg/ml ConA cells were observed at 4, 8, 24, 48 and 72 post-treatment. Controls (0 hours) were not subjected to ConA treatment. At each time cultured cells were observed using an inverted microscope and phase contrast microscopy in order to establish the general "fitness" of cells. Trypanosomes were then harvested, fixed, permeabilised and incubated with DAPI. Slides were examined by both phase contrast (columns 1 and 3) and fluorescence (columns 2 and 4) microscopy. Numbers in the right margin denote time in hours post-treatment with ConA. Bar at the top of each tile denotes 10 μm.


1
2
3
4
Hours
0
4
8
24
48
72

Control cells observed in culture were very active and free-swimming. Cellular aggregates were rare and consisted of no more than 2-3 cells. Phase contrast microscopy of fixed cells revealed a morphology stereotypical of procyclic *T. brucei* (Figure 3.8, column 1, 0 hours). Fluorescence microscopy showed most cells to contain a single kinetoplast and a single nucleus (1K, 1N), while a minority possessed 2K, 1N or 2K, 2N DNA content (column 2, 0 hours).

At 4 hours post treatment cells remained very active, but cellular aggregates had increased both in frequency (column 1, 4 hours) and in the number of cells constituting each (column 3, 4 hours). Although an accurate count was not possible, it was estimated that 80-90% of all cells in culture were aggregated. Analysis of cellular DNA content by fluorescence microscopy became more problematic when cells formed large aggregates, as it proved difficult to distinguish individual trypanosomes by phase contrast and to then correlate with the DAPI stained image (columns 3 and 4, 4 hours). Analysis of smaller aggregates and a few non-aggregated cells suggested an increase in the proportion of 2K, 1N and 2K, 2N cells within the population relative to the control, although the number of cells amenable to such scrutiny was sufficiently low as to prevent accurate quantitation.

At 8 hours post treatment cells in culture had settled out of solution, forming what appeared to be a uniform blanket across the bottom of the flask. All cells within the culture were observed to be associated in large aggregates. Despite the fact that cells were aggregated the overall level of physical activity within the culture at this time remained high. Phase contrast microscopy of fixed cells revealed few cells extraneous to aggregates. Where the outline of individual cells could be discerned, morphology

appeared normal. DNA content of individual cells could not be determined at this time (columns 3 and 4, 8 hours).

At 24 hours post treatment most cells in culture were still observed to constitute large aggregates, although a few cells could be observed either individually of as part of smaller aggregates. Physical activity within the culture was markedly reduced with respect to the control and consisted mainly of locomotion of free flagellae, the "bodies" of trypanosomes remaining relatively motionless. Phase contrast microscopy revealed that the majority of free cells displayed a grossly distorted surface (column 1, 24 hours), with multiple flagellae detected on several occasions. Fluorescence microscopy detected a marked increase in ploidy, with some cells possessing 4 discrete nuclei (column 2, 24 hours). Kinetoplasts were more difficult to identify in these cells than had been the case in controls, a phenomenon that could either be the result of an increased nuclear mass obscuring them, or a reduction in number of kinetoplasts relative to nuclei. The "lumpy" cell surface morphology revealed by phase contrast is thought to be related directly to the increased ploidy revealed by DAPI staining .

At 48 hours cultured cells appeared extremely lethargic, although a degree of flagellar movement was detectable in most. Distribution of cells within the culture was not noticeable altered from the 24 hour time-point. Phase contrast microscopy revealed that the majority of cells remained bound in large agglutinated masses, while a minority of free cells could be detected. Few apparently normal cells were observed, the majority tending to have gross morphological defects such as massive blebbing of the cell surface and multiple flagellae. The individual portrayed in column 1 (48 hours) was a prime example, exhibiting 6 detectable flagellae and a distorted cell surface. Fluorescence microscopy of the same cell (column 2) revealed 8 clearly defined nuclei,

a four-fold increase on the normal maximum number. The majority of individual cells observed displayed a similar increase in number of nuclei, possessing either 4 or 8 and indicating that division of nuclear DNA remained a co-ordinated event. DAPI staining of cell clusters (column 4) revealed a huge increase in quantity of DNA relative to earlier time-points (staining procedure and fluorescent exposure were constant for all slides), consistent with the observations made for individual cells.

Observation of cells in culture at 72 hours post treatment with ConA revealed that the large agglutinated masses reported at previous times had now broken down into smaller cell clusters. Physical activity within the culture at this time was scarce, with a minority of trypanosomes displaying any flagellar movement. Phase contrast observation of fixed cells revealed that the morphological abnormalities reported at 48 hours were now detectable in all cells observed. In addition, fluorescence microscopy showed the nuclei of many cells to be less distinct than at previous times, possibly indicating nuclear degradation.

3.3 Discussion

The work carried out in this chapter was based on the premise that procyclic *T. brucei* treated with ConA die by a process akin to apoptosis in metazoan cells (Welburn et al., 1996), and that *de novo* mRNA synthesis continues throughout this process. The aim of this work was to identify transcripts that were differentially expressed during the 72 hour death period, with a view to better understanding the mechanisms and significance of this phenomenon in a protozoan parasite.

Apoptosis in metazoans involves a series of morphological and biochemical changes within the cell, culminating in the removal of specific cells from a population without the induction of bystander death (Wyllie *et al.*, 1980; Duvall and Wyllie, 1986). Stereotypical apoptotic cells become rounded, display a reduction in volume and dissociate from both substrate and neighbouring cells (Kerr *et al.*, 1972). Concurrent condensation and margination of chromatin within the nucleus, blebbing of the plasma membrane and the eventual formation of apoptotic bodies containing degraded cellular components (and often intact organelles) all typify the morphology of apoptotic cell death (Kerr *et al.*, 1972). At the biochemical level, activation of caspases, oligonucleosomal DNA fragmentation and externalisation of phosphatidylserine are all normally associated with this mode of death [although DNA laddering has not been detected in all cases of apoptosis and has been detected in some cases of necrosis (Dong *et al.*, 1997)]. It is this precisely controlled combination of events that differentiates between this and other forms of cell death.

Induction of cell death in the kinetoplastids *Trypanosoma brucei* (Welburn *et al.*, 1996) *Trypanosoma cruzi* (Ameisen *et al.*, 1995) and *Leishmania amazonensis* (Moreira *et al.*, 1996) has been achieved by addition of the tetrameric lectin ConA, starvation and heat

shock respectively, and in each case produced both morphological and biochemical features similar to apoptosis in metazoans. In all three systems cells displayed a loss of mobility and rounding. Condensation and margination of chromatin within the nucleus was also common to all three models. However, while such nuclear morphology has been widely reported in apoptotic cells, the direct relationship between this phenotype and apoptosis is unclear. It has recently been suggested that chromatin condensation may in fact not be actively associated with apoptotic cell death, but rather represents a passive phenomenon associated with a loss of structural integrity of the euchromatin, nuclear matrix and nuclear lamina (Hendzel *et al.*, 1998).

Terminal deoxytransferase-mediated dUTP nick end labelling (TUNEL) and terminal deoxynucleotidyl transferase (TdT) labelling of *T. cruzi* and *T. brucei* respectively revealed DNA fragmentation late in the cell death process of both (Ameisen *et al.*, 1995; Welburn *et al.*, 1996). While these labelling techniques were not carried out in the *L. amazonensis* study, oligonucleosomal DNA fragmentation patterns were detected in both *L. amazonensis* and *T. brucei* (Moreira *et al.*, 1996; Welburn *et al.*, 1996). Interestingly, in both cases the minicircle kDNA remained intact, indicating that nuclease activity was restricted to the nucleus. The integrity of cytoplasmic organelles also remained intact throughout the death process (as determined by light microscopy) in both *T. brucei* and *L. amazonensis*, again in agreement with apoptosis in metazoans. Oligonucleosomal DNA fragmentation was demonstrated in *T. cruzi*, but only after addition of complement. This result was interpreted as being indicative of an apoptotic phenotype, but, as the complement membrane attack complex creates channels through the plasma membrane, degradation of DNA in this experiment could equally have been the result of secondary activation of endonucleases as has been demonstrated in necrotic cells (Dong *et al.*, 1997).

A major difference between the death of cells studied in these three systems was the occurrence of membrane blebbing, a hallmark of apoptosis in metazoans (Kerr *et al.*, 1972). Electron microscopic study of *T. brucei* revealed a degree of plasma membrane vesiculation, but no blebbing (Welburn *et al.*, 1996). Observations of dying *L. amazonensis* similarly failed to detect plasma membrane blebbing (Moreira *et al.*, 1996). In both cases the presence of a rigid cytoskeleton composed of subpellicular microtubules was proposed as a mechanistic barrier for expression of this phenotype. However, the study carried out on *T. cruzi* revealed substantial membrane blebbing in dying cells (Ameisen *et al.*, 1995), negating this hypothesis.

In the current study procyclic *T. brucei* undergoing death induced by ConA were examined by both phase contrast microscopy and DAPI staining. Due to the facts that ConA is a tetrameric lectin that binds mannose residues (Wassef *et al.*, 1985; Solis *et al.*, 1987), and that the N-linked glycan of EP procyclin terminates in a mannose residue (Roditi *et al.*, 1998), it is perhaps not surprising that *T. brucei* thus treated agglutinate. The occurrence of large aggregated masses of trypanosomes made this study more difficult than it may otherwise have been as it rendered the vast majority of cells unavailable for individual analysis. However, at most time-points a minority of cells were observed extraneous to aggregates, and it was these that were observed most closely. Phase contrast microscopy revealed that over time cells developed gross defects in surface morphology, becoming lumpy and swollen. By 48 hours post-treatment multiple flagellae were apparent on many cells. Fluorescence microscopy of DAPI-stained cells revealed multiple nuclei in many at 48 and 72 hours post-treatment. Taken together these results suggest that rather than the cell cycle arrest and cellular shrinkage commonly reported in apoptotic cells, procyclic *T. brucei* treated with ConA

maintain an active cell cycle in the absence of cytokinesis. It would thus appear that *T. brucei* do not possess a cell cycle checkpoint preventing initiation of mitosis in the absence of a previous round of cytokinesis. The lack of a conventional mitosis to cytokinesis checkpoint in *T. brucei* has previously been suggested (Ploubidou *et al.*, 1999). Interestingly, cells at 24 and 48 hours exhibited either 2, 4 or 8 nuclei, indicating that karyokinesis in ConA-treated *T. brucei* remained a co-ordinated event. It would be of interest to determinate whether other cellular components (kinetoplast, parabasal body, mitochondrion, flagellum) were also duplicated with such fidelity in these cells. During the current study it was not possible to quantify kinetoplasts using DAPI staining of cells during the later stages of ConA treatment due to the comparatively large amount of nuclear DNA present. Transition EM, while providing very detailed images, can only give data pertaining to a section through a cell, rendering such an approach inappropriate for the quantitation of organelles. Thus, it is proposed that immunofluorescence microscopy coupled with organelle specific antibodies would be the approach of choice, as cells could be viewed in their entirety and organelles counted. Monoclonal antibodies detecting the *T. brucei* paraflagellar rod (Robinson and Gull, 1991; Bastin *et al.*, 1998; Kohl *et al.*, 1999) basal bodies (Robinson and Gull, 1991) and mitochondrial HSP70 (Tyler *et al.*, 1997) have been used in previous studies with considerable success.

A recent mutagenesis study conducted on procyclic *T. brucei* identified the N-glycan group of EP PARP as a likely target for ConA binding (Hwa *et al.*, 1999). However, agglutination of cells was demonstrated to be non-essential for induction of death, as the dimeric lectin succinyl-ConA, which did not cause cellular agglutination, induced death as efficiently as its tetrameric counterpart (Hwa *et al.*, 1999). Taken together with the findings presented in this chapter, it is proposed that ConA (or succinyl-ConA) binds to

and cross-links the N-glycan residue of EP PARP, creating a mechanistic barrier to cytokinesis. Following several cell cycles without physical division cells enter crisis and die by an as yet undefined process that produces oligonucleosomal fragmentation of DNA. It seems unlikely that this process is akin to apoptosis in metazoans, although a degree of cellular participation cannot currently be ruled out. In light of these findings, significant changes in gene expression throughout the time-course of death would be expected.

All forms of mRNA differential display are prone to artefacts, and differential display PCR is no exception. The PCR reaction can (and often does) produce artefactual bands, and products that are genuinely differentially expressed may co-migrate with species that do not display differential expression. Subsequent re-amplification can then artefactually select for products that formed minority constituents of the original band. Consequently, confirmation of all differential display products is imperative, either by Northern blot or by semi-quantitative RT-PCR.

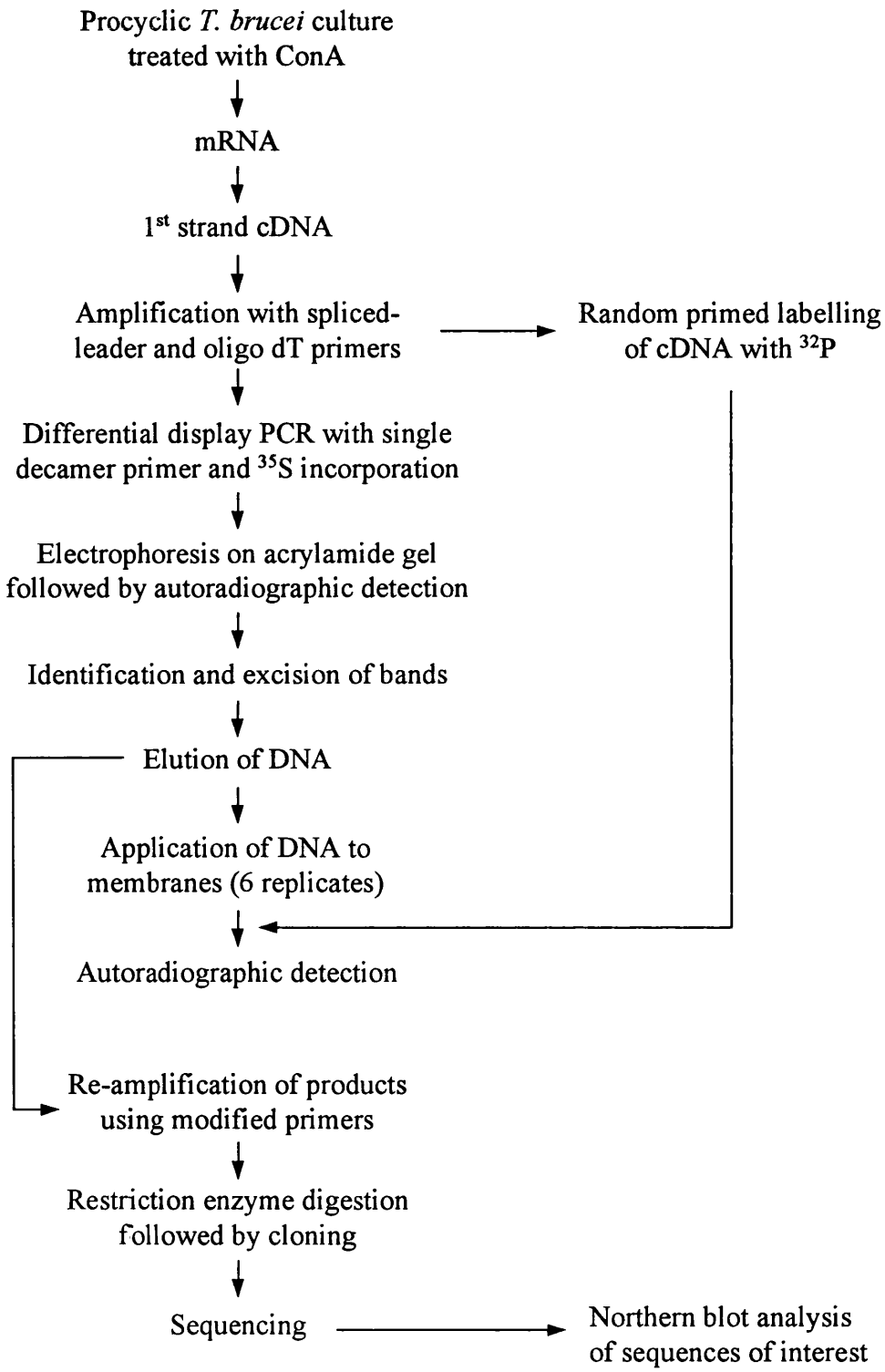
The work carried out in the present study utilised a technique specifically designed for use with kinetoplastids, amplifying first strand cDNA with oligo-dT and spliced-leader specific primers prior to differential amplification with a single arbitrary decamer primer. This meant that minimal template material could be utilised for multiple PCR reactions. An added refinement was the differential amplification of each sample at two different template concentrations, minimising the probability of artefactual bands being identified as differentially expressed. While this safeguard reduced selection of artefactual amplicons to a minimum, re-amplification artefacts were observed as a persistent factor during this study. On several occasions heterologous DNA species were cloned from PCR products thought to constitute a single re-amplified DNA

fragment (as determined by agarose gel electrophoresis). In such cases the cloned insert that approximated the original amplicon most closely was assumed to be the product of interest. However, on two occasions an informed choice was not possible due to the size similarity between products. These results highlight the problems posed by co-migrating bands and artefactual re-amplification of DNA. Furthermore, they raise the possibility that several of the sequences produced during this study may in actuality represent PCR products that were artefactually re-amplified, and not the originally identified amplicon.

Northern blot analysis of 2 genes identified during this study, *QM* and *MOB1*, together with the genes *TRACK* and β-tubulin as controls, failed to substantiate differential expression patterns identified by RADES-PCR. The main drawback faced by this work was the lack of a reliable control with which to compare experimental samples. The *TRACK* transcript had previously been reported to be up-regulated over the time-course of ConA-induced cell death of *T. brucei*, but displayed down-regulation when investigated in the current study. β-tubulin, a transcript commonly used to confirm equal loading on Northern blots, also displayed down-regulation during this study. It was therefore concluded that the behaviour of genes commonly used as controls for Northern blots were likely to be unreliable in dying cells. Coupled with severe time constraints and a lack of template material it was decided that further Northern blot analysis of clones identified by RADES-PCR would not be pursued. Despite not having validated RADES-PCR as a system for identifying transcripts displaying differential expression during cell death, this project proved to be a useful "gene fishing" exercise, identifying a number of novel *T. brucei* genes.

A modification of the RADES-PCR protocol has been envisaged, and is laid out in Figure 3.9. Briefly, the initial stages of the protocol would be as previously with mRNA prepared followed by 1st strand cDNA synthesis and amplification using oligo dT and spliced leader specific primers. Addition of *Pfu* or comparable proof-reading DNA polymerase to the amplification reaction would be of value to ensure representative amplification of larger transcripts. Cycling conditions would have to be altered accordingly. The differential display reaction would be conducted as previously, but ^{35}S incorporated into the reaction mix. PCR products would then be resolved by electrophoresis on a polyacrylamide gel. The resultant increase in resolution should significantly reduce the phenomenon of co-migrating species, and hence artefactual re-amplification, although co-migrating products have been reported as remaining a major cause of artefacts when polyacrylamide gels are utilised (Zhang *et al.*, 1998). Following autoradiographic detection precise sections of the gel would be excised and DNA extracted. This would then be stored at –20°C until required. Once sufficient samples had been accumulated, DNA from each would be spotted onto replicate membranes and hybridised with ^{32}P-labelled cDNA. In this way the expression pattern of selected amplicons could be confirmed prior to the cumbersome tasks of cloning and sequencing. Re-amplification of DNA species of interest would also be modified to further reduce artefacts. Ten to twenty rounds of thermal cycling would be carried out, in contrast to the 40 used previously, reducing the probability of skewing the relative proportions of co-migrating bands and amplicon of interest. Re-amplification would utilise a single primer consisting of (from 5' to 3') 5 random bases, 6 bases corresponding to an *Eco* RI restriction site, and the 10 bases of the decamer primer used in the differential amplification. Restriction enzyme digestion of the entire PCR reaction and precipitation of DNA would precede sticky-end cloning of products into an appropriate plasmid vector. It is recognised that digestion of re-amplification products

Figure 3.9 Flow diagram depicting proposed DDRT-PCR protocol



prior to cloning would, in some cases, result in cleavage within the amplicon rather than purely within the primer sites at the termini. While this phenomenon may reduce the number of products cloned in their entirety, it is thought that the ease of sticky-end cloning (compared to T-vector) combined with the predicted reduction in artefacts make this protocol better than that used in the current study. Sequencing of inserts would be as previously, and Northern blot analysis of genes of interest conducted.

CHAPTER FOUR

Isolation and characterisation of the *Trypanosoma brucei* homologue of the 60S ribosomal protein QM

4.1 Introduction

The *QM* gene was first identified by subtractive hybridisation as a cDNA transcript elevated in a non-tumourigenic Wilm's tumour microcell hybrid relative to the tumourigenic parental cell line (Dowdy *et al.*, 1991). Subsequent Southern blot analysis on both human and mouse DNA indicated that *QM* was a member of a multi-gene family, although to date *QM* is the only member of the family known to be expressed. *QM* cDNAs have now been cloned from 17 eukaryotic species (not including the current work) representing all eukaryotic kingdoms. The human and yeast genes both encode hydrophilic, basic proteins with predicted molecular weights of 24.7 kDa (Dowdy *et al.*, 1991) and 25.4 kDa (Koller *et al.*, 1996) respectively, and which are highly conserved with 63-65% identity over their whole length.

The QM peptide does not contain any obvious membrane spanning or membrane associated regions, nor does it contain any glycosylation sites or active site motifs. A putative nuclear localisation signal is located towards the N-terminus (Dowdy *et al.*, 1991). During a phage display search for proteins that interacted with the transcription factor c-Jun (Jifs) Monteclaro and Vogt (1993) identified the chicken homologue of QM which they named Jif-1. It was reported that Jif-1 could bind c-Jun *in vitro*, and in so doing inhibit the ability of c-Jun to bind DNA. Subsequent work by Inada *et al.* (1997) confirmed the c-Jun/QM interaction and demonstrated that Zn^{2+} ions were required for binding (although QM does not possess any known zinc-binding motif). Jif-1/QM has therefore been proposed to be a negative regulator of the transcription factor c-Jun (Monteclaro and Vogt, 1993), a role that fits well with both its elevation in the non-tumourigenic Wilm's tumour microcell hybrid and its putative nuclear localisation signal.

Tron *et al.* (1995) identified the yeast homologue of *QM* (*QSR1*) during a screen for mutants requiring the otherwise non-essential *QCR6*, a nuclear gene encoding subunit 6 of the cytochrome bc1 complex. *QSR1* was found to encode an essential yeast protein which is concentrated in the cytoplasm, and which appears to be subject to nuclear exclusion. Later work by Dick *et al.* (1997) showed that while QCR6 is retained in the mitochondria of *qsr1-1* mutants, the QSR1 protein remains purely cytosolic. The nature of the relationship between QSR1 and QCR6 remains uncertain, although it has been suggested that other metabolic factors may be involved (Nika *et al.*, 1997). Koller *et al.* (1996) characterised a yeast temperature-sensitive mutant of *GRC5* (*QSR1/QM*), finding aberrant phenotypes related to cell morphology, cell growth and cell proliferation.

Immunofluorescent staining of mammalian cells with antibodies directed against QM reveal a perinuclear cytoplasmic pattern indicative of association with the endoplasmic reticulum (Loftus *et al.* 1997; Nguyen *et al.*, 1998; Mills *et al.*, 1999). Subcellular fractionation experiments carried out in both mammalian cells (Loftus *et al.*, 1997) and yeast (Dick *et al.*, 1997) confirm that QM is localised on the cytoplasmic face of the rough endoplasmic reticulum. Further analysis by Dick *et al.* (1997) revealed that QSR1 was a ribosomal protein peripherally associated with the 60S subunit. Through addition of a hemagglutinin epitope tag to the C-terminus of GRC5, a two-dimensional mobility shift assay of purified 60S ribosomal subunits revealed GRC5 to be the yeast ribosomal protein L9 (Nika *et al.*, 1997). The sequence of GRC5 corresponds to that of the rat ribosomal protein L10 (Chan *et al.*, 1996). Through work with *QSR1* temperature sensitive mutants Eisinger *et al.* (1997) went on to demonstrate that this protein is essential for joining of the 40S and 60S subunits to make a functional 80S

ribosome, with protein synthesis significantly reduced in mutant cells at the restrictive temperature.

Mills *et al.* (1999) analysed QM expression patterns during mouse embryogenesis using whole mount in situ hybridisation and whole mount immunohistochemistry. They observed expression in numerous tissues of the midgestation embryos, which became more restricted throughout development. Moreover, levels of expression were not necessarily highest in actively differentiating cells as had previously been reported (Dowdy *et al.*, 1991) but tended to be linked to cells involved in protein production (with the notable exception of haematopoietic cells). This observation fits well with the ribosomal role of QM in translation, but is contradictory to the earlier hypothesis that QM is a negative regulator of the transcription factor c-Jun - cells that are actively producing protein would be expected to have elevated levels of transcription. It has therefore been proposed by various authors (Loftus *et al.*, 1997; Mills *et al.*, 1999) that the interaction between QM and c-Jun is an *in vitro* artefact and plays no role *in vivo*. This is further supported by the cellular localisation of these proteins, with QM being specifically excluded from the nucleus (Nguyen *et al.*, 1998; Mills *et al.*, 1999).

A trypanosome *QM* gene was isolated during the RADES-PCR screen described in chapter 3. The aim of the work presented in this chapter was to characterise the *Trypanosoma brucei* homologue of *QM*. Southern blot analysis of genomic DNA was carried out in order to determine copy number of the *QM* gene. A recombinant epitope-tagged QM protein was overexpressed in *T. brucei* and the stability/turnover of this protein was investigated by Western blot analysis. Indirect immunofluorescence

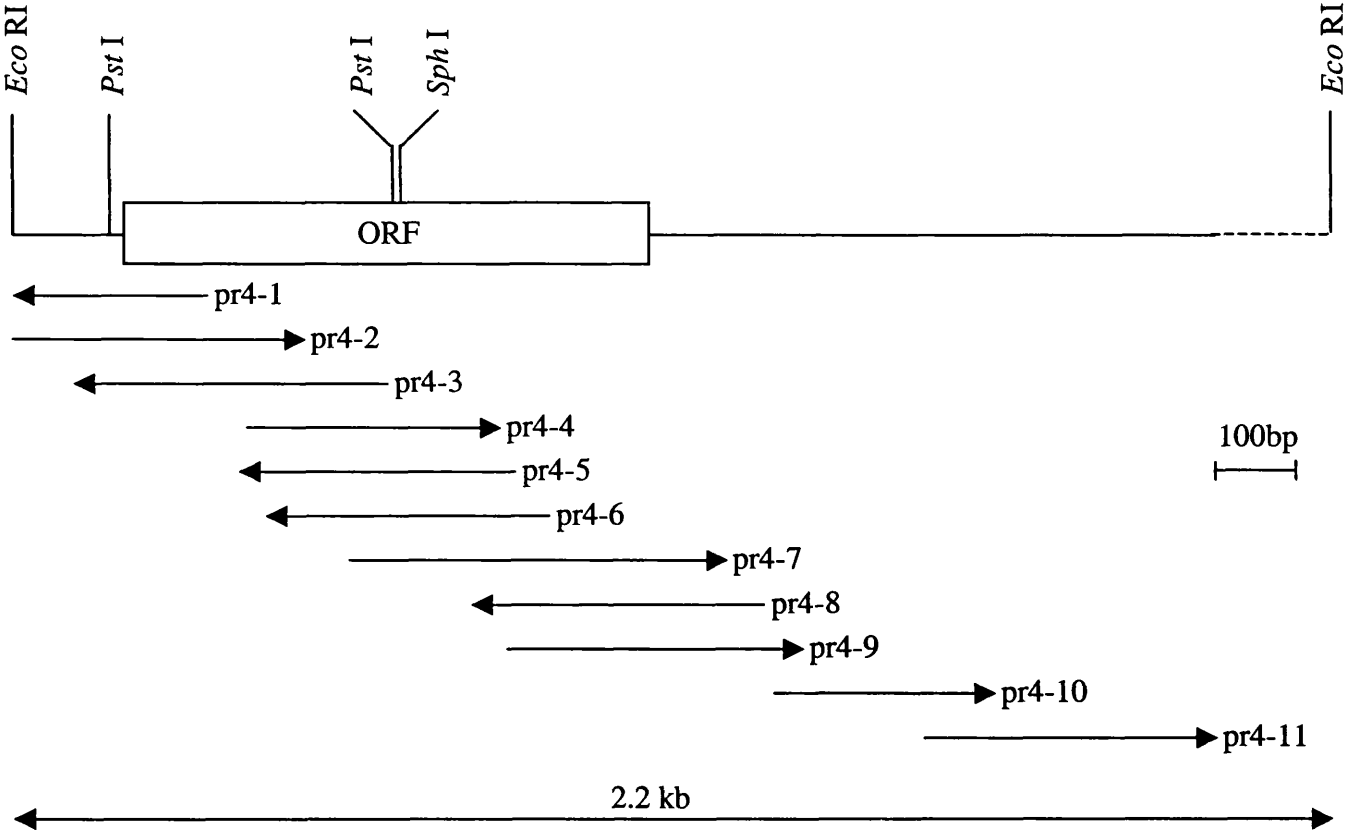
microscopy and subcellular fractionation were used to determine the sub-cellular location of the epitope-tagged QM.

4.2 Results

4.2.1 Isolation and sequence analysis of the *T. brucei QM*

Sequence analysis of clone p1499(5), isolated during a RADES-PCR screen of ConA-treated procyclic trypanosomes (Section 3.2.1), revealed strong homology to the *QM* (*QSR1/GRC5/Jif-1*) gene of other organisms. The full length *T. brucei QM* gene was obtained by screening a *T. brucei* λgt11 library, as described in Section 2.9, using the insert from plasmid p1499(5) as a probe. A 2.2 kb insert was excised from clone λG3QM by restriction digestion with *Eco* RI, sub-cloned into pBluescript to produce plasmid pG3QM and sequenced. The 642 bp open reading frame of the gene was sequenced in both directions in order to improve the accuracy of the data (Figure 4.1). 1553 bp of sequence was obtained from the 2.2 kb clone, and is presented in Figure 4.2 with the position of the RADES product, open reading frame and flanks indicated. Figure 4.3 represents the nucleotide sequence of the *T. brucei QM* open reading frame and the deduced amino acid sequence. The open reading frame of the *T. brucei QM* encodes a protein with a predicted mass of 24.8 kDa, consisting of 213 residues. The protein is rich in highly charged amino acids (23%), and has a predicted isoelectric point of 10.56. These data fit well with earlier reports from human, mouse and yeast (Dowdy *et al.*, 1991; Eisinger *et al.*, 1993; Tron *et al.*, 1995).

Figure 4.1 Schematic representation of *T. brucei* subclone pG3QM



Schematic representation of the *T. brucei QM* gene based on analysis of subclone pG3QM, demonstrating position of overlapping sequences, restriction enzyme sites and open reading frame. Primer sequences (pr4-) are detailed in Appendix II. Dotted line (----) denotes unsequenced region of clone. Diagram (excluding unsequenced region) is to scale.

Figure 4.2 Sequence of *QM* open reading frame and flanks

GAATTCCGTGCACAGTGAATCCTCCATCCATTGTGATGTTAACATAACTTTTTCCTCACAT 61
CTTTCCTCTCTTTCTCTTTGTCTGTACTTTCTTATACTTCTTGTTTCTGCAGTGGTTTGATCG 124
GCCAAAAGATTCGTC**ATGGCTCGCCGTCCCGCACGTTGCTACCGCTTCTGCAAGAAC** 181
AAACCGTATCCTAAGTCACGCTTCTGTCGTGGTGTACCGGACCCGAGGATTCGCACC 238
TTCGACATTGGTAAGCGCCGTGCGCCAGTGGATGAGTTCCCCGTGTGTGTCCATGTT 295
GTGTCCCGTGAACTAGAGCAAATCTCATCTGAGGCATTGGAAGCTGCCCGTATTCAG 352
GCTAACAAGTATATGGTGAAGCGTGCCA<u>ACAAGGAATGTTTCCACATGCGTATCCGC</u> 409
<u>GCCCATCCGTTCCACGTACTTCGCATCAACAAAATGCTTTCGTGTGCTGGCGCCGAT</u> 466
<u>CGTCTGCAGACGGGCATGCGGCAGTCGTACGGCAAACCGAATGGGACCTGCGCCCG</u> 522
<u>CGTGCGGATTGGTCAGATCCTCTTGTCTATGAGGACAAAGGACACATACGTCCCACA</u> 579
<u>GGCACTGGAGTCTCTTCGCCGTGCTAAGATGAAGTTCCCTGGTAGGCAAATCATTGT</u> 636
<u>GATATCGAAATATTGGGGCTTCACAAACATCCTCCGAAATGAGTACGAGGAGTTACG</u> 693
<u>TGACGCAGGGAAGTTGCAACAACGCGGCCTCCACGTGAAGCTGATCACACCAAAGG</u> 749
<u>GTAAGATCACCCCATACAACATCATGGCCTAA</u>ACGACCACGGCTCTTTTGCTCGATTGA 808
GCGTTCATTTCTACTTTTCTGCTGCTTAATGTTCCTTTTAATGATTTTTGCGTTTGTCGTCTT 871
GTGGTGCAACGTTGTTGGACTCTCATGCTGGAACGCAGGGTGTTTGCTAGCATTTATATTT 932
CACTTTTTCTCCTTTTTTTTTCcGCTCTCTCTCTCTCcTTTGcCCCCCCCCCCCTTCAATTTAAC 996
ATACCGACCATCTTTTGATGATATTATGTGTGCTTGCCACTGGTGGAAAGGGgAACCAAA 1056
GGGGCCAACCTTGCGAAAAAATTCCAGGCGGAAGGTGGTCGTACTATCCTCATTGCTTGC 1116
GAACTGTAGTGTCATATTTTGACATTCCATGTACAAGGAGACCACTATGACACCCATACA 1176
TGGGTATACTCTGCCGGTTCCCTTGACAAATGGTGGGTATGCGTCGATGCTGTCCGTCATC 1237
AGGGCTCTTGGGCGGCTTGATCAAACAAGCGAACGTGTCTTTGGTAACATTGAACAGGCT 1297
GTTGAGCGGTCACGCGAAACACTGGAGCAACTTCAGGGACGCATCGCGGTTTGTGCTGAT 1357
CAAGTCGAGCAGCTTCAAGGACGGAGGGAGGCGATGGTGGTGAAGAGTAGGGTTCGATT 1416
TCCAAAACAAAAACACTACACGCTTCCAATAGGGCGCGCACCGTGTGAGGATCGAGGCT 1475
GTGTGCCAAAAAGGGCGTATGCCATGCCAGCAATCATTCAAGCAGATGATTCGGGTGCAT 1535
CACCAGTGGTGGGCAGTA 1553

T. brucei QM gene sequence based on data derived from subclone pG3QM. The open reading frame is in **bold**, the sequence of the RADES product is <u>underlined</u>, and regions flanking the open reading frame are denoted by grey. Numbers on the right refer to base pairs. Letters in lower case denote ambiguity in base identity. The open reading frame was sequenced fully in both directions.

Figure 4.3 Nucleotide and deduced amino acid sequences of the *T. brucei QM* open reading frame

1	ATG	GCT	CGC	CGT	CCC	GCA	CGT	TGC	TAC	CGC	TTC	TGC	
	M	A	R	R	P	A	R	C	Y	R	F	C	12
37	AAG	AAC	AAA	CCG	TAT	CCT	AAG	TCA	CGC	TTC	TGT	CGT	
	K	N	K	P	Y	P	K	S	R	F	C	R	24
73	GGT	GTA	CCG	GAC	CCG	AGG	ATT	CGC	ACC	TTC	GAC	ATT	
	G	V	P	D	P	R	I	R	T	F	D	I	36
109	GGT	AAG	CGC	CGT	GCG	CCA	GTG	GAT	GAG	TTC	CCC	GTG	
	G	K	R	R	A	P	V	D	E	F	P	V	48
145	TGT	GTC	CAT	GTT	GTG	TCC	CGT	GAA	CTA	GAG	CAA	ATC	
	C	V	H	V	V	S	R	E	L	E	Q	I	60
181	TCA	TCT	GAG	GCA	TTG	GAA	GCT	GCC	CGT	ATT	CAG	GCT	
	S	S	E	A	L	E	A	A	R	I	Q	A	72
217	AAC	AAG	TAT	ATG	GTG	AAG	CGT	GCC	AAC	AAG	GAA	TGT	
	N	K	Y	M	V	K	R	A	N	K	E	C	84
253	TTC	CAC	ATG	CGT	ATC	CGC	GCC	CAT	CCG	TTC	CAC	GTA	
	F	H	M	R	I	R	A	H	P	F	H	V	96
289	CTT	CGC	ATC	AAC	AAA	ATG	CTT	TCG	TGT	GCT	GGC	GCC	
	L	R	I	N	K	M	L	S	C	A	G	A	108
325	GAT	CGT	CTG	CAG	ACG	GGC	ATG	CGG	CAG	TCG	TAC	GGC	
	D	R	L	Q	T	G	M	R	Q	S	Y	G	120
361	AAA	CCG	AAT	GGG	ACC	TGC	GCC	CGC	GTG	CGG	ATT	GGT	
	K	P	N	G	T	C	A	R	V	R	I	G	132
397	CAG	ATC	CTC	TTG	TCT	ATG	AGG	ACA	AAG	GAC	ACA	TAC	
	Q	I	L	L	S	M	R	T	K	D	T	Y	144
433	GTC	CCA	CAG	GCA	CTG	GAG	TCT	CTT	CGC	CGT	GCT	AAG	
	V	P	Q	A	L	E	S	L	R	R	A	K	156
469	ATG	AAG	TTC	CCT	GGT	AGG	CAA	ATC	ATT	GTG	ATA	TCG	
	M	K	F	P	G	R	Q	I	I	V	I	S	168
505	AAA	TAT	TGG	GGC	TTC	ACA	AAC	ATC	CTC	CGA	AAT	GAG	
	K	Y	W	G	F	T	N	I	L	R	N	E	180
541	TAC	GAG	GAG	TTA	CGT	GAC	GCA	GGG	AAG	TTG	CAA	CAA	
	Y	E	E	L	R	D	A	G	K	L	Q	Q	192
577	CGC	GGC	CTC	CAC	GTG	AAG	CTG	ATC	ACA	CCA	AAG	GGT	
	R	G	L	H	V	K	L	I	T	P	K	G	204
613	AAG	ATC	ACC	CCA	TAC	AAC	ATC	ATG	GCC	TAA			
	K	I	T	P	Y	N	I	M	A	*			214

The nucleotide sequence (black) and deduced amino acid sequence (red) of the *T. brucei QM* open reading frame are shown. The position of the nucleotides is displayed in the left margin, and the position of the amino acid residues is displayed in the right margin.

The open reading frame of the *T. brucei QM* was used to search databases for homology using the BLAST programme. Homologous genes from other organisms were identified and peptide sequences acquired for comparative alignment using the MegAlign programme [DNASTAR, Madison, Wisconsin, USA]. The resultant alignment between the trypanosome QM and homologues from 17 other species was produced without the introduction of gaps and is displayed in Figure 4.4. The extremely high level of conservation between the sequences of this gene from different organisms is immediately evident, with greater than 60% peptide identity between *Trypanosoma brucei*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Drosophila melanogaster* and *Homo sapiens* (Table 4.1). Not surprisingly the intraspecific conservation within phylogenetically distinct groups such as the kinetoplastids, plants or mammals is markedly higher than the interspecific conservation between such groups. As has previously been reported the high level of conservation displayed by this gene is concentrated in the N-terminal 175 residues of the protein (Farmer *et al.*, 1994), with a total of 74 amino acids (42%) demonstrating complete conservation between the 18 species analysed. The C-terminal region of the protein, which spans between 38 and 53 residues, demonstrates a far higher degree of divergence and contains only two residues conserved throughout the species analysed.

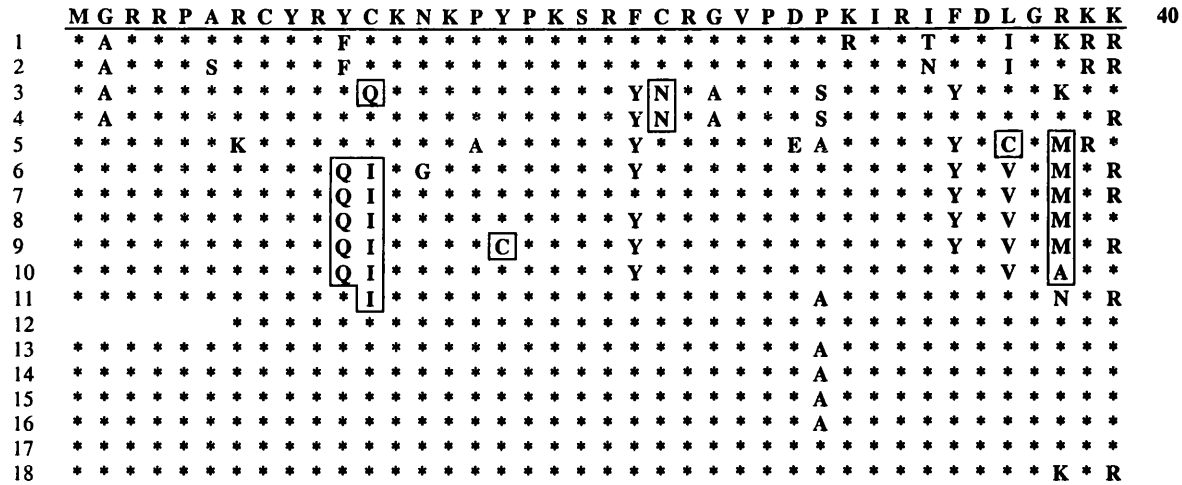
Conserved motifs within the QM protein were sought using the *T. brucei* peptide sequence as template in conjunction with both PROSITE (http://expasy.hcuge.ch/sprot/prosite.html) and MOTIF (http://www.motif.genome.ad.jp) programmes. A putative protein kinase C phosphorylation site thus identified, consisting of S-x-R and situated between residues 137-139 inclusive, was conserved in all 18 species and is indicated in Figure 4.4. This fits well with the reported ability of protein kinase C to

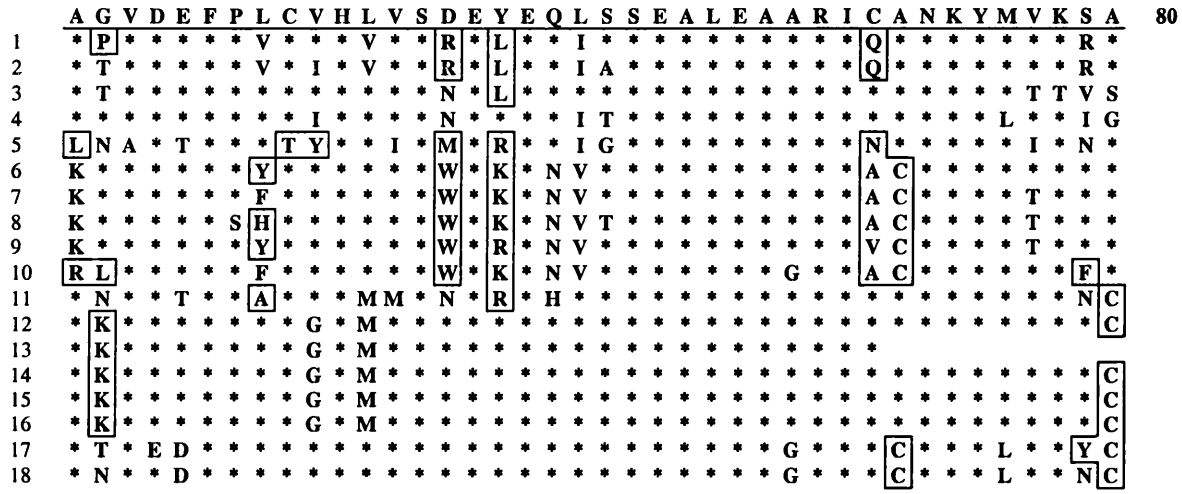
Figure 4.4 Alignment of QM peptide sequence from diverse species

The peptide sequences of QM homologues from diverse organisms were obtained by homology search using the BLAST programme. Consensus sequence runs along the top. Numbers in the left margin denote sequences from:

(1) *Trypanosoma brucei*
(2) *Leishmania mexicana* [4493745]
(3) *Saccharomyces cerevisiae* [1172812]
(4) *Schizosaccharomyces pombe* [4107323]
(5) *Euglena gracilis* [2500352]
(6) *Arabidopsis thaliana* [4262180]
(7) *Solanum melongena* [2500354]
(8) *Oryza sativa* [2500353]
(9) *Zea mays* [1172809]
(10) *Pinus taeda* [2317762]
(11) *Caenorhabditis elegans* [1172807]
(12) *Gallus gallus* [1172808]
(13) *Sus scrofa* [2500351]
(14) *Bos taurus* [4929242]
(15) *Mus musculus* [1172810]
(16) *Homo sapiens* [5174431]
(17) *Drosophila melanogaster* [3123839]
(18) *Bombyx mandarina* [4063389]

Numbers in the right margin denote position of the terminal residue. Residues that match the consensus exactly are denoted by *. Non-conservative substitutions are boxed. Gaps within the sequence represent missing or ambiguous data. The motif highlighted in red denotes a conserved putative protein kinase C phosphorylation site. Numbers in parentheses (above) denote protein sequence identifiers (http://www.ncbi.nlm.nih.gov).


M G R R P A R C Y R Y C K N K P Y P K S R F C R G V P D P K I R I F D L G R K K 40
1 * A * * * * * * * F * * * * * * * * * * * * * * * R * * T * * I * K R R
2 * A * * * S * * * * F * * * * * * * * * * * * * * * * * N * * I * * R R
3 * A * * * * * * * * Q * * * * * * * * Y N * A * * * S * * * * Y * * * K * *
4 * A * * * * * * * * * * * * * * * * * Y N * A * * * S * * * * * * * * * * R
5 * * * * * * K * * * * * * * * A * * * * Y * * * * * E A * * * * Y * C * M R *
6 * * * * * * * * * Q I * G * * * * * * Y * * * * * * * * * * Y * V * M * R
7 * * * * * * * * * Q I * * * * * * * * * * * * * * * * * * * Y * V * M * R
8 * * * * * * * * * Q I * * * * * * * * Y * * * * * * * * * * Y * V * M * *
9 * * * * * * * * * Q I * * * * C * * * * Y * * * * * * * * * * Y * V * M * R
10 * * * * * * * * * Q I * * * * * * * * Y * * * * * * * * * * * V * A * *
11 * * * * * * * * * * I * * * * * * * * * * * * * * * A * * * * * * * N * R
12 *
13 * A * * * * * * * * * *
14 * A * * * * * * * * * *
15 * A * * * * * * * * * *
16 * A * * * * * * * * * *
17 *
18 * K * R


A G V D E F P L C V H L V S D E Y E Q L S S E A L E A A R I C A N K Y M V K S A 80
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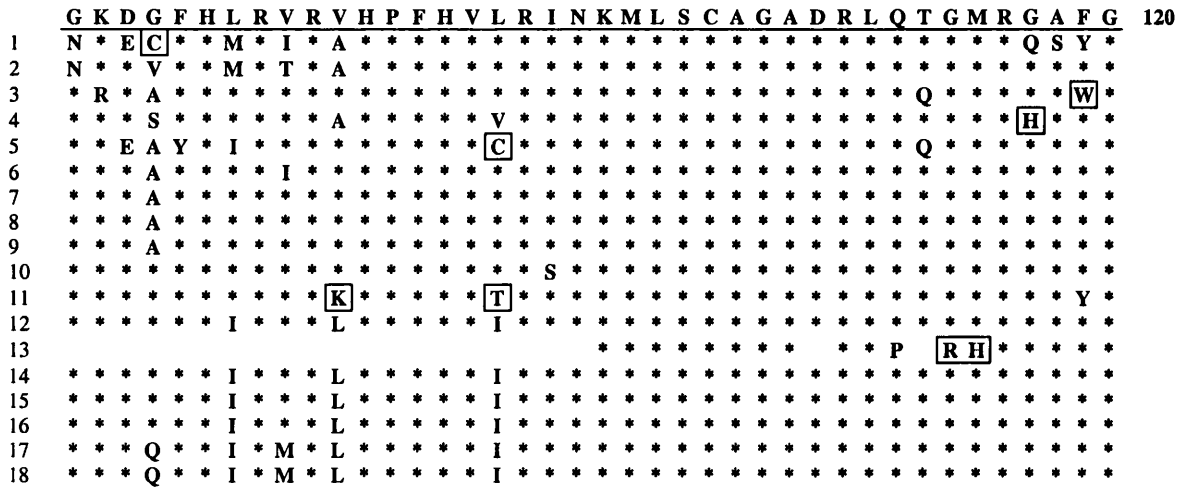
2 * T * * * * * V * I * V * * R * L * * I A * * * * * * * * * Q * * * * * * R *
3 * T * * * * * * * * * * * N * L * * * * * * * * * * * * * * * * * T T V S
4 * * * * * * * * I * * * N * * * * I T * * * * * * * * * * * * * L * * I G
5 L N A * T * * * T Y * * I * M * R * * I G * * * * * * * * * N * * * * * I * N *
6 K * * * * * * Y * * * * * W * K * N V * * * * * * * * * A C * * * * * * * *
7 K * * * * * * F * * * * * W * K * N V * * * * * * * * * A C * * * * T * * *
8 K * * * * * S H * * * * * W * K * N V T * * * * * * * * A C * * * * T * * *
9 K * * * * * * Y * * * * * W * R * N V * * * * * * * * * V C * * * * T * * *
10 R L * * * * * F * * * * * W * K * N V * * * * * * G * * A C * * * * * * F *
11 * N * * T * * A * * * M M * N * R * H * * * * * * * * * * * * * * * * N C
12 * K * * * * * * * G * M * C
13 * K * * * * * * * G * M *
14 * K * * * * * * * G * M * C
15 * K * * * * * * * G * M * C
16 * K * * * * * * * G * M * C
17 * T * E D * G * * * C * * * L * * Y C
18 * N * * D * G * * * C * * * L * * N C


G K D G F H L R V R V H P F H V L R I N K M L S C A G A D R L Q T G M R G A F G 120
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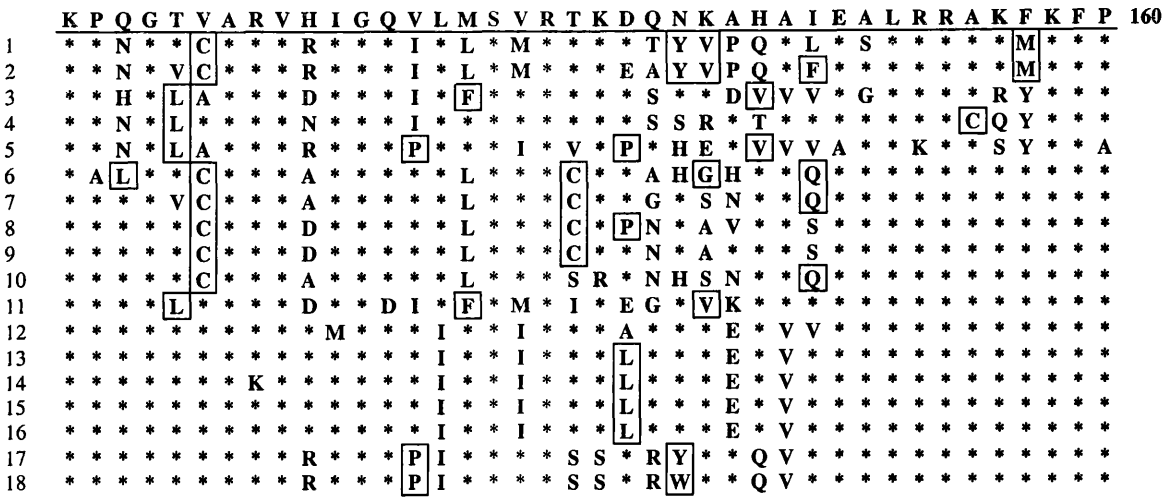
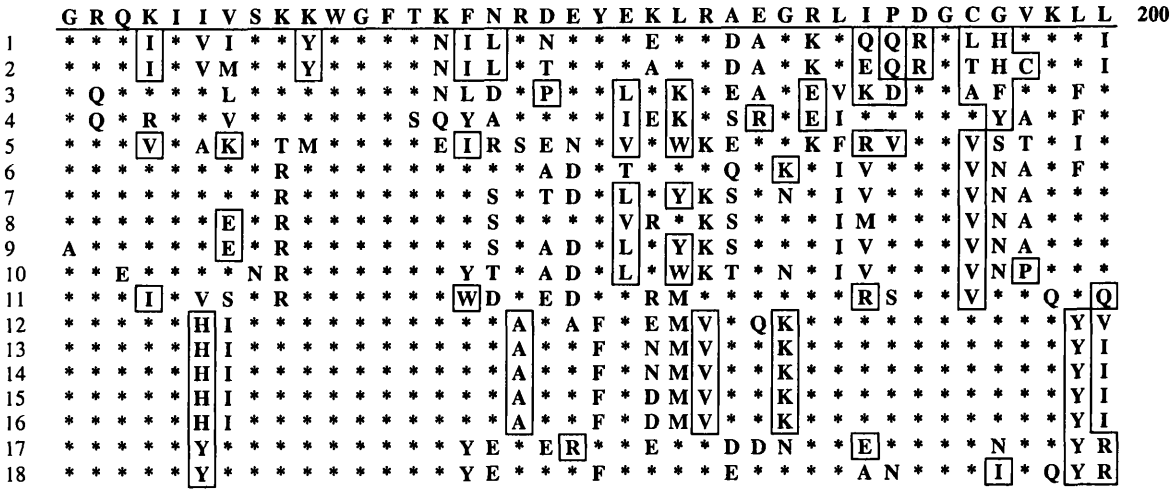
2 N * * V * * M * T * A *
3 * R * A * Q * * * * W *
4 * * * S * * * * * A * * * * V * * * * * * * * * * * * * * * * H * * *
5 * * E A Y * I * * * * * * * * C * * * * * * * * * * * * * * Q * * * * * *
6 * * * A * * * * I *
7 * * * A *
8 * * * A *
9 * * * A *
10 * * * * * * * * * * * * * * * * * S * * * * * * * * * * * * * * * * * *
11 * * * * * * * * * K * * * * T * * * * * * * * * * * * * * * * * * Y *
12 * * * * * I * * * L * * * * I *
13 * P R H * * * * *
14 * * * * * I * * * L * * * * I *
15 * * * * * I * * * L * * * * I *
16 * * * * * I * * * L * * * * I *
17 * * * Q * * I * M * L * * * * I *
18 * * * Q * * I * M * L * * * * I *


K P Q G T V A R V H I G Q V L M S V R T K D Q N K A H A I E A L R R A K F K F P 160
1 * * N * * C * * * R * * * I * L * M * * * * * T Y V P Q * L * S * * * * * M * * *
2 * * N * V C * * * R * * * I * L * M * * * E A Y V P Q * F * * * * * * * M * * *
3 * * H * L A * * * D * * * I * F * * * * * * * S * * D V V V * G * * * * R Y * * *
4 * * N * L * * * * N * * * I * * * * * * * * * S S R * T * * * * * * * C Q Y * * *
5 * * N * L A * * * R * * * P * * * I * V * P * H E * V V V A * * K * * S Y * * A
6 * A L * * C * * * A * * * * * L * * * C * * A H G H * * Q * * * * * * * * * * *
7 * * * * V C * * * A * * * * * L * * * C * * G * S N * * Q * * * * * * * * * * *
8 * * * * * C * * * D * * * * * L * * * C * P N * A V * * S * * * * * * * * * * *
9 * * * * * C * * * D * * * * * L * * * C * * N * A * * * S * * * * * * * * * * *
10 * * * * * C * * * A * * * * * L * * * S R * N H S N * * Q * * * * * * * * * * *
11 * * * * L * * * * D * * D I * F * M * I * E G * V K * * * * * * * * * * * * * *
12 * * * * * * * * * * M * * * I * * I * * * A * * * E * V V * * * * * * * * * * *
13 * * * * * * * * * * * * * * I * * I * * * L * * * E * V * * * * * * * * * * * *
14 * * * * * * * K * * * * * * I * * I * * * L * * * E * V * * * * * * * * * * * *
15 * * * * * * * * * * * * * * I * * I * * * L * * * E * V * * * * * * * * * * * *
16 * * * * * * * * * * * * * * I * * I * * * L * * * E * V * * * * * * * * * * * *
17 * * * * * * * * * R * * * P I * * * * S S * R Y * * Q V * * * * * * * * * * * *
18 * * * * * * * * * R * * * P I * * * * S S * R W * * Q V * * * * * * * * * * * *


G R Q K I I V S K K W G F T K F N R D E Y E K L R A E G R L I P D G C G V K L L 200
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2 * * * I * V M * * Y * * * * N I L * T * * * A * * D A * K * E Q R * T H C * * I
3 * Q * * * * L * * * * * * * N L D * P * * L * K * E A * E V K D * * A F * * F *
4 * Q * R * * V * * * * * * S Q Y A * * * * I E K * S R * E I * * * * Y A * F *
5 * * * V * A K * T M * * * * E I R S E N * V * W K E * * K F R V * * V S T * I *
6 * * * * * * * * R * * * * * * * * * A D * T * * Q * K * I V * * * V N A * F *
7 * * * * * * * * R * * * * * * S * T D * L * Y K S * N * I V * * * V N A * * *
8 * * * * * * E * R * * * * * * S * * * * V R * K S * * * I M * * * V N A * * *
9 A * * * * * E * R * * * * * * S * A D * L * Y K S * * * I V * * * V N A * * *
10 * * E * * * * N R * * * * * * Y T * A D * L * W K T * N * I V * * * V N P * * *
11 * * * I * V S * R * * * * * * W D * E D * * R M * * * * * R S * * V * * Q * Q
12 * * * * * H I * * * * * * * * * * A * A F * E M V * Q K * * * * * * * * * Y V
13 * * * * * H I * * * * * * * * * * A * * F * N M V * * K * * * * * * * * * Y I
14 * * * * * H I * * * * * * * * * * A * * F * N M V * * K * * * * * * * * * Y I
15 * * * * * H I * * * * * * * * * * A * * F * D M V * * K * * * * * * * * * Y I
16 * * * * * H I * * * * * * * * * * A * * F * D M V * * K * * * * * * * * * Y I
17 * * * * * Y * * * * * * * * * Y E * E R * * E * * D D N * * E * * * N * * Y R
18 * * * * * Y * * * * * * * * * Y E * * * F * * * * E * * * A N * * * I * Q Y R

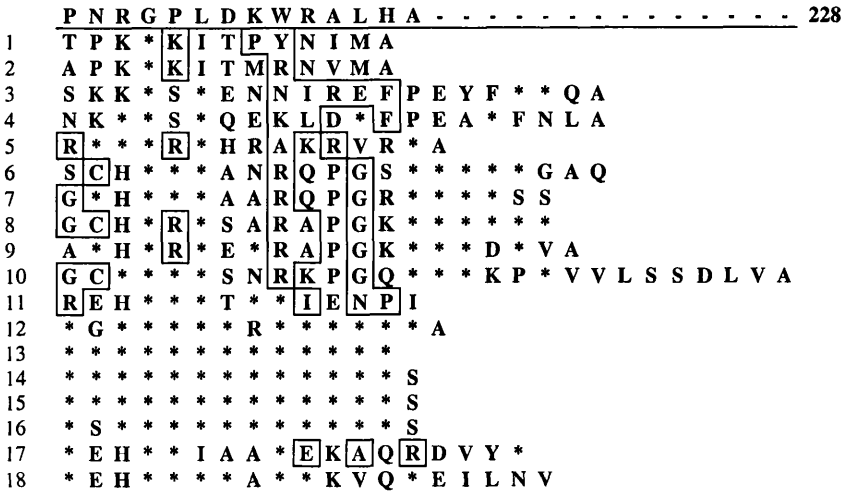

P N R G P L D K W R A L H A - - - - - - - - - - - - - - - - 228
1 T P K * K I T P Y N I M A
2 A P K * K I T M R N V M A
3 S K K * S * E N N I R E F P E Y F * * Q A
4 N K * * S * Q E K L D * F P E A * F N L A
5 R * * * R * H R A K R V R * A
6 S C H * * * A N R Q P G S * * * * * G A Q
7 G * H * * * A A R Q P G R * * * * S S
8 G C H * R * S A R A P G K * * * * *
9 A * H * R * E * R A P G K * * * D * V A
10 G C * * * * S N R K P G Q * * * K P * V V L S S D L V A
11 R E H * * * T * * I E N P I
12 * G * * * * * R * * * * * * A
13 * * * * * * * * * * * * *
14 * * * * * * * * * * * * S
15 * * * * * * * * * * * * S
16 * S * * * * * * * * * * S
17 * E H * * I A A * E K A Q R D V Y *
18 * E H * * * * A * * K V Q * E I L N V

Table 4.1 Percentage peptide identity between QM homologues from diverse species

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
2	85																
3	61	61															
4	61	64	71														
5	54	56	60	58													
6	61	63	63	63	59												
7	61	64	64	64	61	88											
8	60	63	64	64	61	85	88										
9	60	62	65	63	61	86	89	90									
10	58	61	61	61	59	82	85	81	81								
11	63	64	64	63	59	66	69	67	69	66							
12	60	61	63	66	59	65	66	66	65	65	69						
13	50	51	54	55	49	55	56	55	55	54	59	78					
14	61	63	65	67	60	66	68	67	67	66	72	93	84				
15	61	63	65	68	60	67	68	68	67	66	73	94	84	99			
16	61	63	65	68	60	67	68	68	67	66	73	94	83	99	99		
17	63	65	63	66	57	67	67	65	65	67	68	76	64	77	77	77	
18	62	63	64	66	60	66	67	65	66	67	71	77	66	79	79	79	88

Table indicating percentage identity between QM homologues from 18 different species, as calculated by the Megalign programme. Numbers in the margins denote:

(1) *Trypanosoma brucei*

(2) *Leishmania mexicana* [4493745]

(3) *Saccharomyces cerevisiae* [1172812]

(4) *Schizosaccharomyces pombe* [4107323]

(5) *Euglena gracilis* [2500352]

(6) *Arabidopsis thaliana* [4262180]

(7) *Solanum melongena* [2500354]

(8) *Oryza sativa* [2500353]

(9) *Zea mays* [1172809]

(10) *Pinus taeda* [2317762]

(11) *Caenorhabditis elegans* [1172807]

(12) *Gallus gallus* [1172808]

(13) *Sus scrofa* [2500351]

(14) *Bos taurus* [4929242]

(15) *Mus musculus* [1172810]

(16) *Homo sapiens* [5174431]

(17) *Drosophila melanogaster* [3123839]

(18) *Bombyx mandarina* [4063389]

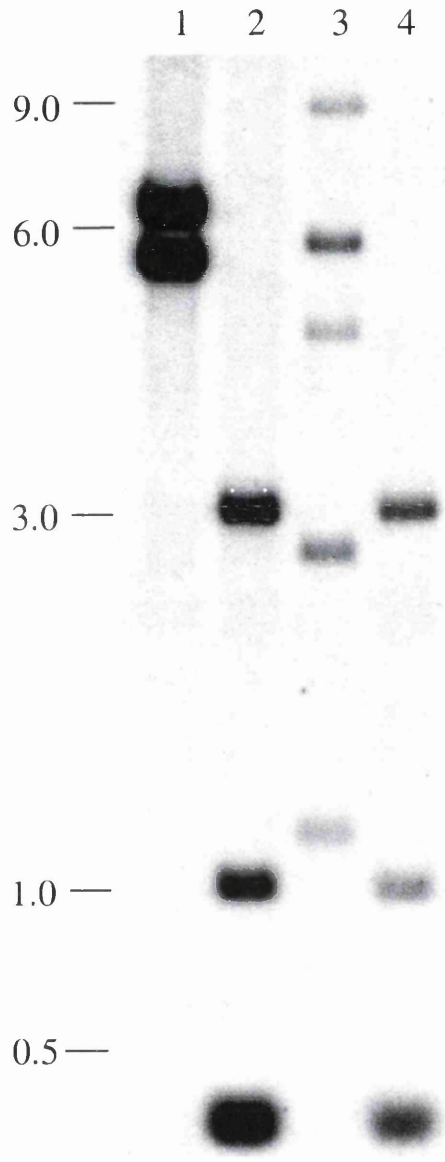
phosphorylate QM *in vitro* (Inada *et al.*, 1997). Despite the high level of conservation demonstrated by this gene no other conserved motifs were found.

4.2.2 Southern blot analysis of *T. brucei* QM

In order to determine the number of copies of *QM* in the *T. brucei* genome 10 μg genomic DNA was subjected to double digestion with *Bam* HI/*Bgl* II, *Bgl* II/*Pst* I, *Bgl* II/*Sph* I or *Pst* I/*Sph* I. Following agarose gel electrophoresis Southern blots were hybridised with a ^{32}P-labelled probe, derived from the insert of plasmid pTyQMGEM-2, (Section 4.2.3) which constituted the open reading frame of *QM*. Sequence analysis revealed that *Pst* I cut 27 bases 5' of the open reading frame and again at base 334 of the open reading frame. *Sph* I cut at base 344 of the open reading frame. No sites are evident for either *Bam* HI or *Bgl* II within the 1553 bases sequenced (Figure 4.1). Signal was detected autoradiographically and is presented in Figure 4.5.

Digestion with *Bam* HI/*Bgl* II produced DNA fragments of 6.3 and 5.8 kb (lane 1) indicating the presence of either two copies of *QM* in the genome or a single copy gene with variable flanks. Digestion with *Bgl* II/*Pst* I produced DNA fragments of 3.1, 1.0 and 0.36 kb (lane 2). Sequence analysis predicted the presence of the 360 base pair fragment (see above). The remaining fragments are the result of either *Bgl* II or *Pst* I cutting at the denoted distances 3' of the open reading frame, and as above indicate either that there are two copies of the *QM* gene or that there is a single copy with further variation in the flanking region. Digestion with *Pst* I/*Sph* I (lane 4) produced fragments of the same size as resulted from the *Bgl* II/*Pst* I digest, indicating that in both cases the banding pattern was the result of *Pst* I digestion alone. *Bgl* II sites must therefore lie outwith the *Pst* I sites. Digestion with *Bgl* II/*Sph* I (lane 3) produced fragments of 9.0,

Figure 4.5 Southern blot analysis of *T. brucei* QM



Southern blot analysis of *T. brucei* DNA demonstrating the presence of multiple DNA fragments hybridising to the probe. 10 µg of genomic DNA was subjected to double digestion with (1) *Bam* HI/*Bgl* II, (2) *Bgl* II/*Pst* I, (3) *Bgl* II/*Sph* I or (4) *Pst* I/*Sph* I as denoted, blotted, and hybridised with a ^{32}P-labelled probe derived from plasmid pTyQMGEM-2 and representing the open reading frame of the gene. Markers denote DNA fragment size in kilobases.

5.9, 4.8, 2.7 and 1.5 Kb. As *Sph* I cuts once within the open reading frame (see above) the data suggest the *T. brucei* genome contains are two copies of *QM*, with variation for *Sph* I in one of the flanks of one of these genes.

4.2.3 Introduction of an epitope tag sequence to the *T. brucei QM*

In order to gain insight into the function of the *T. brucei QM* and its location within the cell, it was decided to add an epitope tag to the protein. Inducible overexpression in the appropriate *T. brucei* cell line could then be carried out and the behaviour of the epitope-tagged protein under various conditions monitored. Due to time constraints this approach was preferable to the production of a recombinant QM protein and subsequent raising of polyclonal antisera. The epitope tag used in this study was a 10 amino acid section from the major structural protein of the *Saccharomyces cerevisiae* Ty1 virus-like particle. Two monoclonal antibodies are available which recognise this epitope, and which have been used in conjunction with trypanosomes without cross-reaction (Bastin *et al.*, 1996).

Based on the reduced conservation of residues in the C-terminus of QM proteins compared to the N-terminus (Figure 4.4), coupled with the fact that a previous study had produced functional GRC5 with a C-terminal HA tag (Nika *et al.*, 1997), it was decided that introduction of the Ty epitope tag sequence would be appropriate at the 3' end of the *QM* gene. Oligonucleotides pr4-12 and pr4-13 were designed for this purpose and are detailed in Figure 4.6. PCR amplification of the *QM* gene contained in plasmid pG3QM was carried out using *Pfu* proof reading DNA polymerase [Stratagene] in order to minimise the probability of introduced errors.

Figure 4.6 Oligonucleotide sequences for introduction of Ty epitope tag into *T. brucei QM*, and schematic representation of PCR product

Forward primer (pr4-12)

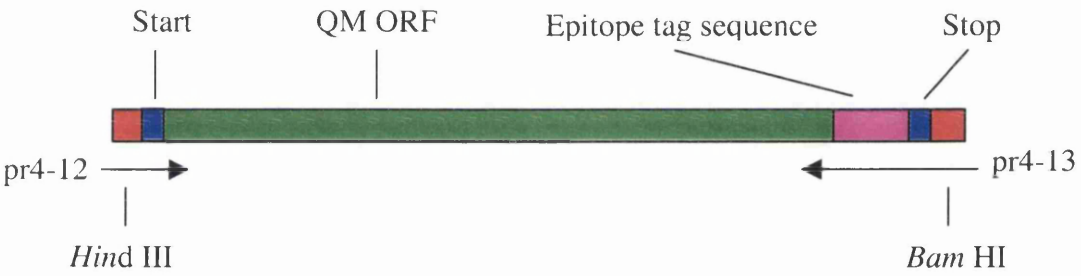
5'AGAAAAGCTTATGGCTCGCCGTCCCGCACG 3'

Reverse primer (pr4-13)

5'GTAGAGGATCCTTAGTCAAGTGGATCCTGGTTAGTATGGACCTCGGCCTAG

ATGTTGTATGGGGTG 3'

PCR product



Oligonucleotide sequences for the introduction of a Ty epitope tag into the *T. brucei QM* was based upon sequence analysis of the open reading frame of the gene (section 4.2.1) and the published sequence of the Ty1 epitope (Bastin *et al.*, 1996). Regions in red denote restriction enzyme sites [*Hind* III in forward and *Bam* HI in reverse], regions in blue denote the start and stop codon, the region in pink denotes sequence encoding the Ty epitope tag and regions in green denote regions matching the *QM* gene sequence respectively.

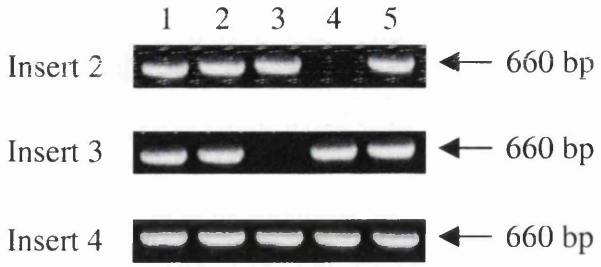
Following PCR amplification T-vector cloning was facilitated by addition of dATP to the product. The single product was cloned into pGEM®-T Easy vector, and ligations used to transform competent JM109 cells. Following blue/white colour selection four

white colonies were grown overnight in LB medium supplemented with 50 μg/ml ampicillin.

Plasmid clones pTyQMGEM 1-4 were digested with *Bam* HI and *Hind* III in order to release the insert for sub-cloning into the expression vector, then subjected to agarose gel electrophoresis. Clone pTyQMGEM 1 failed to produce an insert, while clones pTyQMGEM 2-4 all produced an insert of the predicted size. Inserts were excised from the gel and purified. Plasmid pHD675 (Biebinger *et al.*, 1997) was digested with *Bam* HI and *Hind* III, then subjected to agarose gel electrophoresis together with uncut plasmid in order to confirm that digestion was complete. Plasmid DNA was excised and purified. Quantitation of purified fragments (both pHD675 and inserts) was by agarose gel electrophoresis with known amounts of DNA ladder. Inserts from pTyQMGEM 2-4 were ligated with pHD675 using a Rapid DNA Ligation Kit [Boehringer Mannheim] as directed by the manufacturer. Competent JM109 were transformed, plated on LB agar plates supplemented with 50 μg/ml ampicillin and cultured overnight.

Five colonies from each plate were screened for the presence of insert by colony PCR using the oligonucleotides pr4-12 and pr4-13 (Figure 4.6). Results are shown in Figure 4.7. All but 2 clones (insert 2 clone 4 and insert 3 clone 3) produced PCR products of the predicted size.

Figure 4.7 Colony PCR for plasmids containing epitope-tagged *QM* insert



PCR amplification (pr4-12 and pr4-13) of inserts from 5 colonies (1-5) derived from ligation of inserts from pTyQMGEM 2-4 into pHD675. These clones will henceforth be referred to as pTyQMHD675 (2-4)(clones 1-5).

Sequence data derived from plasmid stocks pTyQMGEM 2-4 was of insufficient quality to determine with certainty whether errors had been introduced during PCR amplification of the epitope tagged insert. However, sequence analysis did reveal a significant flaw in the amplification and cloning approach employed thus far. The DNA sequence of the Ty tag detailed in Figure 4.6 was found to have a *Bam* HI site within it. Consequently, the pTyQMHD675 plasmids, which had been cloned using *Bam* HI, were all found to be missing a portion of the epitope tag and the stop codon. In order to correct the defect two oligonucleotides were designed (Figure 4.8) encoding the missing region of the epitope tag, the stop codon, a unique *Xho* I site and *Bam* HI ends.

Figure 4.8 Oligonucleotides designed for replacement of 3' end of Ty-tag

Oligonucleotide pr4-14

5' GAT CCA CTT GAC TAA CTC GAG G 3'

3' GT GAA CTG ATT GAG CTC CCT AG 5'

Oligonucleotide pr4-15

Oligonucleotides used to insert part of the Ty tag and stop codon into pTyQMHD675-2 clone 1. Pink denotes the fragment of the Ty tag absent in pTyQMHD675-2 clone 1. Blue denotes the stop codon. Black denotes the unique *Xho* I site. At each ends are overhangs compatible with *Bam* HI digested DNA.

In order to anneal oligonucleotides pr4-14 and 4-15 together, 200 µl dH_2O containing 1 nanomole of each oligonucleotide was heated to 96°C for 5 minutes and then allowed to cool to room temperature overnight. Ligations were set up containing 15 ρmol, 5 ρmol and 0.5 ρmol annealed oligonucleotides with 20 ng *Bam* HI linearised pTyQMHD675-2 clone 1. A control ligation was set up consisting of *Bam* HI linearised pTyQMHD675-2 clone 1 without oligonucleotides. Ligations were used to transform competent JM109, and cells were plated on LB agar supplemented with 50 µg/ml ampicillin.

The plate corresponding to the control ligation produced more than 100 colonies while those corresponding to the experimental ligations produced around 40 each. This result had been predicted as the non-phosphorylated oligonucleotide doublet was expected to reduce ligation efficiency. 30 colonies from the 15 ρmol plate were screened for presence of insert in the correct orientation by colony PCR using pr4-15 and pr4-16 as primers. Following agarose gel electrophoresis of PCR products five colonies were determined to be positive. These were cultured overnight in LB broth supplemented

with 50 μg/ml ampicillin and plasmid DNA prepared as described previously. pHD675 contains a unique *Not* I site 858 bases upstream of the multiple cloning site, and pTyQMHD675-2 clone 1 containing the oligonucleotide doublet had a unique *Xho* I site immediately downstream of the *QM* open reading frame. Accordingly, plasmids were digested with *Not* I/*Xho* I to confirm oligonucleotide insertion. Agarose gel electrophoresis demonstrated that all 5 colonies produced an insert of the predicted size. Sequencing of one of these clones (henceforth referred to as pTyQMHD675-2 clone 1-1) confirmed presence of the oligonucleotide doublet in the correct orientation.

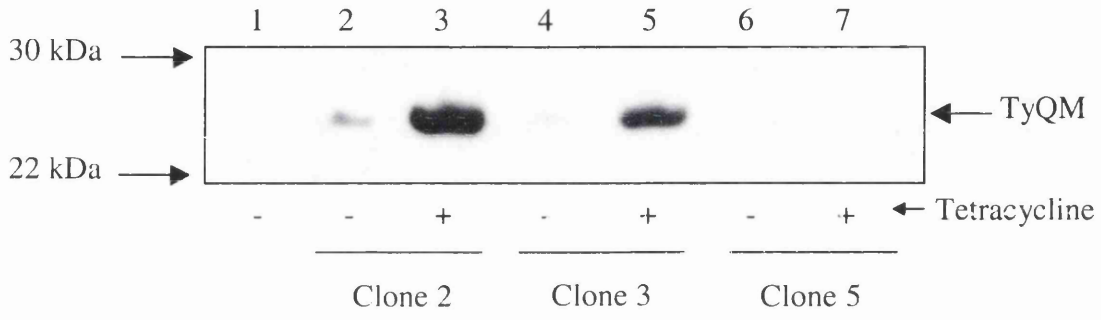
Plasmid pTyQMHD675-2 clone 1-1 was prepared as described previously, linearised by digestion with *Not* I, cleaned twice with phenol/chloroform then ethanol precipitated. Following resuspension in sterile dH_2O DNA was quantified spectrophotometrically at 260 nm. Linearised plasmid was subjected to agarose gel electrophoresis together with uncut control in order to determine whether digestion was complete. Linear plasmid was then used to transfect *Trypanosoma brucei brucei* stock EATRO 795 stably transfected with plasmid construct pHD 449 (Biebinger *et al.*, 1997)(kindly supplied by J. Hellemond). Following overnight recovery populations were cloned by limiting dilution with appropriate antibiotic selection.

4.2.4 Inducible expression of Ty-tagged QM in *T. brucei*

Six tTyQM cell lines from a 96-well plate survived antibiotic selection and were therefore deemed clonal. Phenotypic analysis was carried out on clones tTyQM-2, -3 and -5. Following overnight induction with 20 ng/ml tetracycline as described in section 2.16, 1 x 10^8 cells were harvested and whole cell lysate prepared. Protein from 1 x 10^7 trypanosomes was subjected to SDS-PAGE and Western blotting, using the

mouse monoclonal BB2 α-Ty as the primary antibody (Bastin *et al.*, 1996) and α-mouse IgG/HRP conjugate [Promega] as secondary antibody. Detection of antibody was through use of the SuperSignal® system [Pierce].

Figure 4.9 Immunoblot analysis of procyclic clonal populations stably transfected with pTyQMHD675-2 clone 1-1



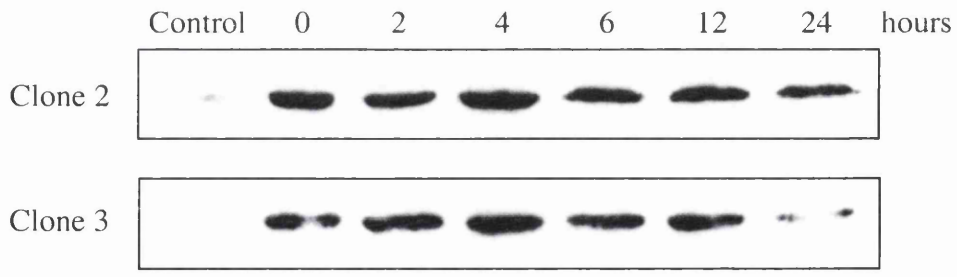
Immunoblot of total protein from 1 x 10^7 procyclic trypanosomes / lane probed with monoclonal antibody BB2. Cells were (1) untransfected control, (2) tTyQM-2 minus induction, (3) tTyQM-2 with induction, (4) tTyQM-3 minus induction, (5) tTyQM-3 with induction, (6) tTyQM-5 minus induction and (7) tTyQM-5 with induction.

The predicted size of the TyQM protein is 26 kDa. As can be seen in Figure 4.9 tTyQM clones 2 and 3 both produced protein which was detected at this size, and produced significantly higher levels of tagged protein in the induced cells compared to uninduced cells. The BB2 antibody did not cross react with proteins from untransfected control cells (lane 1), confirming previously published results (Bastin *et al.*, 1996). Clonal population 5 did not produce any tagged protein in either induced or non-induced cells (lanes 6 and 7).

Stability of TyQM *in vivo*

In order to determine the stability of the Ty-tagged QM protein within procyclic trypanosomes, tTyQM clones 2 and 3 were induced to produce protein overnight by addition of 20 ng/ml tetracycline. Cells were harvested, washed once with fresh SDM 79 to remove tetracycline then resuspended at 1 x 10^6 cells/ml in fresh complete SDM 79. At timepoints 0, 2, 4, 6, 12 and 24 hours after removal of tetracycline 1 x 10^8 cells were harvested and frozen at –20°C for temporary storage. Control cells were of the same cell line as experimental cells but were not exposed to tetracycline induction. Protein was subjected to electrophoresis, Western blotting and immunodetection as described previously.

Figure 4.10 Turnover of TyQM protein within procyclic cells over time



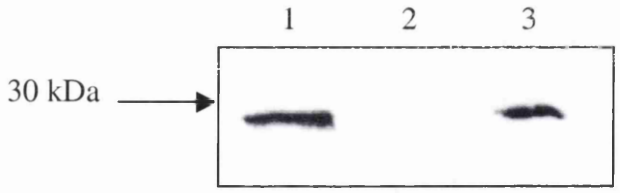
tTyQM clones 2 and 3 were induced overnight, harvested, washed and resuspended at 1 x 10^6 cells/ml in fresh complete SDM 79. At timepoints 0, 2, 4, 6, 12 and 24 hours after removal of tetracycline 1 x 10^8 cells were harvested and frozen at –20°C for temporary storage. Protein equivalent to 1 x 10^7 trypanosomes was subjected to immunoblot analysis. Controls were from the same clonal line as experimental cells, but were not subjected to tetracycline induction.

The TyQM protein expressed by tTyQM clones 2 and 3 appears very stable (assuming *de novo* synthesis of tagged protein ceases shortly after removal of tetracycline), with no detectable reduction for 12 hours following removal of induction signal (Figure 4.10). It should be noted that an equal volume of cells were harvested at each time-point. Consequently, the reduced level of protein observed in both clones at 24 hours may in part be due to dilution of tagged protein rather than turnover, as cell density had increased almost 3-fold by this time-point.

Cell fractionation analysis of TyQM protein

tTyQM clone 2 cells were induced to express protein overnight by addition of 20 ng/ml tetracycline. 2 x 10^8 cells were harvested, pelleted and resuspended in 1 ml LSGI for 30 minutes on ice. Lysate was centrifuged at 100 000 x g for 45 minutes at 4°C in a Beckman Optima™ TL Ultracentrifuge. Supernatant was aspirated and stored for later use and the pellet washed 4 times with 1 ml LSGI before being resuspended in 75 µl 0.25% Triton-X 100 and 25 µl 4 x Laemmli buffer. Protein from total cell extract, the soluble fraction and the insoluble fraction equivalent to 5 x 10^6 cells was subjected to protein gel electrophoresis then Western blotting and immunodetection with α-Ty monoclonal antibody BB2.

Figure 4.11 Cell fractionation analysis of TyQM protein



tTyQM clone 2 cells were induced to express TyQM protein overnight by adition of 20 ng/ml tetracycline. Cells were harvested, lysed in LSGI then fractionated by ultracentrifugation at 100 000 x g. Protein equivalent to 5 x 10^6 trypanosomes from (1) total cell lysate, (2) soluble fraction and (3) insoluble fraction was subjected to immunoblot analysis with the BB2 α-Ty antibody.

The tagged QM protein was found to be present in total cell lysate (lane 1) and the insoluble fraction (lane 3), but not in the soluble fraction (lane 2) resulting from the S100 fractionation (Figure 4.11). As LSGI contains 1% Triton-X 100, membranes throughout the trypanosome should be dispersed during lysis, effectively making soluble all cellular constituents not part of large complexes. Following ultracentrifugation these soluble components should remain within the supernatant, while large protein complexes such as ribosomes form the pellet. The fact that the TyQM locates purely in the pellet suggests that it is part of such a large complex.

4.2.5 Immunofluorescence microscopic analysis to determine subcellular location of the TyQM protein

Despite the fact that tTyQM clone 2 gave the higher level of induced expression (Figure 4.9, lane 3) it was decided that immunofluorescence work focus on tTyQM clone 3, based on its lower level of background expression (Figure 4.9, lane 4). Immunofluorescence microscopy was carried out as described in section 2.18 using the mouse BB2 (α-Ty) as primary antibody, and goat α-mouse IgG/FITC conjugate [Sigma] as secondary antibody. Because of the predicted association between QM and the endoplasmic reticulum colocalisation was carried out using a primary antibody against *T. brucei* BiP, an Hsp70 family member located in the lumen of the endoplasmic reticulum (Bangs *et al.*, 1993; Matlack *et al.*, 1999). The BiP primary antibody was derived from rabbits (Bangs *et al.*, 1993), so goat α-rabbit/TRITC conjugate [Sigma] was used as the secondary antibody. In both cases primary antibody was used at a dilution of 1:50, while secondary antibody was used at 1:100. Control cells were treated with secondary antibody only.

The TyQM protein displayed a cytoplasmic distribution with distinct nuclear and kinetoplast exclusion (Figure 4.12, tiles 3 and 5). The punctate pattern is suggestive of endoplasmic reticulum (ER) localisation. Comparable levels of TyQM were observed in cells representing all stages of the cell cycle (as determined by nucleus and kinetoplast content). One cell was observed which did not express protein detected by the BB2 α-Ty antibody (Figure 4.12, tile 3, white arrow), indicating that tTyQM clone 2 cells were no longer clonal at the time of this experiment. BiP displayed a similar distribution throughout the cell to that demonstrated by TyQM, although the two were not a perfect match (tiles 4 and 6). Furthermore, BiP was not as completely excluded

Figure 4.12 Immunolocalisation of TyQM protein in *T. brucei* with BiP

Fixed permeabilised procyclic cells were treated with mouse α-Ty and rabbit α-BiP as primary antibodies, then goat α-mouse/FITC conjugate and goat α-rabbit/TRITC conjugate as secondary antibodies. DAPI was added to cells during incubation with secondary antibody. Tiles are;

(1) phase contrast

(2) DAPI

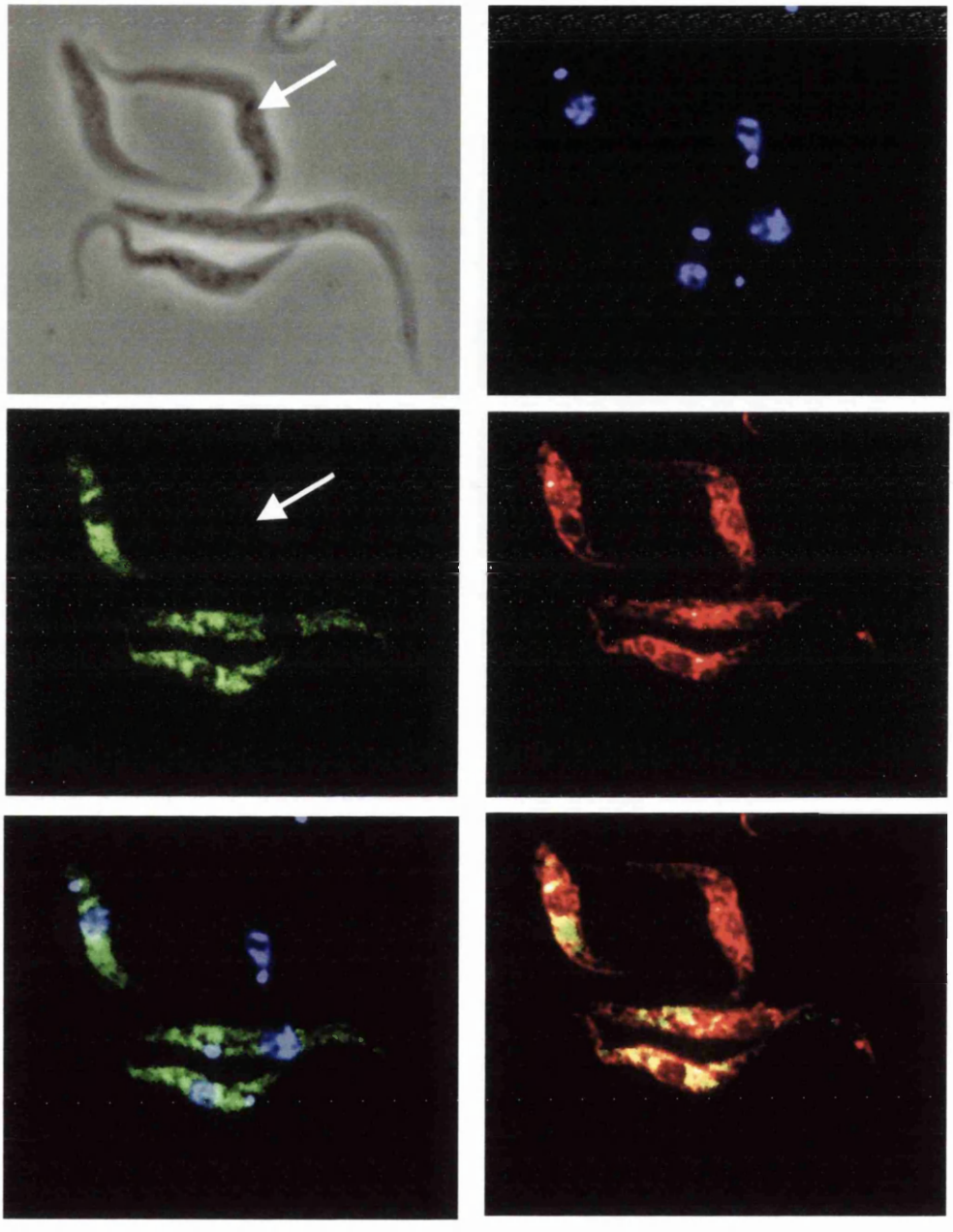
(3) BB2 α-Ty

(4) α-BiP

(5) BB2 α-Ty merged with DAPI

(6) BB2 α-Ty merged with α-BiP

White arrow indicates trypanosome which does not express protein detected by BB2 α-Ty antibody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from nucleus or kinetoplast as was the case for QM, although in most cells its levels appeared lower in these regions. The lack of co-localisation between the TyQM and BiP was possibly the result of QM being concentrated in ribosomes on the cytoplasmic face of the rough ER while BiP was ER luminal. Alternatively, it is possible that due to overexpression the stable tagged QM protein (Figure 4.10) accumulated in the cytoplasm and that the pattern of expression observed was not related to ER localisation. However, were this the case a more homogenous distribution of protein throughout the cytoplasm of the cell would be expected, rather than the distinctly punctate pattern observed. In order to further investigate these possibilities a second round of co-localisation was carried out with antibody raised to the *Leishmania mexicana* ER associated protein, GPI8 (kindly supplied by J. Hilley). The α-GPI8 is a polyclonal antibody raised in rabbits against a recombinant *L. mexicana* GPI8 protein. While the specificity of this antibody has not been fully characterised it is known to detect a *T. brucei* protein of 36 kDa, the predicted size of the *L. mexicana* GPI8. Preparation of samples for immunofluorescence was exactly as described previously, with rabbit α-GPI8 and BB2 used as primary antibodies.

The cytoplasmic distribution of Ty-tagged protein was found to be the same as previously described (Figures 4.12 and 4.13), with nuclear and kinetoplast exclusion (Figure 4.13, tiles 3 and 5). The fluorescent signal produced by the α-GPI8 antibody overlapped to a large degree the TyQM (tiles 3, 4 and 6), indicating clearly that the target of the α-GPI8 antibody co-localises very closely with the TyQM. This result suggests that the pattern observed for TyQM is not the result of random aggregation of protein within the cytoplasm. However, it does raise the possibility that the α-GPI8 antibody is cross-reacting with over-expressed TyQM. To investigate this possibility

Figure 4.13 Immunolocalisation of TyQM protein in *T. brucei* with GPI8

Fixed permeabilised procyclic cells were treated with mouse α-Ty and rabbit α-GPI8 as primary antibodies, then goat α-mouse/FITC conjugate and goat α-rabbit/TRITC conjugate as secondary antibodies. DAPI was added to cells during incubation with secondary antibody. Tiles are;

(1) phase contract

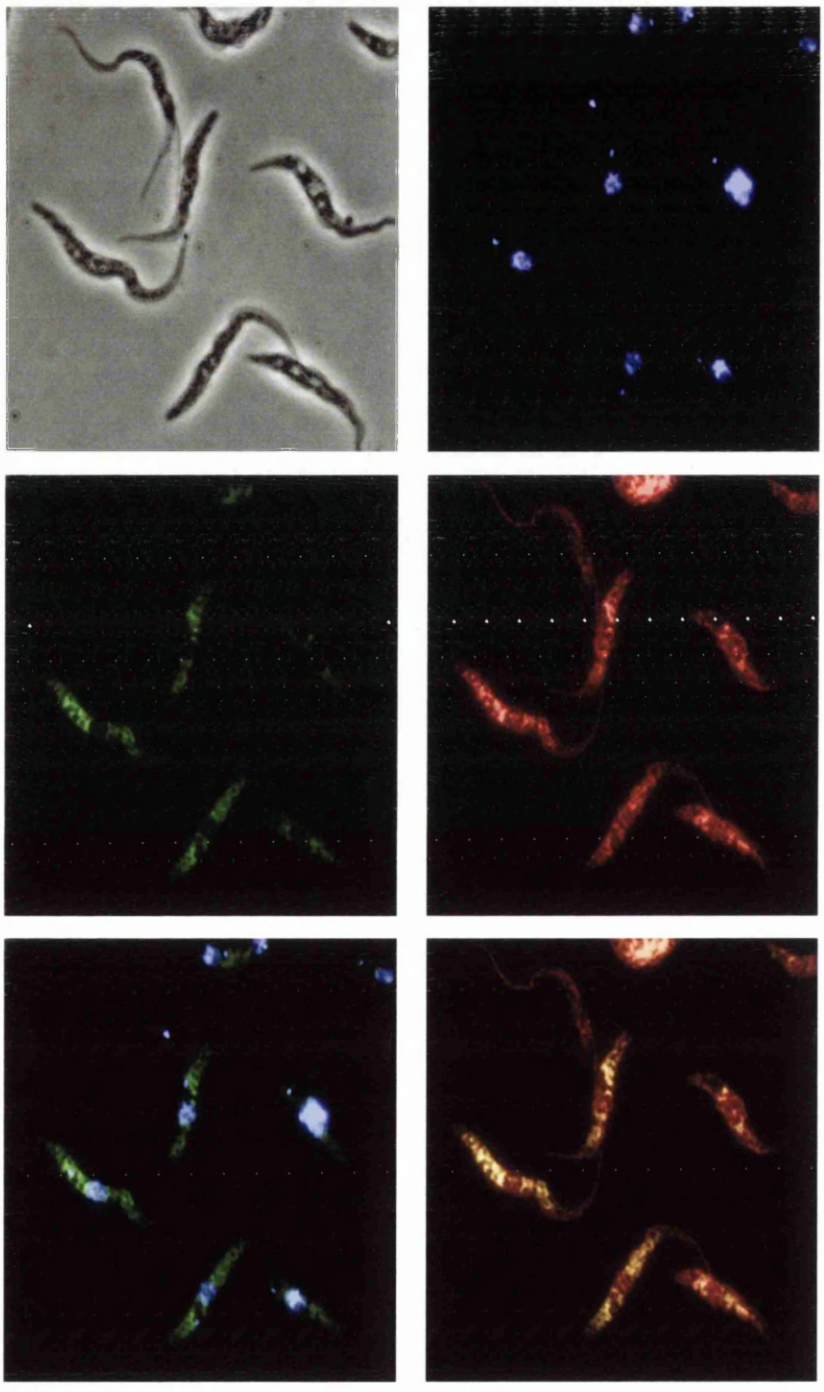
(2) DAPI

(3) BB2 α-Ty

(4) α-GPI8

(5) BB2 α-Ty merged with DAPI

(6) BB2 α-Ty merged with α-GPI8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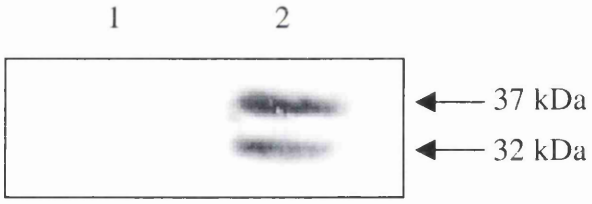
the two fractions from the QM S100 assay (Figure 4.11) were subjected to immunoblot analysis, using the α-GPI8 as primary antibody, and α-rabbit/HRP conjugate as secondary. This resulted in detection of protein at approximately 32 and 37 kDa in the insoluble fraction (Figure 4.14, lane 2) as compared to the single band of 26 kDa previously reported for TyQM (Figure 4.11). Unless the fixation conditions employed for immunofluorescence microscopy result in alternative TyQM epitope presentation, the α-GPI8 antibody does not cross-reacting with the TyQM. This result does not rule out cross-reaction between the α-GPI8 and another ribosomal protein, or ribosome-associated protein.

Figure 4.14 Immunoblot analysis of TyQM S100 assay with α-GPI8 antibody



Soluble (lane 1) and insoluble (lane 2) fractions from the TyQM S100 assay (Figure 4.11) were subjected to immunoblot analysis with rabbit α-GPI8 antibody raised against recombinant *Leishmania mexicana* GPI8 protein. Secondary antibody was goat α-mouse/HRP conjugate.

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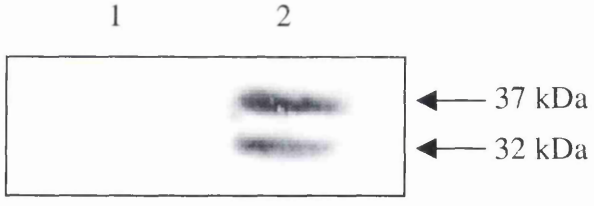

Soluble (lane 1) and insoluble (lane 2) fractions from the TyQM S100 assay (Figure 4.11) were subjected to immunoblot analysis with rabbit α-GPI8 antibody raised against recombinant *Leishmania mexicana* GPI8 protein. Secondary antibody was goat α-mouse/HRP conjugate.

4.3 Discussion

At the time of its discovery by Dowdy *et al.* (1991) *QM* was a novel gene with no sequence homologues on any DNA databases. During the intervening eight years 17 sequences with extremely high homology to the human *QM* have been published on various databases, and span vertebrates, invertebrates, plants and yeast. The human *QM* encodes a 214 amino acid basic protein (Dowdy *et al.*, 1991) with a predicted pI of 10.5 and a predicted mass of 24 kDa (Stanbridge *et al.*, 1994). In agreement with these data the *T. brucei* homologue of the human *QM* gene encodes a 213 amino acid protein with a predicted pI 10.56 and a predicted mass of 24.6 kDa .

Multiple alignment of all published QM sequences confirmed the extremely high conservation displayed by this gene throughout evolution. Sequence analysis revealed the presence of a conserved putative protein kinase C phosphorylation site in the middle of the protein, but despite the presence of several blocks of complete identity between diverse organisms this was the only conserved motif detected. Furthermore, as had previously been reported for the human QM (Farmer *et al.*, 1994) no matches were found between domains of the *T. brucei* homologue and any other non-QM proteins currently lodged on mainstream public databases. While the data suggest that the function of the QM protein is likely to have been conserved throughout evolution, the sequence of the gene itself does not give any indication as to what that function may be.

Southern blot analysis of both human and mouse genomes demonstrated *QM* to be part of a multigene family (Dowdy *et al.*, 1991). By contrast, the *Drosophila* and yeast homologues of QM are single copy genes (Tron *et al.*, 1995: Koller *et al.*, 1996: Nguyen *et al.*, 1997). Sequence analysis of three additional members of the human *QM*

family showed that all were the result of retrotranspositional events, that all maintained the complete *QM* open reading frame and that at least one had a bias against mutations resulting in codon change (Stanbridge *et al.*, 1994). However, there is currently no tangible evidence to suggest that any but the originally identified *QM* is expressed.

Southern blot analysis of the *T. brucei* genome has revealed evidence in favour of the presence of 2 copies of the *QM* gene. There is currently no evidence to suggest whether both copies of the gene are actively transcribed, or whether one represents a pseudogene or a homologue. While sequencing of additional lambda clones could be used for immediate clarification of the situation, the *T. brucei* sequencing projects currently underway are likely to resolve the issue before long.

In the current work the *T. brucei QM* was amplified by PCR using a primer incorporating the Ty1 epitope tag into its 3' terminus, cloned into an inducible expression vector and used to transfect procyclic trypanosomes expressing the tetracycline repressor protein (tet^r). Following antibiotic selection and cloning, cells were induced to express protein by addition of tetracycline. Three clones were investigated, of which 2 produced significantly higher levels of protein when induced compared to uninduced controls. The variation in levels of inducible expression between clonal populations is likely to be the result of a combination of factors. Firstly, constitutive expression of the tet^r protein is likely to impose a metabolic burden on cells. While the presence of the zeocin resistance gene within the same construct allows for initial selection of successfully transfected trypanosomes, continuing zeocin selection will only act to preserve the integrity of the zeocin resistance gene, and not of the construct as a whole. Thus, over time a disparity may arise in levels of tet^r protein

produced by trypanosomes within the same culture. This in turn will result in differing levels of 'leaky' expression of the protein of interest (in this case TyQM), as differing levels of tet^r production between different clones will result in various levels of promoter suppression. Higher levels of non-induced expression may then result in a second metabolic burden, selecting for cells which either possess tighter control of construct expression, or which manage to eliminate physically or functionally part of the construct encoding the protein of interest while retaining the hygromycin resistance gene. This hypothesis fits well with the observed lack of TyQM expression exhibited by tTyQM clone 5 (Figure 4.9, lanes 6 and 7), and the single trypanosome in Figure 4.12 which is plainly visible under phase contrast, DAPI and α-BiP, but which is not detected by α-Ty.

Once expressed the TyQM protein was demonstrated to be very stable over a 24 hour period in both clones investigated. As the cell cycle of exponentially growing *T. brucei* procyclic cells is around 8.5 hours (Matthews and Gull, 1994), turnover of TyQM is not cell cycle regulated. This data is in stark contrast to a Ty-tagged version of the *T. brucei* cell cycle-regulated cyclin 2, TyCyc2, which was demonstrated to be stable for 6 hours but completely degraded by the proteosome within 9 hours post induction (J. Van Hellemond, personal communication).

The Western blot analysis of TyQM stability was non-quantitative. In order to investigate the half-life of TyQM *in vivo* a pulse-chase experiment would have to be carried out, whereby cells would be incubated in medium containing ^{35}S-methionine during overnight tetracycline induction. Following removal of tetracycline/^{35}S-methionine, cells would be harvested at time-points as previously and TyQM

immunoprecipitated. Quantitation would be with a liquid scintillation counter. Negative control cells would be incubated in ^{35}S-methionine without tetracycline induction.

Work on QSR1, the yeast homologue of QM, demonstrated that the protein encoded by this gene was associated with 60S ribosomes (Dick *et al.*, 1997). Concurrent work by another research group confirmed this result and went on to show that QSR1p associates peripherally on the cytoplasmic face of the rough endoplasmic reticulum (Loftus *et al.*, 1997). Subsequently it was discovered that the QSR1p is required for joining of the 60S subunit to its 40S counterpart to form a functional 80S ribosome. Furthermore, mutation of QSR1p did not result in failure of the entire 60S subunit to assemble (as is the case for most 60S ribosomal protein subunits), consistent with QSR1p being a peripheral 60S ribosomal protein (Eisinger *et al.*, 1997). Immunofluorescent analysis of QM expression in both yeast and mammalian cells has clearly demonstrated that the gene product has a punctate perinuclear localisation within the cytoplasm, with distinct nuclear exclusion (Loftus *et al.*, 1997: Nguyen *et al.*, 1998: Mills *et al.*, 1999). This localisation pattern is indicative of association with the endoplasmic reticulum. The nuclear exclusion observed for the QM protein is inconsistent with its proposed function as a transcription factor, but is consistent with a protein added at a late stage of 60S ribosome assembly (core ribosomal proteins are assembled in the nucleolus (Woolford, 1991: Mélèse and Xue, 1995)). It is now suspected that the interaction between QM and c-Jun was an *in vitro* artefact (Loftus *et al.*, 1997: Mills *et al.*, 1999).

Indirect immunofluorescence microscopy was carried out on procyclic *T. brucei* induced to produce TyQM. A punctate distribution of protein throughout the cytoplasm

was observed with distinct nuclear and kinetoplast exclusion. This observation confirms previous immunofluorescence studies of QM at the cellular level in both mammals (Nguyen *et al.*, 1998: Mills *et al.*, 1999) and yeast (Loftus *et al.*, 1997) where a perinuclear staining pattern and nuclear exclusion were observed. The similarity between these observations alone is enough to suggest that the tagged *T. brucei* QM is localising to the endoplasmic reticulum. If, as suspected, this protein is interacting with ribosomes, the nuclear exclusion observed mirrors the behaviour of homologues in other species, being added to the ribosome at a late stage of assembly (Nguyen *et al.*, 1998).

Additional evidence for a ribosomal location was provided by subcellular fractionation studies. Cells induced to produce tagged protein were lysed using a buffer containing 1% Triton X-100, following which lysates were subjected to ultracentrifugation at 100 000 x g. Immunoblot analysis of the fractions revealed the TyQM to be present in only the insoluble phase, as had been described in previous studies with both mammalian and yeast cells (Loftus *et al.*, 1997: Dick *et al.*, 1997). As cell membranes are dispersed by the lysis buffer, an association between the TyQM and a large protein complex is suggested. While this result fits well with the observations of QM associating with ribosomes, it could equally indicate association with another large protein complex or even formation of insoluble aggregates. Collection of a greater range of fractions (for example 1,000, 5,000, 20,000 and 100,000 x g) would be a first step in resolving this issue. Alternatively, purification of ribosomes from these cells would enable a more precise determination of the relationship between the tagged QM and 40S, 60S and 80S ribosomal subunits. The ideal for future work would be to obtain an antibody specific for a *T. brucei* 60S ribosomal protein, enabling confirmation (or not) of co-sedimentation with the tagged QM under a variety of experimental conditions. One

such condition worthy of investigation would be the ability of either 1 M NaCl or 0.5 M KCl to dissociate the tagged QM from the protein complex with which it associates. This approach has been employed previously in both yeast (Dick *et al.*, 1997: Eisinger *et al.*, 1997) and mammalian cells (Loftus *et al.*, 1997) to demonstrate the peripheral association between QM and ribosomes.

Co-localisation of the TyQM with BiP, a luminal ER associated protein, failed to produce an identical match. While the expectation was for both proteins to be localised to the ER, a perfect match between them was not predicted. QM has been reported as associating with ribosomes on the cytoplasmic face of the ER (Loftus *et al.*, 1997), while BiP is sequestered within the lumen of the ER (Vogel *et al.*, 1990). The localisation pattern of BiP was as had been expected, and is reported as "characteristic of the ER morphology of trypanosomes" (McDowell *et al.*, 1998). The pattern displayed by the tagged QM, while of a very similar nature to that of BiP, fails to match at certain 'hot spots', as evidenced by the merged images (Figure 4.12, tile 6). This could result either from the aforementioned differences between the ER lumenal location of BiP and the presence of QM on the ER cytoplasmic face, or could represent QM associating with aggregations of free ribosomes in the cytoplasm (no evidence for this has as yet been published).

The GPI8 protein is part of a transamidase complex responsible for addition of glycosylphosphatidylinositol (GPI) anchors to newly synthesised proteins in the ER (Benghezal *et al.*, 1996). In yeast GPI8 is a membrane glycoprotein located on the luminal face of the ER (Benghezal *et al.*, 1996). The sub-cellular location of GPI8 in *T. brucei* is currently unknown, but is suspected to be similar to its homologue in yeast (J.

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Hilley, personal communication). An α-GPI8 antibody raised to a recombinant *L. mexicana* protein was used to co-localise the tagged QM to the ER membrane. By merging the tagged QM and GPI8 expression patterns (Figure 4.13, tile 6) it was determined that these proteins share an almost identical distribution throughout the cell, with the notable exception of the nucleus. While the TyQM is distinctly excluded from this organelle, the α-GPI8 antibody does detect protein within this compartment, albeit at lower levels than elsewhere in the cell. There are two likely interpretations of these data. Firstly, co-localisation may be a result of TyQM residing in ribosomes on the cytoplasmic face of the rough ER, while the GPI8-transamidase complex is most abundant on corresponding areas of the ER luminal face. Such an association between the cells' protein synthesis machinery and a complex involved in processing a subset of the resultant protein is not unreasonable. However, this does not explain why the α-GPI8 antibody detects protein within the nucleus, an organelle in which detection of GPI8 would not be predicted. A second possibility is that the α–GPI8 antibody cross-reacts with a ribosomal protein other than QM. This would explain the almost identical patterns of expression throughout the cytoplasm, as well as the lack of identity with respect to the nucleus. As ribosomal subunits are assembled within the nucleolus (Woolford, 1991) detection of ribosomal proteins within this organelle would be expected. The data gathered during the course of this work are insufficient to determine which of these hypotheses holds greatest merit. However, the current data are consistent with evidence gathered in both the mammalian and yeast systems, leading to the suggestion that TyQM localises to the endoplasmic reticulum where it associates with large protein complexes, most probably ribosomes.

In summary, a *T. brucei* homologue of *QM* has been identified and sequenced, demonstrating a high level of conservation with *QM* from other species. An epitope tag was added to the *T. brucei* QM, which was then overexpressed. Subcellular fractionation indicated an association between the TyQM and a large protein complex. Immunofluorescence microscopy revealed a punctate distribution within the cytoplasm and nuclear exclusion, as had previously been reported for QM in both yeast and mammals.

CHAPTER 5

Isolation and characterisation of the *Trypanosoma brucei* homologue of the *Saccharomyces cerevisiae* gene *MOB1*

5.1 Introduction

5.1.1 The eukaryotic cell cycle

The term cell cycle refers to the progression of a cell through cell growth, DNA replication and cell division. This cycle consists of four consecutive phases; a gap phase (G1), a DNA synthesis phase (S) during which the entire nuclear DNA content of the cell is duplicated, a second gap phase (G2) and a mitotic phase (M) during which the cell undergoes karyokinesis then cytokinesis, resulting in two daughter cells. The length of each phase of the cell cycle depends not only on the cell type but also on the environmental conditions in which the cell is growing (Alberts *et al.*, 1994). Due to the complexity of eukaryotic cells, a precise ordering of events throughout the cell cycle is necessary (Alberts *et al.*, 1994: Stillman, 1996). In recent years it has become evident that the mechanisms governing progression through the cell cycle are conserved between divergent eukaryotes. Protein kinases are central to this control, participating in complex pathways of protein phosphorylation. The cyclin dependent kinases (CDKs) are a conserved family of serine-threonine kinases that play a major role in cell cycle regulation (and possibly other cellular control processes), and which rely on binding of a cyclin partner to regulate their activity. The yeast *Saccharomyces cerevisiae* possesses 22 cyclins, each of which binds one of five CDKs (see tables in Pines, 1995 and Andrews and Measday, 1998). By contrast, nine CDKs and 16 cyclins have so far been identified in mammalian cells (reviewed in Johnson and Walker, 1999), only some of which are known to participate in cell cycle transitions. The greater number of CDKs in mammalian cells is thought to correspond to the greater complexity of the cell cycle in higher eukaryotes.

5.1.2 The cell cycle in trypanosomes

To date five CDKs (TBCRK1-5) and three cyclins (CYC1-3) have been identified in *Trypanosoma brucei*, with the ongoing *T. brucei* sequencing project expected to identify more in the near future. It has been remarked that this is a surprisingly large number for a unicellular eukaryote (Mottram and Smith, 1995), and could correspond to the complexity of the *T. brucei* lifecycle. The roles of each CDK have yet to be determined both during the trypanosome cell cycle and throughout its digenetic lifecycle. As yet only one CDK-cyclin interaction has been demonstrated, between CRK3 and CYC2 (J. van Hellemond, personal communication).

Control of cell division in the kinetoplastids differs slightly from that in other eukaryotes in that the cell must not only duplicate and segregate its nuclear DNA, but must also co-ordinate the duplication and accurate segregation of its other single copy organelles (Woodward and Gull, 1990). Furthermore, digenetic trypanosomatids must also control the interplay between cell cycle progression and differentiation, as parasites alternate between proliferative and non-proliferative stages throughout the lifecycle. Procyclic form *T. brucei* have been studied in detail and the timing of a number of characteristic cell cycle events established (Sherwin and Gull, 1989; Gull *et al.*, 1990; Woodward and Gull, 1990) *Trypanosoma brucei* cells in early G1 have a single copy of the nucleus, the kinetoplast (mitochondrial genome), the basal body and the flagellum. Initiation of replication of kinetoplast DNA closely precedes that of nuclear DNA (Woodward and Gull, 1990). The first ultrastructurally detectable cell cycle event is the maturation of the probasal body, which occurs almost coincidently with initiation of nuclear S-phase (Sherwin and Gull, 1989; Woodward and Gull, 1990). Outgrowth of a daughter flagellum from the newly elongated basal body begins shortly thereafter, as

does generation of a new probasal body for each of the two mature basal bodies now in the cell. Daughter flagellum elongation continues for a large portion of the cell cycle (Robinson *et al.*, 1995). Nuclear and kinetoplast G2-phases overlap for a short while before basal body segregation occurs. Due to a structural link between the basal body and the kinetoplast, segregation of these organelles and the flagellae are concomitant (Robinson and Gull, 1991), and occur before the daughter flagellum has extended to its mature length (Kohl and Gull, 1998). Nuclear G2 phase continues for a while before the chromosomes are segregated. Segregation of the large chromosomes involves the action of kinetochore microtubules, while the minichromosomes segregate by another method that may involve the action of microtubule motor proteins (Ersfeld and Gull, 1997). Karyokinesis follows and the 2 daughter nuclei move apart. A cleavage furrow is initiated at the anterior end of the cell and proceeds to the posterior, producing daughter cells with one each of the single copy organelles (Sherwin and Gull, 1989; Robinson *et al.*, 1995).

A checkpoint can be thought of as a cellular surveillance system responsible for temporarily halting progression through the cell cycle in response to a perceived defect within the cell, such as DNA damage or the faulty alignment of chromosomes on the spindle (Paulovich *et al.*, 1997). During the ensuing arrest cells have time to rectify the defect prior to continuation of the cell cycle. Disruption of microtubule-mediated events in many eukaryotes results in a mitotic block and thus inhibition of cytokinesis. However, this appears not to be the case in *T. brucei* where treatment of cells with the antimicrotubule agent rhizoxin results in mis-segregation of the nucleus during cytokinesis and the formation of cells with either 1 kinetoplast and 2 nuclei (1K2N) or 1 kinetoplast and no nuclei (1K0N). Additionally, under this drug regimen some cells fail

to undergo mitosis entirely with the formation of seemingly normal 1K1N cells following cytokinesis, together with their aploid 1K0N partners. It therefore seems possible that the microtubule-related checkpoints of other eukaryotes are either altered or absent in *T. brucei* (Robinson *et al.*, 1995).

5.1.3 The spindle assembly checkpoint of budding yeast

The spindle assembly checkpoint of budding yeast detects defects in the spindle pole body (equivalent of the centrosome), microtubules, kinetochore proteins, centromeric DNA or microtubule motors (Rudner and Murray, 1996). Upon activation, the checkpoint inhibits exit from mitosis by preventing activation of a ubiquitin ligase, the anaphase promoting complex (APC), the function of which is to catalyse ubiquination of proteins such as the mitotic cyclin CLB2, thus targeting them for proteolysis (Murray, 1995). Reduction of CLB2/CDC28 kinase activity is known to be a prerequisite for exit from mitosis (Surana *et al.*, 1993). Studies carried out over the past decade have revealed a number of interacting proteins involved in the spindle checkpoint, and work is currently underway to further characterise their mode of action. MPS1 is a dual specificity protein kinase essential for duplication of the spindle pole body (SPB) during G1 phase of the cell cycle (Winey *et al.*, 1991). Unlike other proteins involved in SPB duplication (KAR1, NDC2, MPS2 and CDC31), MPS1 also plays a non-essential role in the spindle assembly checkpoint during M-phase (Weiss and Winey, 1996). *MPS1-1* mutants are therefore observed not only to fail in duplication of the SPB, but also to fail to arrest the cell cycle in response to this defect, the result being polyploid cells whose increasing DNA content rapidly becomes lethal. Overexpression of MPS1 results in mitotic arrest due to constitutive activation of the checkpoint, rather than in response to any spindle deficit. This arrest is prevented by

mutation in any of *MAD1-3* (**m**itotic **a**rrest **d**eficient) or *BUB1-3* (**b**udding **u**ninhibited by **b**enzimidazole), suggesting that these otherwise non-essential genes function downstream of MPS1 at the spindle checkpoint. MAD1 has been shown to be an *in vivo* substrate for MPS1 kinase (Hardwick *et al.*, 1996), and also to interact physically with MAD2 (Chen *et al.*, 1999). BUB3 has been shown to physically interact with and be a substrate for the protein kinase BUB1 (Roberts *et al.*, 1994). Taken together with the fact that the vertebrate homologues of MAD2 and BUB1 both locate to the kinetochore (Chen *et al.*, 1996; Taylor and McKeon, 1997), it has been proposed that the MAD and BUB proteins, in association with MPS1, may form a spindle-monitoring complex at this location (Rudner and Murray, 1996; Farr and Hoyt, 1998). The mechanism by which this putative complex blocks cell cycle progression is thought to be through the action of phosphorylated MAD2, which binds to and inactivates the CDC20/APC, preventing targeting and degradation of PDS1 or mitotic cyclins and thus blocking mitotic exit (Hwang *et al.*, 1998).

Several lines of evidence exist to suggest that BUB2 acts in a checkpoint pathway distinct to that described above. Firstly, unlike other MAD and BUB proteins, BUB2 is not required for the cell cycle delay caused by impaired kinetochore function (Wang and Burke, 1995). Secondly, of the checkpoint associated genes *BUB2Δ* alone prevents the mitotic arrest induced in $cdc20^{ts}$ cells (Shirayama *et al.*, 1998). Finally BUB2 has been localised to the SPB (Fraschini *et al.*, 1999), as opposed to the other checkpoint proteins which are thought to be present on the kinetochore. BUB2 activity negatively regulates the DBF2 protein kinase (Fesquet *et al.*, 1999) and overexpression of MPS1 also inhibits DBF2 kinase activity. It has therefore been suggested that BUB2 may not

participate in an entirely separate checkpoint pathway, but may in fact be a member of a novel branch of the existing pathway under the control of MPS1.

DBF2 kinase activity is required for progression through late mitosis, and becomes essential in the absence of its homologue DBF20 (Toyn *et al.*, 1991). The substrate(s) for DBF2 and its precise cellular roles are currently unknown, but it has been shown to interact physically with members of the transcription regulatory CCR4 complex, including CCR4 and CAF1 (Liu *et al.*, 1997), suggesting a role in regulation of transcription as well as cell cycle.

5.1.4 The MOB1 protein

Knockout experiments have revealed *MOB1* to be an essential gene in *Saccharomyces cerevisiae*, and mutational analysis demonstrated it to be required for completion of mitosis, with conditional mutants arresting as large budded cells with a 2N DNA content when moved to the restrictive temperature (Luca and Winey, 1998). When maintained at the permissive temperature these cells retained a stable haploid phenotype if the mutant gene was expressed from a low copy plasmid, but stable integration into the genome resulted in apparent diploidisation of cells. MOB1 is therefore also implicated as possessing a role in maintenance of ploidy (Luca and Winey, 1998).

The MOB1 protein was first discovered during a 2-hybrid screen aimed at identifying interacting partners of MPS1 (Luca and Winey, 1998), or DBF2 (Komarnitsky *et al.*, 1998). In both cases the 2-hybrid interaction was confirmed by immunoprecipitation. MOB1 has been shown to be a phosphoprotein *in vivo* and a substrate for MPS1 kinase activity *in vitro* (Luca and Winey, 1998). Furthermore, MOB1 has been shown to

interact with DBF2 *in vivo*, and to have 2-hybrid interactions with several members of the CCR4 transcription regulatory complex, namely CAF1, CAF16 and CAF17 (Komarnitsky *et al.*, 1998). Several observations suggest that MOB1 function during M phase is facilitated through its interaction with DBF2. Firstly, overexpression of DBF2 complements *mob1^{ts}* mutant phenotype, but is incapable of complementing *MOB1Δ*. Secondly, overexpression of MOB1 complements both defects associated with *dbf2^{ts}* mutants, and also lethality associated with *DBF2Δ/DBF20Δ*. Finally, m*ob1^{ts}* and *dbf2^{ts}* double mutants result in lethality (Komarnitsky *et al*, 1998).

Mutations in *MOB1* exhibit lethality when combined with several other mutant genes possessing similar late mitotic arrest phenotypes to itself. These include the protein kinases *CDC5* and *CDC15* and the GDP/GTP exchange factor *LTE1* (Luca and Winey, 1998). Genetic interactions have been demonstrated between various combinations of *CDC5*, *CDC14*, *CDC15*, *DBF2* and *TEM1*, suggesting a functional link between many of these genes during mitosis. Furthermore, an assay carried out to quantify APC cyclin-ubiquitin ligase activity in arrested mutant strains revealed that levels were negligible (except in *cdc14-1* mutants), indicating that these late mitotic proteins are required for activation of the APC toward mitotic cyclins (Jaspersen *et al.*, 1998).

The function of the MOB1 protein *in vivo* remains unclear, either in its association with MPS1 or during late mitosis. The aim of the work described in this chapter was to clone and characterise the MOB1 gene of *T. brucei*. Southern blot analysis was carried out in order to determine copy number of the *MOB1* gene. Two *MOB1* genes were identified and sequences ascertained. A recombinant epitope-tagged protein was overexpressed in *T. brucei* and its stability/turnover investigated by Western blot. Indirect

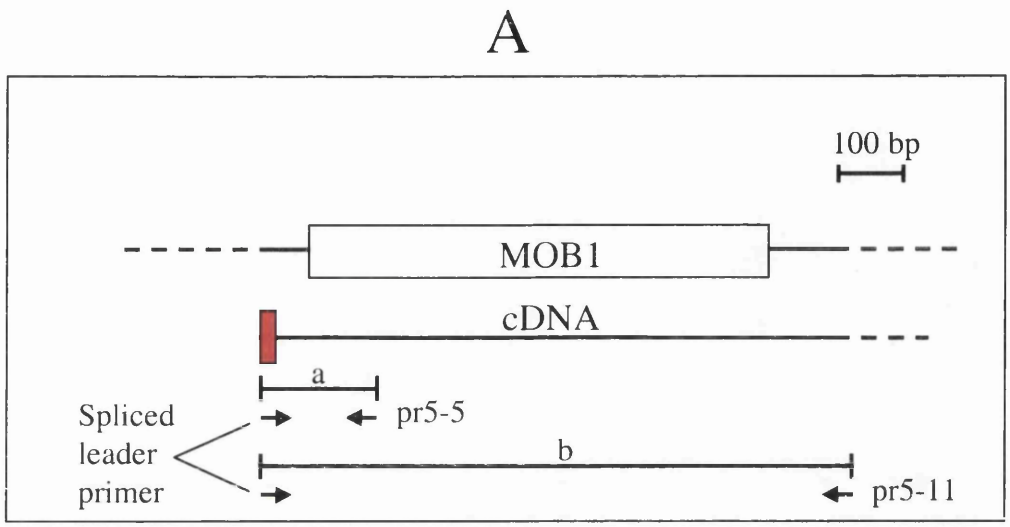
immunofluorescence microscopy and subcellular fractionation were employed to determine the sub-cellular location of the epitope-tagged MOB1. Inducible expression of both antisense and double-stranded RNA was used in attempt to disrupt gene function, and alterations in phenotype sought. A recombinant MOB1 polypeptide was produced, and polyclonal antisera raised.

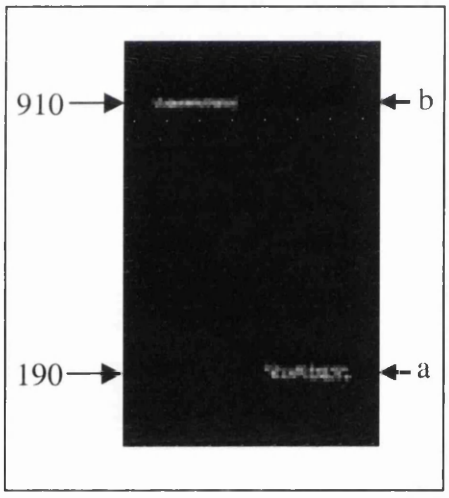
5.2 Results

5.2.1 Southern blot and sequence analysis of *T. brucei MOB1*

Sequence analysis of clone p1365(2), isolated during a RADES-PCR screen of ConA-treated procyclic trypanosomes (Section 3.2.1), revealed strong homology to the *MOB1* gene of other organisms. To isolate a full length *T. brucei MOB1* gene, a *T. brucei* λgt11 library was screened as described in Section 2.9, using the insert of plasmid p1365(2) as a probe. A single positive plaque was isolated and a 4.3 kb insert excised by restriction digestion with *Eco* RI. This was subcloned into pUC18 to give plasmid pMOB1. Sequencing was carried out with commercial M13-20 and M13 reverse primers, and with internal primers based on the sequence of p1365(2). While the internal primers extended the gene sequence to 1.4 kb, the commercial primers failed on two occasions and were abandoned. At this stage the known sequence encoded the majority of the open reading frame (as determined by comparison to MOB1 sequences from other organisms) and 800 bp of the 3' UTR. Sequencing reactions in a 3' → 5' orientation (i.e. from the *MOB1* ORF towards the 5' flank) repeatedly failed. Consequently, 5' RACE was carried out using internal primer pr5-33 in conjunction with the spliced leader primer (Appendix II) on 1st strand cDNA using *Pfu* DNA polymerase. A single fragment of 910 bp was detected following agarose gel electrophoresis (Figure 5.1). This amplicon was used as template for a nested PCR using a second internal primer, pr5-34, in conjunction with the spliced leader primer. A single fragment of the predicted size of 190 bp was detected, confirming the identity of the original amplicon, which was then T-vector cloned into pGEM-T Easy vector [Promega] to give plasmid pMOBME, and sequenced using T7 and SP6 primers to identify the splice acceptor site and start codon of the *MOB1* gene (Figure 5.1).

Figure 5.1 Results of *MOB1* 5' RACE utilising nested PCR primers





C

AGCATTGTGGTAAAATAAATTCCAC
AAGCGGCATAGAAAAATATTCTTC
ATGGAAAAGCATAAGGAAGGAAC
TGAACGTTACAACCTCCACAAGT
TTGCCAAATCACTTGTGCGCTCA
GGTGACCTCAGTGCTGCTGTAAA
GCTACCGCAAGGCACAGATCTGA
ATCATTGGCTTTCCGTACACACTG
TAGATTTCTACAACATCACTAATG
TTTTGTACGGCTCTCTAACAGAGT
TTTGCACGAACAGCAGTTGTCCT
GTCATGTCGTCAGGTCCACGCTA
TGAGTATTTATGGCGTGACCCGC
CGGAATATCC

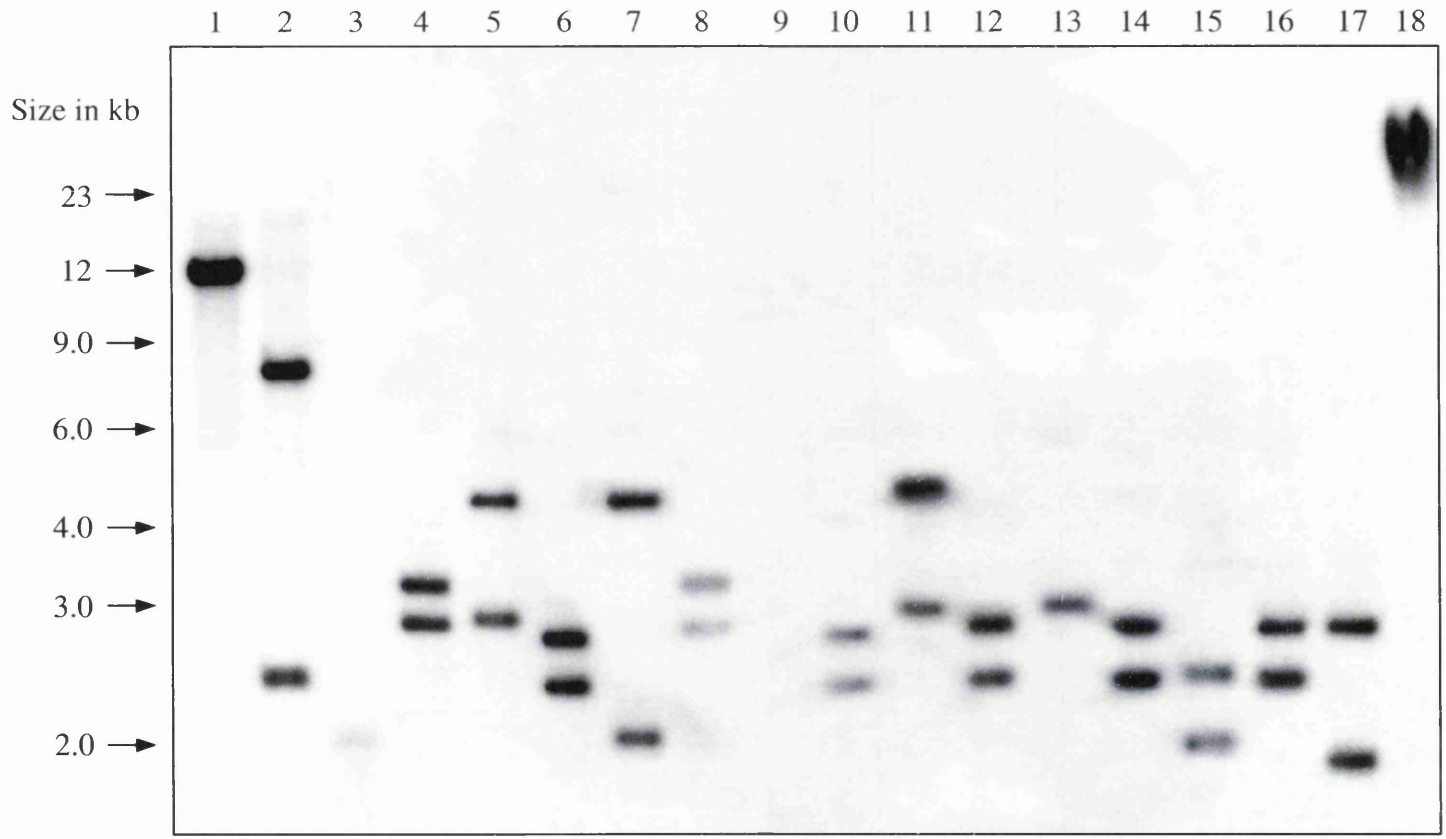
5' RACE was carried out (plate A) using the spliced leader primer in conjunction with nested primers pr5-11 and pr5-5 on first strand cDNA template. Results are presented (plate B). Sequence data derived from fragment (a) is presented (plate C) with the splice acceptor site denoted by red, the 5' UTR denoted by grey and the open reading frame denoted by black. Primer sequences are in Appendix II.

Sequence data pertaining to the open reading frame of *MOB1* derived from pMOB1 and the 5' RACE was confirmed by subsequent sequencing of pPCR23Mob (see later in this Section) and was used as the basis for later experiments.

The failure to sequence the full *MOB1* ORF contained within plasmid pMOB1 led to two hypotheses: either pMOB1 contained two *MOB1* ORFs, each with a different 5' terminus, but common termination site and 3' UTR, or the insert of pMOB1 contained substantial levels of secondary structure coinciding with the 5' terminus of the gene. In order to address these possibilities the number of copies of *MOB1* in the *T. brucei* genome was investigated by subjecting genomic DNA to a variety of single and double restriction enzyme digests. Following agarose gel electrophoresis Southern blots were hybridised with a ^{32}P-labelled probe, derived from the insert of plasmid pTyMobGEM-1 (Section 5.2.3), which contained the open reading frame of *MOB1*. Results are displayed in Figure 5.2 and Table 5.1.

Digestion with *Bcl* I alone produced a fragment of approximately 12 kb. Single digests with *Cla* I, *Hae* II, *Mlu* I and *Pvu* II all produced two bands, indicating that either there were two copies of the *MOB1* gene, or that all four of these enzymes cut within the open reading frame. Digestion with *Fok* I failed to produce any bands that hybridised with the probe, indicating very frequent cutting by this enzyme to produce fragments that ran off the bottom of the gel, possibly as the result of multiple recognition sites within the open reading frame or star activity. Double digestion with *Mlu* I/*Pst* I produced fragments of 5.0 and 1.9 kb. As digestion with *Mlu* I alone produced fragments of 5.0 and 3.0 kb this result indicated that *Pst* I cut within the smaller of the two *Mlu* I

Figure 5.2 Southern blot analysis of the *T. brucei MOB1* gene



5 µg genomic DNA was subjected to restriction enzyme digestion with (1) *Bcl* I, (2) *Cla* I, (3) *Fok* I, (4) *Hae* II, (5) *Mlu* I, (6) *Pvu* II, (7) *Mlu* I/*Pst* I, (8) *Hae* II/*Apa* I, (9) *Fok* I/*Nco* I, (10) *Pvu* II/*Nco* I, (11) *Mlu* I/*Pvu* I, (12) *Pvu* II/*Sal* I, (13) *Mlu* I/*Bgl* II, (14) *Pvu* II/*Eco* RI, (15) *Pvu* II/*Bam* HI, (16) *Pvu* II/*Hin*d III and (17) *Pvu* II/*Sph* I. Lane 18 was 5 µg undigested genomic DNA. Southern blots were hybridised with a ^{32}P-labelled probe, derived from the insert of plasmid pTyMobGEM, (Section 5.2.3) which contained the open reading frame of *MOB1*.

Table 5.1 Sizes of genomic DNA fragments corresponding to the *MOB1* gene following restriction enzyme digestion

	Bcl I	*Cla* I	*Hae* II	*Mlu* I	*Pvu* II
Bcl I	12.0				
Cla I		7.5 2.5			
Hae II			3.3 2.9		
Mlu I				5.0 3.0	
Pvu II					2.8 2.3
Pst I				5.0 1.9	
Apa I			3.3 2.9		
Nco I					2.8 2.3
Pvu I				5.0 3.0	
Sal I					2.8 2.3
Bgl II				3.0 0.2	
Eco RI					2.8 2.3
Bam HI					2.3 1.8
Hind III					2.8 2.3
Pst I					2.8 1.7

Fragment sizes of *T. brucei* genomic DNA subjected to both single and double digestion with a selection of restriction enzyme prior to hybridisation with a *MOB1*-specific probe. Numbers in the table denote fragment sizes in kb.

fragments, but probably not within the open reading frame (or at least not near its centre). Digestion with *Hae* II/*Apa* I produced fragments of 3.3 and 2.9 kb. As these were the fragment sizes produced by *Hae* II alone this result indicated that *Apa* I cut out-with the *Hae* II fragments. Double digestion with *Pvu* II/*Nco* I, *Pvu* II/*Sal* I, *Pvu* II/*Eco* RI and *Pvu* II/*Hin*d III all produced fragments of 2.8 and 2.3 kb, the size produced by single digestion with *Pvu* II. This indicated sites for *Pvu* II flanking the *MOB1* gene(s) in closer proximity than *Nco* I, *Sal* I, *Eco* RI and *Hind* III. Furthermore, *Nco* I, *Sal* I, *Eco* RI and *Hind* III did not cut within the open reading frame of the gene. Double digestion with *Mlu* I/*Pvu* I produced fragments of 5.0 and 3.0 kb, the same size as were produced by single digestion with *Mlu* I. This indicated that *Mlu* I cut closer to the gene(s) than *Pvu* I. Double digestion with *Mlu* I/*Bgl* II produced fragments of 3.0 and (approximately) 0.2 kb (not shown). The result of this particular digest was not fully understood, but was indicative of a site(s) for *Bgl* II in the larger of the *Mlu* I fragments. Double digestion with *Pvu* II/*Hin*d III produced fragments of 2.3 and 1.8 kb, indicating either that *Hin*d III cut within 500 bp of one end of each of the *Pvu* II fragments, or that it cut at 1 kb into the larger of the two *Pvu* II fragments. Double digestion with *Pvu* II/*Pst* I produced fragments of 2.8 and 1.7 kb, indicating a recognition site for *Pst* I within 400 bp of one of the termini of the smaller *Pvu* II fragment.

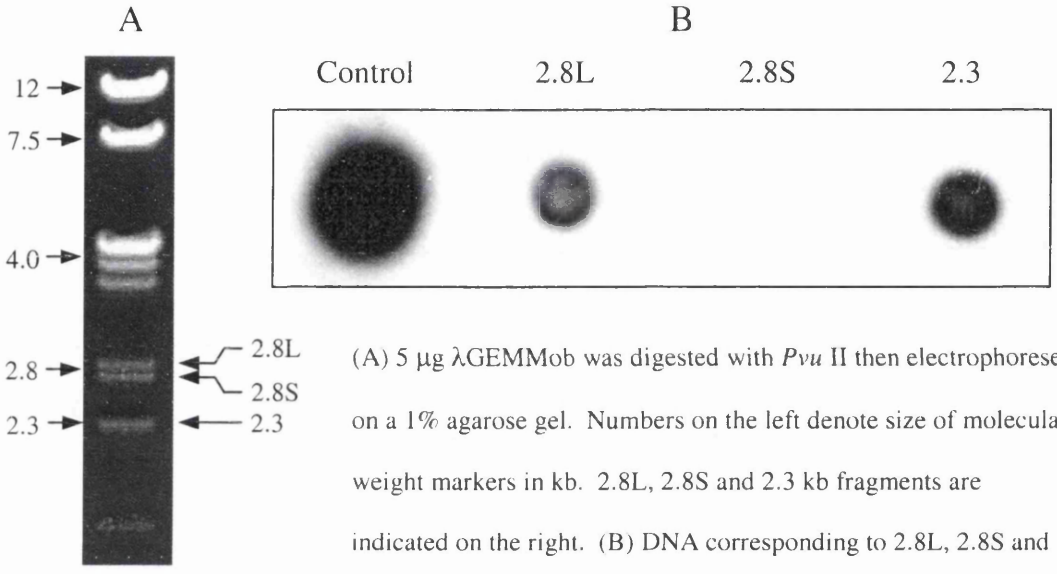
These data digests indicated very strongly that there were two copies of the *MOB1* gene within the *T. brucei* genome, or one copy with a close homologue. Furthermore, these genes were demonstrated to be within the 12 kb of the *Bcl* I fragment. However, insufficient data was collected to allow more informative restriction mapping to be carried out. At this stage it was decided that screening of a genomic lambda library,

sequencing of the two (putative) *MOB1* genes and subsequent restriction mapping of genomic DNA based on these sequences would be an appropriate approach.

A *T. brucei* library consisting of ILTat 1.2 genomic DNA partially digested with *Sau* 3A, size selected for fragments of 11-19 kb and cloned into LambdaGEM-12 (produced by N. Burman) was screened for clones hybridising to a ^{32}P-labelled probe corresponding to the insert of clone p1365(2) (Section 3.2.1). 10 positive clones were identified, and phage DNA prepared from 4 of these. Agarose gel electrophoresis of purified phage DNA indicated that only one of the 4 clones, subsequently referred to as λGEMMob, had produced a yield of DNA suitable for subsequent analysis. Based on Southern blot data (Figure 5.2, Table 5.1), phage DNA was digested with *Pvu* II. Restriction digested DNA was analysed by agarose gel electrophoresis, and revealed two fragments of approximately 2.8 kb and one fragment of approximately 2.3 kb (in addition to a variety of other fragments, the sizes of which were not calculated). The 2.8 kb fragments will subsequently referred to as 2.8L and 2.8S for the Larger and Smaller fragments respectively. Restriction mapping (MapDraw) of the arms of LambdaGEM-12 demonstrated that no fragments within the range 1.9 - 3.7 kb would be produced by *Pvu* II digestion of the vector, indicating that the 2.8 and 2.3 kb fragments were derived from the insert DNA. Appropriate bands were excised from the gel and DNA purified. 1 μl purified DNA from each band together with 100 ng p1365(2) were heat denatured then spotted onto Hybond-N+ membrane. DNA was fixed by UV cross-linking then hybridised with a ^{32}P-labelled probe derived from the insert of p1365(2). Autoradiographic detection (Figure 5.3) revealed that the probe had hybridised to the control, the 2.8L and the 2.3 kb fragments. This confirmed, as had been indicated by

Bcl I digestion of *T. brucei* genomic DNA, that the *MOB1* genes were located in close proximity to each other in the genome.

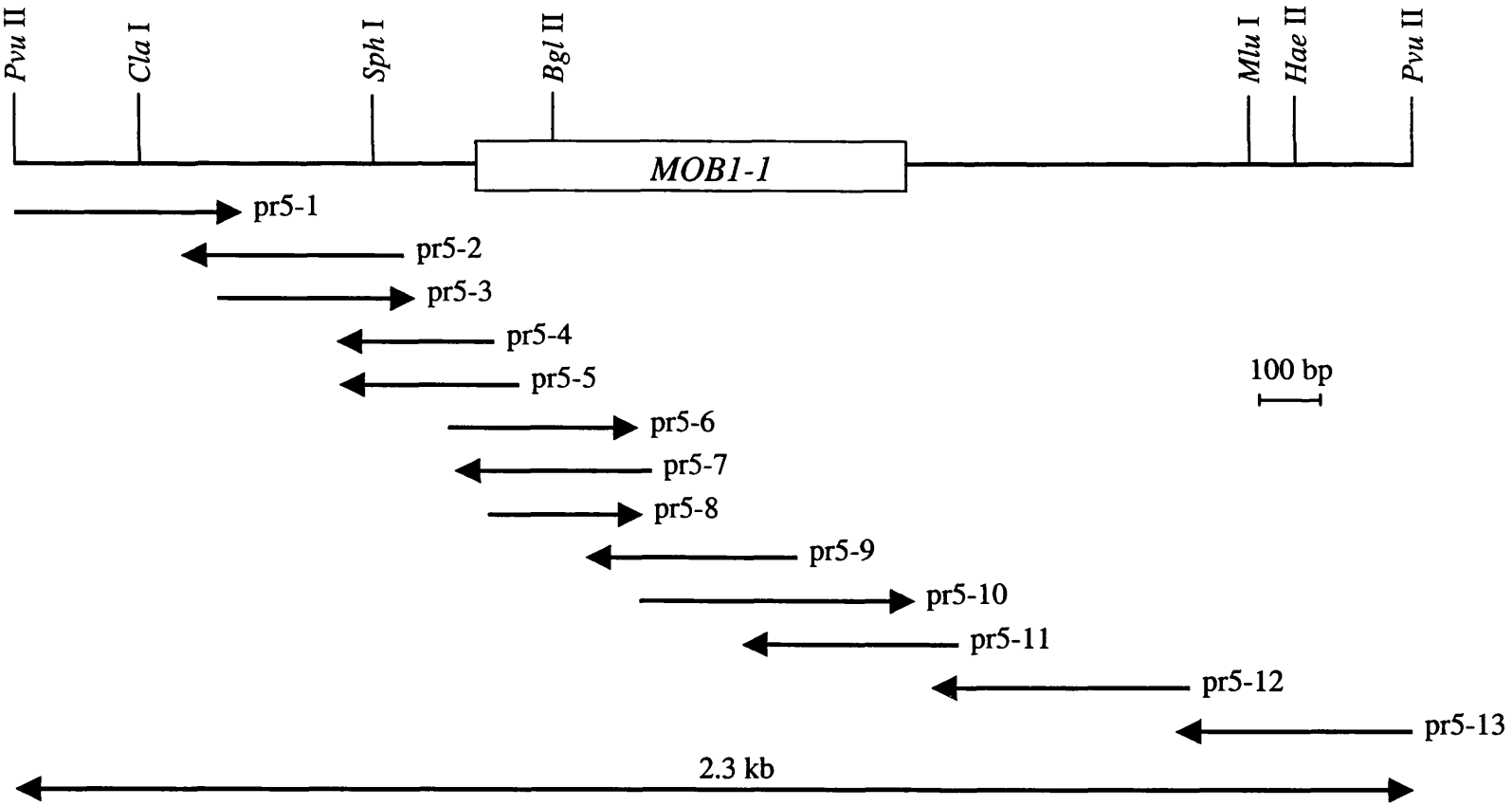
Figure 5.3 *MOB1* fragments excised from λGEMMob



(A) 5 µg λGEMMob was digested with *Pvu* II then electrophoresed on a 1% agarose gel. Numbers on the left denote size of molecular weight markers in kb. 2.8L, 2.8S and 2.3 kb fragments are indicated on the right. (B) DNA corresponding to 2.8L, 2.8S and 2.3 kb fragments was heat denatured at 95°C for 5 minutes then spotted into Hybond-N+ membrane. Following UV cross-linking the membrane was hybridised with a ^{32}P-labelled probe derived from the insert of p1365(2). Control was 100 ng p1365(2). Autoradiographic detection followed.

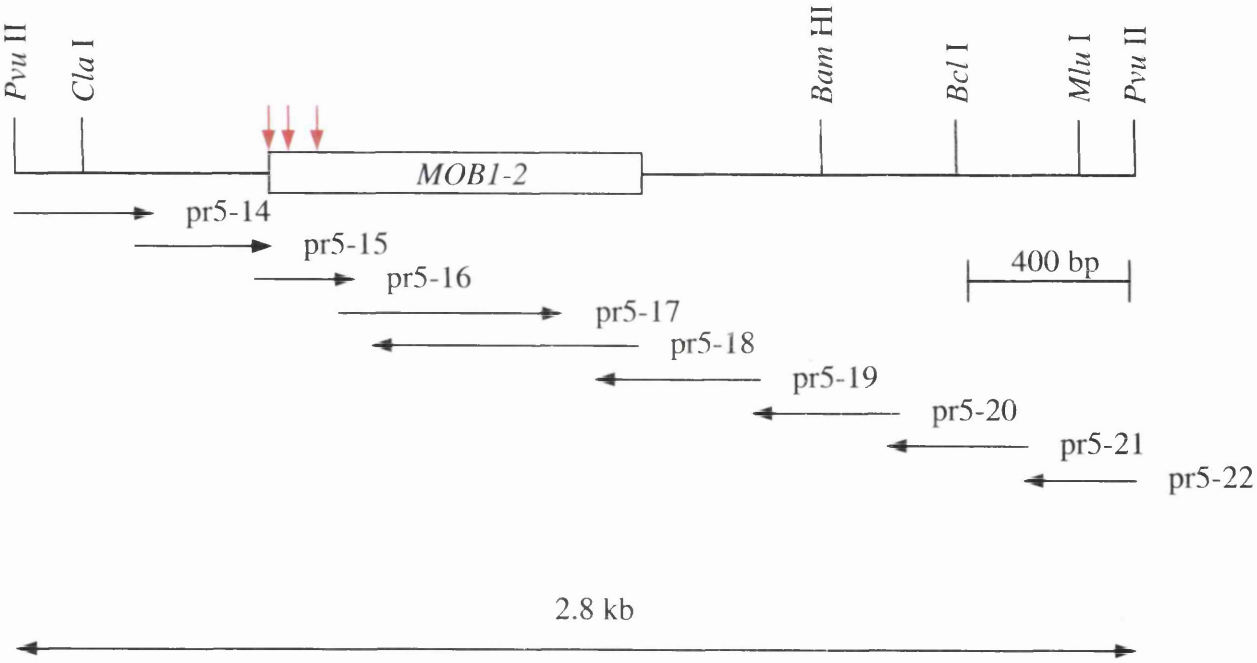
Pvu II digested DNA has blunt ends. Consequently, the 2.8L and 2.3 kb fragments were cloned into the *Srf* I site of PCR-Script™ Amp [Stratagene] according to the manufacturers guidelines to produce plasmids pPCR28Mob1 and pPCR23Mob1. Plasmid DNA was prepared, and the presence of inserts confirmed by digestion with *Eco* RI/*Not* I (cloning of 2.8L and 2.3 kb fragments was carried out in collaboration with M. McLaughlin). The full length of pPCR23Mob1 and pPCR28Mob1 were

Figure 5.4 Schematic representation of *T. brucei* subclone pPCR23Mob1



Schematic representation of the *T. brucei MOB1-1* gene based on sequence analysis of subclone pPCR23Mob1, demonstrating position of overlapping sequences, restriction enzyme sites deduced from the sequence and open reading frame. Primer sequences (pr5-) are detailed in Appendix II. Diagram is to scale.

Figure 5.5 Schematic representation of *T. brucei* subclone pPCR28Mob1



Schematic representation of the *T. brucei MOB1-2* gene based on sequence analysis of subclone pPCR28Mob1, demonstrating position of overlapping sequences, restriction enzyme sites deduced from the sequence and putative open reading frame. Red arrows indicate position of 3 putative methionine start codons. Primer sequences (pr5-) are detailed in Appendix II. Diagram is to scale.

sequenced as indicated in Figures 5.4 and 5.5 respectively. The full length of the *MOB1* open reading frame contained within pPCR23Mob1 was sequenced fully on both strands. The *MOB1* open reading frame contained in pPCR28Mob1 was sequenced only partially in both directions (i.e. not complete sequence on both strands) due to time constraints. The open reading frames contained within pPCR23Mob1 and pPCR28Mob1 were demonstrated to have notable differences (Figures 5.8 and 5.9), and will henceforth be referred to as *MOB1-1* and *MOB1-2* respectively. Subsequent work centred around the sequence of *MOB1-1*.

2316 bp of sequence data was derived from clone pPCR23Mob1, and is presented in Figure 5.6 with the position of the RADES product, open reading frame and flanks indicated. The nucleotide and deduced amino acid sequences of the *MOB1-1* open reading frame are presented in Figure 5.7. Sequence data pertaining to pPCR28Mob1 is lodged in Appendix IV. A schematic comparison of the pPCR23Mob1 and pPCR28Mob1 sequences is presented in Figure 5.8. The MOB1 open reading frame encoded by pPCR23Mob1was highly conserved in the pPCR28Mob1 clone, with both sequences sharing a common stop codon. The immediate 5' and 3' flanks were dissimilar. At the 5' terminus of each clone was another highly conserved section of DNA, but homology searches failed to produced a matches between this and sequences currently lodged on databases.

The open reading frame of *MOB1-1* encoded a protein consisting of 208 residues with a predicted mass of 24.6 kDa and isoelectric point of 7.0. The open reading frame of *MOB1-2* encoded a protein of either 290, 265 or 226 residues corresponding to 3 putative start codons at the 5' terminus of the ORF. None of the putative start codons of

Figure 5.6 Sequence of *MOB1-1* open reading frame and flanks

TCCACGCCTGAACAAACCAGTAACCGAGTAATTGTTGTGGTTGATTGAAAGTTTGCGA 58
AGCAAATCTACCACCTTCAGTTGTGTCCTTGTGCACCGAACCTCTCGGTTTCAGTGAAT 117
GTTGTGTGGTGATTGTTTTCTTTCCTTTTCTTTGTTCTTTTCGTTCTTTCCGTGTCATATC 178
TCCTCTCTTTTCCTCGAACAGATGATGTTTATCGATTTCGAGACTTACTCACACTTGAG 237
GCTCCTGCGACCAGGACCCTCTTTCCTGTTTTTTCTGGATGGTGGAGGATTTATGAATC 296
ATTGTGGCTGTGCAACATATCTGTTTAACACCTCTACTGCACTCATCGTCCCTCCACAA 355
TTATGTTCCGTTGTTTCCTTAATTTGCTTTTAGAAAGGTAAGCTCTGGTAGTCGTTTGCT 415
CTTCACAATATGTCGGGGAAACATATTTTCACTTGTTATGACGATGCTGCTGCTTGCTT 474
TTGCGTTGAGCAAATCAATCACCCGTCGCAACACTGTTAAAACTAAACTGTGACGCGA 532
GAACAAAACCACACTACCAGTACGCTGGAGGTGTTGTTTTGCATGCCTAAATGTATCT 590
GTAAACCAAAGCTGGTGAATGCTCCGTACCTAAATGTGAAAAAGTTGGATGTGCAAG 647
GAGGCAGCTAGCTAGCTTATCTCCTAGCGAGTCGGATCTTCTTAATATATTTCTTCTCT 706
TTCCCATAATGACGTTCAATGCGAGCACTCGGGCACAAGAAAAAAAAGAATGTTCCCT 764
ACTTTCATCCCTCCCTTTATTCAATTTGTTTAAAATAAAAGCATTGTGGTAAAATAAAT 823
TCCACAAGCGGCATAGAAAAATATTCTTC**ATGGAAAAGCATAAGGAAGGAACTGAA** 879
CGTTACAACCTCCACAAGTTTGCCAAATCACTTGTGCGCTCAGGTGACCTCAGTG 934
CTGCTGTAAAGCTACCGCAAGGCACAGATCTGAATCATTGGCTTTCCGTACACAC 989
TGTAGATTTCTACAACATCACTAATGTTTTGTACGGCTCTCTAACAGAGTTTTGCA 1045
CGAACAGCAGTTGTCCTGTCATGTCGTCAGGTCCACGCTATGAGTATTTATGGCG 1100
TGACCCGCCGGAATATCCAAAAGCAACGAAGGTGAGCGCACCGGAGTATGTGAG 1154
ATTGTTGATGGAGTGGATCGAACGGCAGATCAATGATGAACGTGTGTTTCCGTCT 1209
GAGGATCGTAATCCCTATCCACCAGATTTCGCGGATAGGGTGAAGGCGTGCTTC 1263
AAGCGACTGTTCCGCGTTTATGCACACGTTTACTATTCCCACTTTGCGAAGATTC 1318
GTGAGTTGCAGGAGGAATCTCACATCAACACCGCACTGAAACATTTTATGTATTT 1373
TGTGTGGGAGTTTGATCTGATTCCTCGTGAGGAGGTGTCTCCGCTGCGTGAATTG 1428
CTGGTAAACTTAATGGGTCAGCGCGCGAAGGAAAAGTTGGAGGTTCCGTAAACA 1482
ATTTGTGTGCGCGCCCTTGTGTTGGTGCCTTCTTCTAGCAGGGATGATTTTTGCTCATTT 1542
TTTAATAAATCGAAGTGCACATCCTCGTTGGTTGATGGTAATGTTGGTCCCTGAATGTT 1601
ATTTCTCTTTATTAATCAATTTTTGCAGTGACAATTGGTAGAGCAAGTGATGGGTTGAT 1660
TCAAAGCCTTCACTTTXGTCCCAACTCACGCAGTTATTAATGATGGTGAAATCTTTTTT 1719
TTAAATCACTGACCGGAGAAGAACCACTGGGTTGCTTTACCTTTCATACACCTTCGTTT 1778
TGGTATGGCGAAGCCTGAAACTTCTGTCCGCCTTACAAAAAGGCTGTTTCCATTCACTT 1837
TCTCTTTTATATTGATTCGCAACTCGATTTTTATTGGATATTGTTGTGTTGGGCTCGGAC 1897
GGGTCTAATTAATAGTCCGGATGACGGTTGTTGTGGCAGAATGGACATGTTTGGCTTG 1955
TACGAGGGAGAGGTTTGTACACGCGTGTTTCACATCCTTTTTACGTAATTGTTGCAATC 2014
GTAAAGTGAATTTATTTTGTGGATGATGGGATGTACTGAGGAAGGTGAGGAGTCGTTT 2072
ATAGACATGTTGTGGAAAGCTCCACGGTGTGAAGCGTTCTAAGTGGCGCTTCTTCTTA 2130
AGAGTGAACGGATTTAATGGTAAGTGTGCTCCTACTTGGTGGATAGTGTCACGTTATT 2188
TTTCGCAGAAGGGGTGCCTCTATTTGTGCAGTGCACATTGATTTATGCCCTTGATAGGA 2247
AATTTATGTATATCATTAATTGTTGATTGAAAAAGAAAAATTAGTTTGGCTCCACAGA 2305
CTCTTCAGGGG 2316

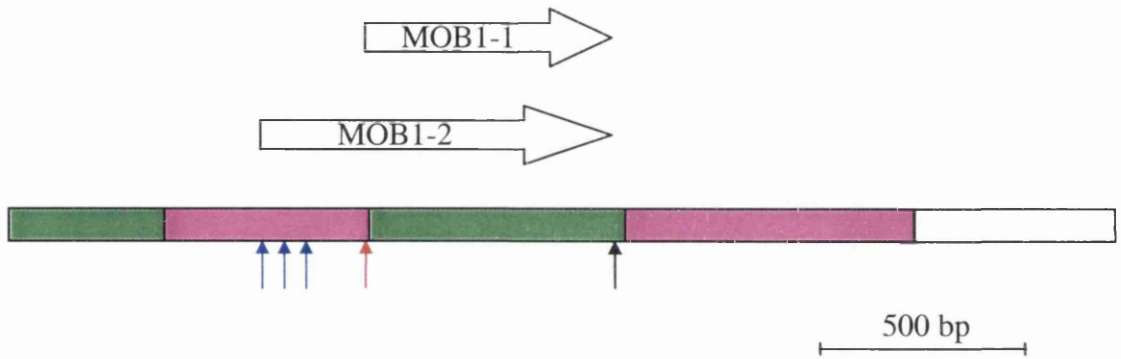
T. brucei MOB1-1 gene sequence based on data derived from subclone pPCR23Mob1. The open reading frame is in **bold**, the sequence of the RADES product is underlined, the regions flanking the open reading frame are denoted by grey. Numbers on the right refer to base pairs. Letters in lower case denote ambiguity in base identity. The splice acceptor site is denoted in red.

Figure 5.7 Nucleotide and deduced amino acids sequences of the *T. brucei MOB1-1* open reading frame

1	ATG	GAA	AAG	CAT	AAG	GAA	GGA	ACT	GAA	CGT	TAC	AAC	
	M	E	K	H	K	E	G	T	E	R	Y	N	12
37	CTC	CAC	AAG	TTT	GCC	AAA	TCA	CTT	GTG	CGC	TCA	GGT	
	L	H	K	F	A	K	S	L	V	R	S	G	24
73	GAC	CTC	AGT	GCT	GCT	GTA	AAG	CTA	CCG	CAA	GGC	ACA	
	D	L	S	A	A	V	K	L	P	Q	G	T	36
109	GAT	CTG	AAT	CAT	TGG	CTT	TCC	GTA	CAC	ACT	GTA	GAT	
	D	L	N	H	W	L	S	V	H	T	V	D	48
145	TTC	TAC	AAC	ATC	ACT	AAT	GTT	TTG	TAC	GGC	TCT	CTA	
	F	Y	N	I	T	N	V	L	Y	G	S	L	60
181	ACA	GAG	TTT	TGC	ACG	AAC	AGC	AGT	TGT	CCT	GTC	ATG	
	T	E	F	C	T	N	S	S	C	P	V	M	72
217	TCG	TCA	GGT	CCA	CGC	TAT	GAG	TAT	TTA	TGG	CGT	GAC	
	S	S	G	P	R	Y	E	Y	L	W	R	D	84
253	CCG	CCG	GAA	TAT	CCA	AAA	GCA	ACG	AAG	GTG	AGC	GCA	
	P	P	E	Y	P	K	A	T	K	V	S	A	96
289	CCG	GAG	TAT	GTG	AGA	TTG	TTG	ATG	GAG	TGG	ATC	GAA	
	P	E	Y	V	R	L	L	M	E	W	I	E	108
325	CGG	CAG	ATC	AAT	GAT	GAA	CGT	GTG	TTT	CCG	TCT	GAG	
	R	Q	I	N	D	E	R	V	F	P	S	E	120
361	GAT	CGT	AAT	CCC	TAT	CCA	CCA	GAT	TTC	GCG	GAT	AGG	
	D	R	N	P	Y	P	P	D	F	A	D	R	132
397	GTG	AAG	GCG	TGC	TTC	AAG	CGA	CTG	TTC	CGC	GTT	TAT	
	V	K	A	C	F	K	R	L	F	R	V	Y	144
433	GCA	CAC	GTT	TAC	TAT	TCC	CAC	TTT	GCG	AAG	ATT	CGT	
	A	H	V	Y	Y	S	H	F	A	K	I	R	156
469	GAG	TTG	CAG	GAG	GAA	TCT	CAC	ATC	AAC	ACC	GCA	CTG	
	E	L	Q	E	E	S	H	I	N	T	A	L	168
505	AAA	CAT	TTT	ATG	TAT	TTT	GTG	TGG	GAG	TTT	GAT	CTG	
	K	H	F	M	Y	F	V	W	E	F	D	L	180
541	ATT	CCT	CGT	GAG	GAG	GTG	TCT	CCG	CTG	CGT	GAA	TTG	
	I	P	R	E	E	V	S	P	L	R	E	L	192
577	CTG	GTA	AAC	TTA	ATG	GGT	CAG	CGC	GCG	AAG	GAA	AAG	
	L	V	N	L	M	G	Q	R	A	K	E	K	204
613	TTG	GAG	GTT	CCG	TAA								
	L	E	V	P	*								208

The nucleotide sequence (black) and deduced amino acid sequence (red) of the *T. brucei MOB1-1* open reading frame is based on data derived from subclone pPCR23Mob1. The position of the nucleotides is displayed in the left margin, and the position of the amino acid residues is displayed in the right margin.

Figure 5.8 Schematic diagram depicting overlapping regions of the 2.3 and 2.8 kb *MOB1* clones



Nucleotide sequences of the 2.3 and 2.8 kb *Pvu* II fragments encoding *T. brucei MOB1* genes were aligned using the MegAlign programme. Regions in green denote greater than 95% identity. Regions in pink denote less than 30% identity. The blank region denotes sequence pertaining to only the 2.8 kb clone. The region in black denotes the largest putative open reading frame encoded by the 2.8 kb clone. Blue arrows denote putative positions of methionine residues of *MOB1-2*. The red arrow denotes the methionine start codon of *MOB1-1*. The black arrow denotes the common stop codon.

Figure 5.9 Alignment of MOB1 protein sequences from diverse species

The protein sequences of MOB1 homologues from diverse species were obtained by homology search using the BLAST programme. Consensus sequence runs along the top. Numbers in the left margin denote sequences from:

(1) *Trypanosoma brucei* (MOB1-1)

(2) *Trypanosoma brucei* (MOB1-2)

(3) *Leishmania major*

(4) *Saccharomyces cerevisiae* [731855]

(5) *Schizosaccharomyces pombe* [3947877]

(6) *Dictyostelium discoideum* [2282525]

(7) *Arabidopsis thaliana* [2832633]

(8) *Gossypium hirsutum* [5048261]

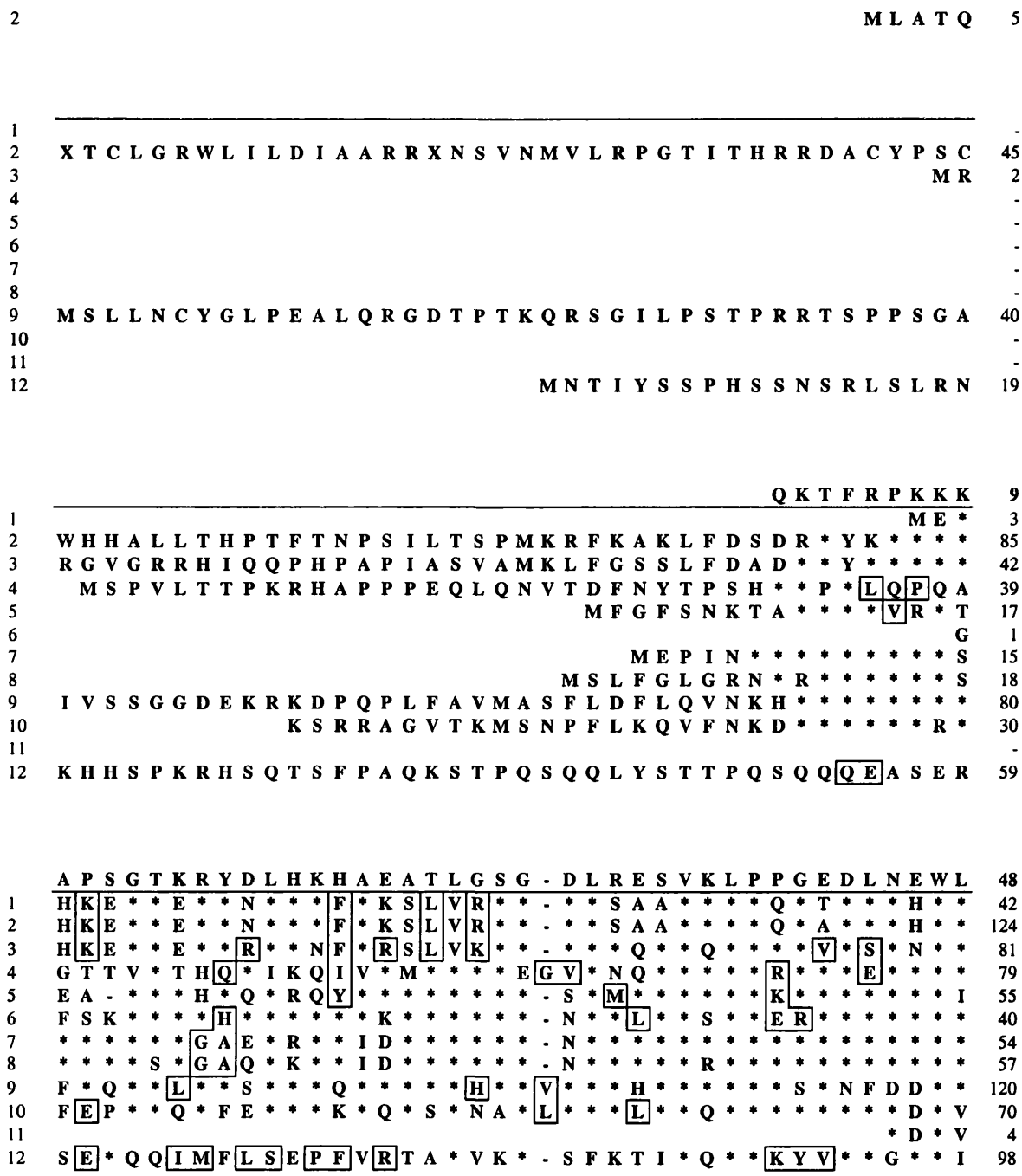
(9) *Caenorhabditis elegans* [4262618]

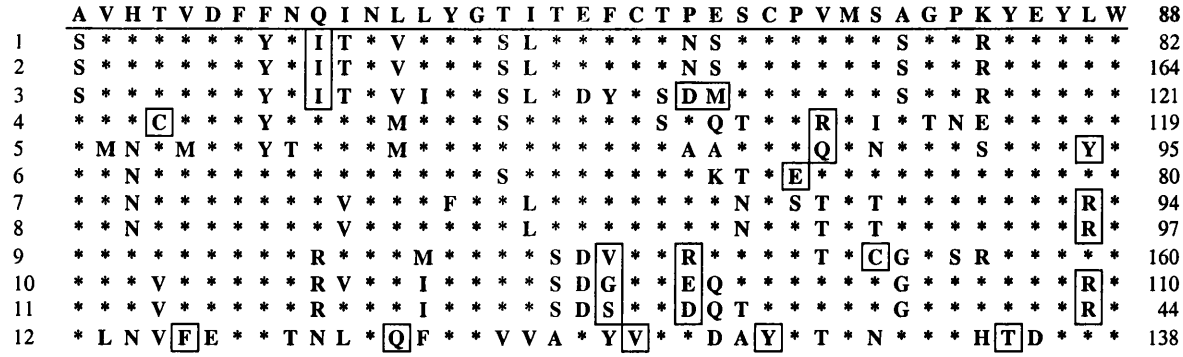
(10) *Homo sapiens* [3342738]

(11) *Paralichthys olivaceus* [5039530]

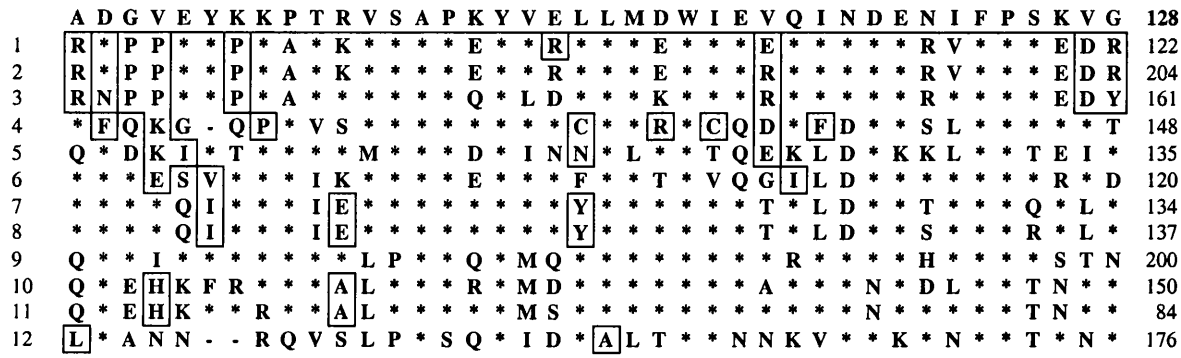
(12) *Saccharomyces cerevisiae* Mob2 [1175942]

Numbers in the right margin denote position of the terminal residue. Residues that match the consensus exactly are denoted by *. Non-conservative substitutions are boxed. Gaps represent missing sequence data. Dashes (-) represent gaps introduced to improve the alignment. The motif highlighted in red denotes a conserved putative casein kinase II phosphorylation site. Numbers in parentheses [above] denote sequence identifiers (http://www.ncbi.nlm.nih.gov). The *T. brucei* MOB1-2 sequence is displayed from the first of the putative methionine start residues.

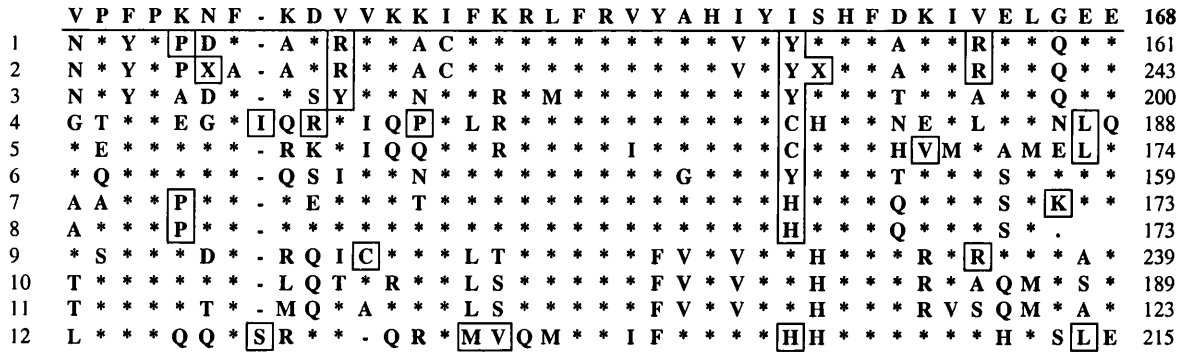




	A	V	H	T	V	D	F	F	N	Q	I	N	L	L	Y	G	T	I	T	E	F	C	T	P	E	S	C	P	V	M	S	A	G	P	K	Y	E	Y	L	W	88
1	S	*	*	*	*	*	*	Y	*	I	T	*	V	*	*	*	S	L	*	*	*	*	*	N	S	*	*	*	*	*	S	*	*	R	*	*	*	*	*	82
2	S	*	*	*	*	*	*	Y	*	I	T	*	V	*	*	*	S	L	*	*	*	*	*	N	S	*	*	*	*	*	S	*	*	R	*	*	*	*	*	164
3	S	*	*	*	*	*	*	Y	*	I	T	*	V	I	*	*	S	L	*	D	Y	*	S	D	M	*	*	*	*	*	S	*	*	R	*	*	*	*	*	121
4	*	*	*	C	*	*	*	Y	*	*	*	*	M	*	*	*	S	*	*	*	*	*	*	S	*	Q	T	*	*	R	*	I	*	T	N	E	*	*	*	*	119
5	*	M	N	*	M	*	*	Y	T	*	*	*	M	*	*	*	*	*	*	*	*	*	*	*	A	A	*	*	*	Q	*	N	*	*	*	S	*	*	*	Y	*	95
6	*	*	N	*	*	*	*	*	*	*	*	*	*	*	*	*	S	*	*	*	*	*	*	*	*	K	T	*	E	*	*	*	*	*	*	*	*	*	*	*	80
7	*	*	N	*	*	*	*	*	*	*	V	*	*	*	F	*	*	L	*	*	*	*	*	*	*	*	N	*	S	T	*	T	*	*	*	*	*	*	*	R	*	94
8	*	*	N	*	*	*	*	*	*	*	V	*	*	*	*	*	*	L	*	*	*	*	*	*	*	*	N	*	*	T	*	T	*	*	*	*	*	*	*	R	*	97
9	*	*	*	*	*	*	*	*	*	R	*	*	*	M	*	*	*	*	S	D	V	*	*	R	*	*	*	*	T	*	C	G	*	S	R	*	*	*	*	*	160
10	*	*	*	V	*	*	*	*	*	R	V	*	*	I	*	*	*	*	S	D	G	*	*	E	Q	*	*	*	*	*	G	*	*	*	*	*	R	*	110
11	*	*	*	V	*	*	*	*	*	R	*	*	*	I	*	*	*	*	S	D	S	*	*	D	Q	T	*	*	*	*	G	*	*	*	*	*	R	*	44
12	*	L	N	V	F	E	*	*	T	N	L	*	Q	F	*	*	V	V	A	*	Y	V	*	*	D	A	Y	*	T	*	N	*	*	*	H	T	D	*	*	*	138



	A	D	G	V	E	Y	K	K	P	T	R	V	S	A	P	K	Y	V	E	L	L	M	D	W	I	E	V	Q	I	N	D	E	N	I	F	P	S	K	V	G	128
1	R	*	P	P	*	*	P	*	A	*	K	*	*	*	*	E	*	*	R	*	*	*	E	*	*	*	E	*	*	*	*	*	R	V	*	*	*	E	D	R	122
2	R	*	P	P	*	*	P	*	A	*	K	*	*	*	*	E	*	*	R	*	*	*	E	*	*	*	R	*	*	*	*	*	R	V	*	*	*	E	D	R	204
3	R	N	P	P	*	*	P	*	A	*	*	*	*	*	*	Q	*	L	D	*	*	*	K	*	*	*	R	*	*	*	*	*	R	*	*	*	*	E	D	Y	161
4	*	F	Q	K	G	-	Q	P	*	V	S	*	*	*	*	*	*	*	C	*	*	R	*	C	Q	D	*	F	D	*	*	S	L	*	*	*	*	*	T	148
5	Q	*	D	K	I	*	T	*	*	*	M	*	*	*	D	*	I	N	N	*	L	*	*	T	Q	E	K	L	D	*	K	K	L	*	*	T	E	I	*	135
6	*	*	*	E	S	V	*	*	*	I	K	*	*	*	E	*	*	*	F	*	*	T	*	V	Q	G	I	L	D	*	*	*	*	*	*	*	R	*	D	120
7	*	*	*	*	Q	I	*	*	*	I	E	*	*	*	*	*	*	*	Y	*	*	*	*	*	*	T	*	L	D	*	*	T	*	*	Q	*	L	*	134
8	*	*	*	*	Q	I	*	*	*	I	E	*	*	*	*	*	*	*	Y	*	*	*	*	*	*	T	*	L	D	*	*	S	*	*	R	*	L	*	137
9	Q	*	*	I	*	*	*	*	*	*	L	P	*	*	Q	*	M	Q	*	*	*	*	*	*	*	R	*	*	*	H	*	*	*	S	T	N	200
10	Q	*	E	H	K	F	R	*	*	*	A	L	*	*	*	R	*	M	D	*	*	*	*	*	*	A	*	*	*	N	*	D	L	*	*	T	N	*	*	150
11	Q	*	E	H	K	*	*	R	*	*	A	L	*	*	*	*	*	M	S	*	*	*	*	*	*	*	*	*	*	N	*	*	*	*	*	T	N	*	*	84
12	L	*	A	N	N	-	-	R	Q	V	S	L	P	*	S	Q	*	I	D	*	A	L	T	*	*	N	N	K	V	*	*	K	*	N	*	*	T	*	N	*	176



	V	P	F	P	K	N	F	-	K	D	V	V	K	K	I	F	K	R	L	F	R	V	Y	A	H	I	Y	I	S	H	F	D	K	I	V	E	L	G	E	E	168
1	N	*	Y	*	P	D	*	-	A	*	R	*	*	A	C	*	*	*	*	*	*	*	*	*	*	V	*	Y	*	*	*	A	*	*	R	*	*	Q	*	*	161
2	N	*	Y	*	P	X	A	-	A	*	R	*	*	A	C	*	*	*	*	*	*	*	*	*	*	V	*	Y	X	*	*	A	*	*	R	*	*	Q	*	*	243
3	N	*	Y	*	A	D	*	-	*	S	Y	*	*	N	*	*	R	*	M	*	*	*	*	*	*	*	*	Y	*	*	*	T	*	*	A	*	*	Q	*	*	200
4	G	T	*	*	E	G	*	I	Q	R	*	I	Q	P	*	L	R	*	*	*	*	*	*	*	*	*	*	C	H	*	*	N	E	*	L	*	*	N	L	Q	188
5	*	E	*	*	*	*	*	-	R	K	*	I	Q	Q	*	*	R	*	*	*	*	I	*	*	*	*	*	C	*	*	*	H	V	M	*	A	M	E	L	*	174
6	*	Q	*	*	*	*	*	-	Q	S	I	*	*	N	*	*	*	*	*	*	*	*	*	G	*	*	*	Y	*	*	*	T	*	*	*	S	*	*	*	*	159
7	A	A	*	*	P	*	*	-	*	E	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	H	*	*	*	Q	*	*	*	S	*	K	*	*	173
8	A	*	*	*	P	*	*	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	H	*	*	*	Q	*	*	*	S	*	.			173
9	*	S	*	*	*	D	*	-	R	Q	I	C	*	*	*	L	T	*	*	*	*	*	F	V	*	V	*	*	H	*	*	*	R	*	R	*	*	*	A	*	239
10	T	*	*	*	*	*	*	-	L	Q	T	*	R	*	*	L	S	*	*	*	*	*	F	V	*	V	*	*	H	*	*	*	R	*	A	Q	M	*	S	*	189
11	T	*	*	*	*	T	*	-	M	Q	*	A	*	*	*	L	S	*	*	*	*	*	F	V	*	V	*	*	H	*	*	*	R	V	S	Q	M	*	A	*	123
12	L	*	*	*	Q	Q	*	S	R	*	*	-	Q	R	*	M	V	Q	M	*	*	I	F	*	*	*	*	H	H	*	*	*	*	*	*	H	*	S	L	E	215

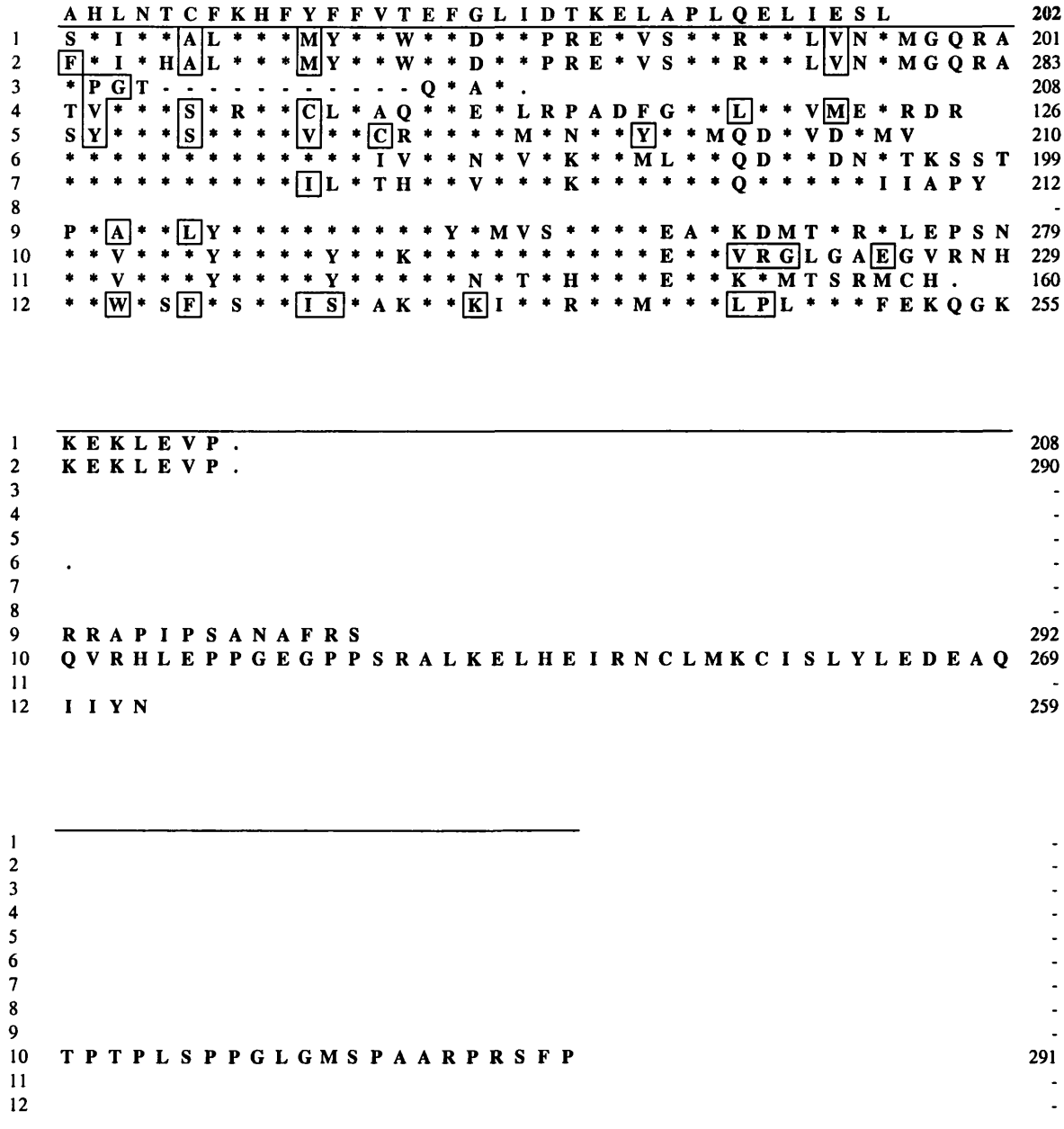

A H L N T C F K H F Y F F V T E F G L I D T K E L A P L Q E L I E S L 202
1 S * I * * A L * * * M Y * * W * * D * * P R E * V S * * R * * L V N * M G Q R A 201
2 F * I * H A L * * * M Y * * W * * D * * P R E * V S * * R * * L V N * M G Q R A 283
3 * P G T - - - - - - - - - - - Q * A * . 208
4 T V * * * S * R * * C L * A Q * * E * L R P A D F G * * L * * V M E * R D R 126
5 S Y * * * S * * * * V * * C R * * * * M * N * * Y * * M Q D * V D * M V 210
6 * * * * * * * * * * * * I V * * N * V * K * * M L * * Q D * * D N * T K S S T 199
7 * * * * * * * * * * I L * T H * * V * * * K * * * * * * Q * * * * * I I A P Y 212
8 -
9 P * A * * L Y * * * * * * * * * Y * M V S * * * * E A * K D M T * R * L E P S N 279
10 * * V * * * Y * * * * Y * * K * * * * * * * * * * E * * V R G L G A E G V R N H 229
11 * * V * * * Y * * * * Y * * * * * N * T * H * * * E * * K * M T S R M C H . 160
12 * * W * S F * S * * I S * A K * * K I * * R * * M * * * L P L * * * F E K Q G K 255
1 K E K L E V P . 208
2 K E K L E V P . 290
3 -
4 -
5 -
6 . -
7 -
8 -
9 R R A P I P S A N A F R S 292
10 Q V R H L E P P G E G P P S R A L K E L H E I R N C L M K C I S L Y L E D E A Q 269
11 -
12 I I Y N 259
1 -
2 -
3 -
4 -
5 -
6 -
7 -
8 -
9 -
10 T P T P L S P P G L G M S P A A R P R S F P 291
11 -
12 -

Table 5.2a Percentage amino acid identity between MOB1 homologues from diverse species

	1	2	3	4	5	6	7	8	9	10	11
2	69										
3	59	47									
4	35	28	31								
5	40	28	35	41							
6	50	35	40	42	50						
7	47	34	40	42	52	62					
8	39	30	42	36	43	53	73				
9	32	35	32	28	30	35	35	30			
10	34	36	31	26	32	34	36	30	45		
11	35	25	29	28	32	41	38	37	36	44	
12	20	20	23	28	30	27	29	22	22	24	22

Table 5.2 b Percentage amino acid identity between conserved region of MOB1 homologues from diverse species

	1	2	3	4	5	6	7	8	9	10	11
2	94										
3	61	67									
4	41	40	35								
5	42	40	35	48							
6	51	50	41	49	51						
7	49	50	42	49	54	65					
8	41	39	41	42	44	52	74				
9	47	48	44	38	44	50	49	43			
10	49	48	43	38	46	49	52	43	63		
11	47	45	38	43	43	52	51	41	52	81	
12	25	29	26	33	37	35	38	29	29	33	36

Tables indicating percentage identity between protein sequences of MOB1 homologues from 10 different species, together the *Saccharomyces cerevisiae* MOB2 sequence. Table 5.2a indicates identity between entire protein sequences, as determined by the number of identical residues divided by the number of residues in the larger of two sequences. Table 5.2b indicates identity between the consensus region of proteins, as determined by the number of identical residues within the consensus region divided by the number of residues within this region (the consensus region of 202 residues is indicated in Figure 5.9). Identities for sample 11 (*Paralichthys olivaceus*) in Table 5.2b were calculated on the basis of 157 being encoded by the consensus region, as sequence data pertaining to the first 45 residues of the consensus were not available. Numbers in the margins denote:

(1) *Trypanosoma brucei* (MOB1-1)
(2) *Trypanosoma brucei* (MOB1-2)
(3) *Leishmania major*
(4) *Saccharomyces cerevisiae* [731855]
(5) *Schizosaccharomyces pombe* [3947877]
(6) *Dictyostelium discoideum* [2282525]
(7) *Arabidopsis thaliana* [2832633]
(8) *Gossypium hirsutum* [5048261]
(9) *Caenorhabditis elegans* [4262618]
(10) *Homo sapiens* [3342738]
(11) *Paralichthys olivaceus* [5039530]
(12) *Saccharomyces cerevisiae* MOB2 [1175942]

MOB1-2 corresponded to the start methionine of *MOB1-1*. Due to the fact that only single pass sequencing was conducted over this region the data must be interpreted with caution.

The open reading frame of the *T. brucei MOB1-1* was used to homology search databases using the BLAST algorithm. Amongst the sequences identified was a *Leishmania major* EST [AI034929], originally isolated during a screen of a promastigote cDNA library (Cambridge Institute for Medical Research), encoding the 5' terminus of the *Leishmania major MOB1* homologue. A λZAP-II clone containing the full-length *L. major* cDNA was kindly supplied by J. Blackwell through the WHO *Leishmania* Genome Initiative. Single pass sequencing of the insert was conducted in conjunction with M. McLaughlin using primers Leish 1-3 (Appendix II).

Homologous sequences from a variety of organisms were identified and protein sequences acquired for comparative alignment using the MegAlign programme. The resultant alignment is presented in Figure 5.9. The extreme N- and C-terminal regions of the proteins were dissimilar. A consensus region consisting of 202 residues is indicated and covers the conserved portion of the gene. Percentage identities between MOB1 (and MOB2) sequences were calculated for both the whole gene and the consensus region, and resultant data are presented in Tables 5.2a and 5.2b respectively. Comparison of the *T. brucei* MOB1-1 and MOB1-2 sequences revealed 78% identity between the proteins, with 94% identity over the consensus region. Analysis revealed 5 residue substitutions (not including the start residue of MOB1-1), 2 ambiguous residues in MOB1-2, and 6 residues of MOB1-2 within the consensus region but prior to the N-terminal residue of MOB1-1. Of the residue substitutions all but one are conservative,

the exception being serine-161 of MOB1-1 substituted as phenylalanine-244 of MOB1-2. The full length sequence of both the *Dictyostelium discoideum* and the *Paralichthys olivaceus* proteins were not available. The sequence of the *Saccharomyces cerevisiae* MOB2 protein was included for comparison. Data pertaining to *P. olivaceus* (sequence 11) are included for completeness, but are of limited value due to the absence of the proteins N-terminus and will not be discussed further. Data derived from comparison of the consensus region displays (predictably) a higher percentage identity than data derived from whole sequence comparisons. Values from the former range from 35% between the *L. major* sequence and those of *S. cerevisiae* and *Schizosaccharomyces pombe*, and 67% between the *L. major* and *T. brucei* MOB1-2.

Conserved motifs within the MOB1 protein were sought using *T. brucei* MOB1-1 peptide sequence as a template in conjunction with both PROSITE (http://expasy.hcuge.ch/sprot/prosite.html) and MOTIF (http://www.motif.genome.ad.jp) programmes. A putative casein kinase II phosphorylation site was discovered, consisting of S/T-x-x-D/E, where x denotes any non-basic residue. This site was situated between residues 65-68 inclusive of the consensus and was conserved between all MOB1 homologues. This site was not present on the *S. cerevisiae* MOB2 sequence. No other conserved motifs were found.

5.2.2 Production of a construct for expression of *MOB1-1* antisense RNA in *T. brucei*

Antisense RNA is complementary to, and forms duplexes with, either mature or precursor mRNA. Interaction between these two molecules is gene specific, involving base pairing between the antisense molecule and its target. mRNA transcripts bound in such RNA:RNA hybrids are blocked at one of the several translatory steps involved in protein synthesis. Gene function is therefore interrupted (Weiss *et al.*, 1999). Antisense-mediated gene silencing by expression of antisense RNA in transfected cells has become a complementing alternative to gene disruption or gene knockouts by homologous recombination (Lichtenstein and Nellen, 1997). This approach is now commonly used where no other gene disruption technique is available or complete gene knockout may result in a lethal phenotype.

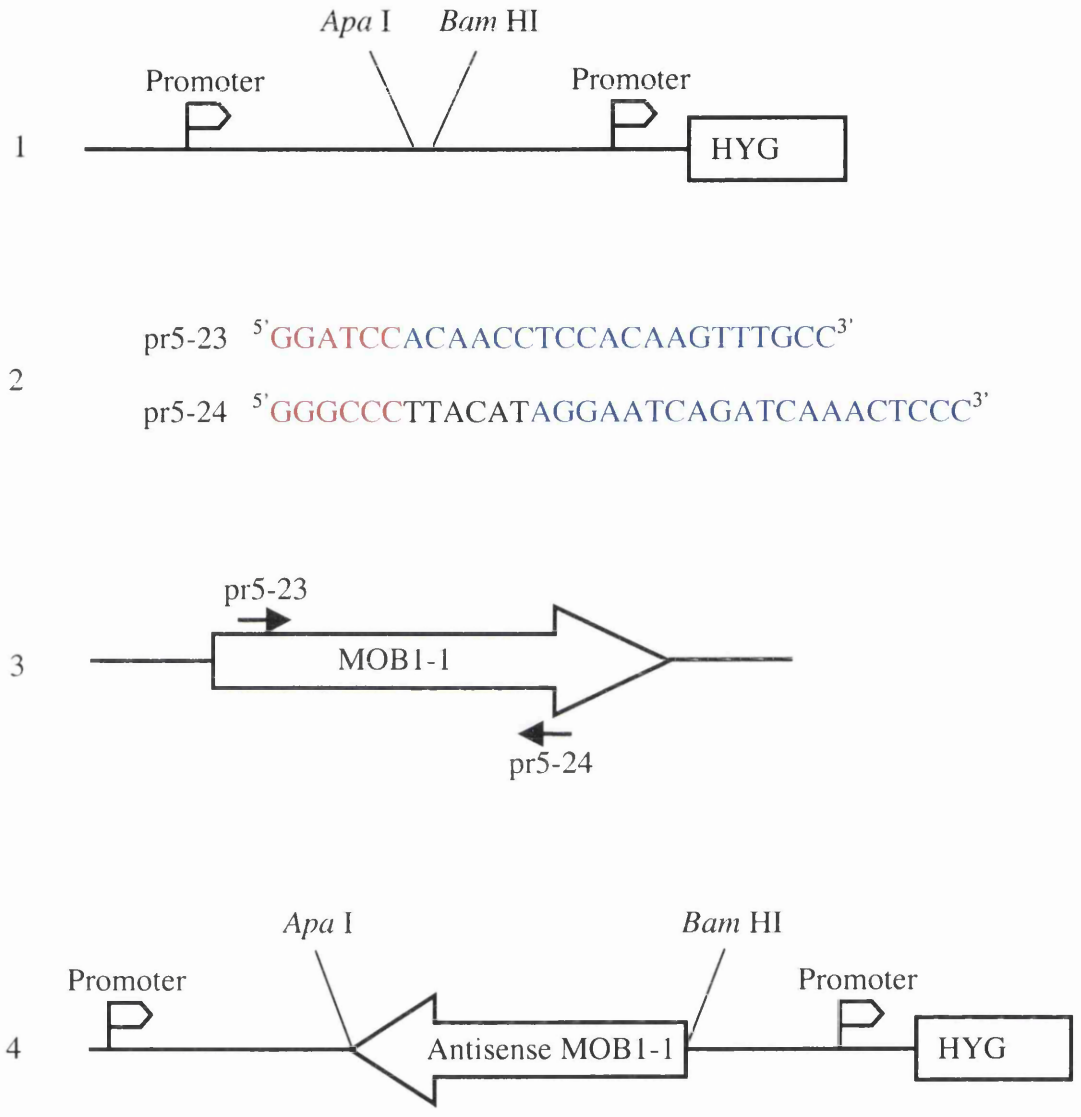
Due to the fact that 2 copies of the *T. brucei MOB1* gene (*MOB1-1* and *MOB1-2*) had been detected by Southern blot analysis it was not possible to conduct conventional gene knockouts immediately as a full map and sequence of the whole MOB1-1 and MOB1-2 loci was not available. Consequently, it was decided that inducible expression of an antisense *MOB1-1* fragment would be attempted in order to inhibit production of MOB1-1 protein. It was hoped that subsequent phenotypic analysis would give insights into gene function. Sequence data pertaining to *MOB1-2* was unavailable at this stage of the work, so all experimentation involving antisense (and subsequently double stranded) constructs was carried out using *MOB1-1* as the template sequence.

Primers pr5-23 and pr5-24 were designed to amplify bases 32-544 of the *MOB1-1* open reading frame (this represented the only region of the open reading frame for which

double stranded sequence was available at the time). Oligonucleotide sequences are detailed in Figure 5.10. PCR amplification of the *MOB1-1* fragment was carried out with *Pfu* DNA polymerase, using plasmid pPCR23Mob1 as template.

Following PCR amplification a single band at the predicted size (526 bp) was cloned into pGEM®-T Easy vector to give plasmid pAntiMobGEM. Plasmid DNA was produced from an overnight culture and digested sequentially with *Apa* I and *Bam* HI, producing fragments which were confirmed to be the correct size by agarose gel electrophoresis. The identity of the insert within plasmid pAntiMobGEM was confirmed by sequencing. The insert from pAntiMobGEM was cloned into the *Apa* I and *Bam* HI sites of plasmid pHD675 (Biebinger *et al.*, 1997). This produced construct pAntiMobHD675 in which the insert was in an antisense orientation with respect to the promoter, as detailed in Figure 5.10.

Figure 5.10 Schematic representation of subunits constituting pAntiMobHD675



Schematic representation of (1) the multiple cloning site of plasmid pHD675 with respect to the PARP promoter and HYG resistance gene. (2) Oligonucleotide sequences based upon analysis of the *MOB1-1* gene sequence. Regions in red denote restriction enzyme sites [*Bam* HI in pr5-23 and *Apa* I in pr5-24], and regions in blue denote sequence matching the open reading frame of the gene. (3) *MOB1-1* gene with PCR primers aligned. (4) 514 bp PCR product cloned into the multiple cloning site of pHD675 in an antisense orientation (pAntiMobHD675).

Introduction and inducible expression of plasmid pAntiMobHD675 in *T. brucei*

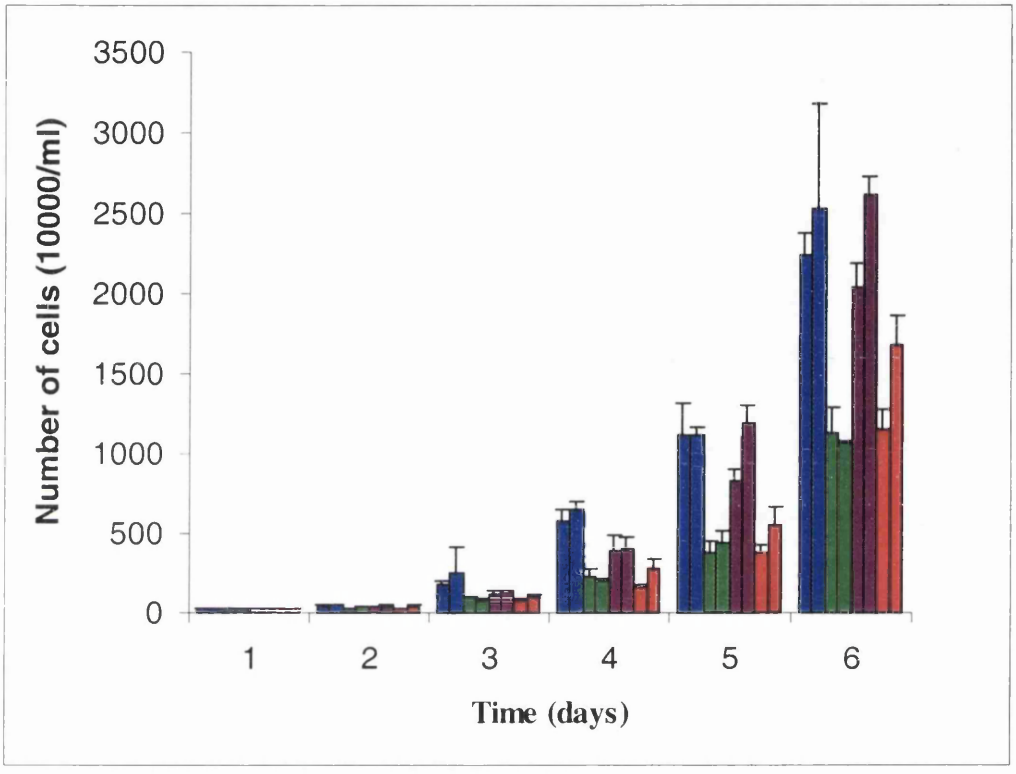
pAntiMobHD675 was linearised by digestion with *Not* I, extracted twice with phenol/chloroform then ethanol precipitated. Following resuspension in sterile dH_2O DNA was quantified spectrophotometrically at 260 nm. 20 μg linear pAntiMobHD675 was used to transfect *T. b. brucei* stock EATRO 795 containing integrated construct pHD449 (Biebinger *et al.*, 1997). Following overnight recovery populations were cloned by limiting dilution with appropriate antibiotic selection.

Two clonal populations survived selection, and will henceforth be referred to as tAntiMob clone 1 and 2. Cells from each population, together with untransfected controls, were induced overnight by addition of 20 ng/ml tetracycline to medium. Living cells were observed by phase contrast light microscopy at 200 x magnification. No obvious differences were detected between induced, non-induced or untransfected control cells. Cells from the same populations were harvested and slides prepared for viewing by DAPI staining as described previously. Fluorescence microscopy revealed no difference between cellular DNA content of induced, non-induced or untransfected controls.

In order to determine if growth rate was affected by expression of antisense *MOB1-1*, a 24 well plate was set up containing 6 replicates each of the following; tAntiMob clone 1, tAntiMob clone 2, cells containing construct pHD449, and cells containing constructs pHD449 and pHD675 (kindly supplied by T. Hammarton). Density in each well at the start of experimentation was 2 x 10^5 cells/ml. Three replicates from each clone were induced with 50 ng/ml tetracycline, while the other three replicates were not induced.

Cell density in each well was quantified daily using a haemocytometer. The experiment was terminated on the 6th day after initiation. Results are displayed in Figure 5.11.

Figure 5.11 Graphical representation of cell numbers over time in tAntiMob and control populations +/- tetracycline induction



Cells were cultured in complete SDM79 containing either 50 ng/ml tetracycline (first column of each doublet) or no tetracycline (second column of each doublet). Each column represents the mean value of three replicates. Error bars represent standard deviation. Blue columns represent control cells containing construct pHD449. Green columns represent control cells containing constructs pHD449 and pHD675. Purple columns represent tAntiMob clone 1. Red columns represent tAntiMob clone 2.

Statistical significance of the data was determined using a Repeated Measures Analysis Of Variance (RMANOVA). This test can be used to determine not only whether there is a significant difference between data sets, but also whether that difference changes with time. For control cells containing construct pHD449 the effect of tetracycline

induction on cell density was not significant ($F_{1,4} = 1.789$, $p = 0.825$). Similarly, control cells containing both constructs pHD449 and pHD675 showed no significant difference in cell density between induced and non-induced cells ($F_{1,4} = 0.251$, $p = 0.643$). By contrast, both tAntiMob clone 1 and tAntiMob clone 2 displayed significantly fewer cells in induced populations relative to non-induced counterparts ($F_{1,4} = 18.813$, $p = 0.012$ and $F_{1,4} = 37.496$, $p = 0.004$ respectively). Furthermore, in both tAntiMob clone 1 and tAntiMob clone 2 the difference between induced and non-induced populations was shown to increase with time ($F_{5,20} = 18.832$, $p < 0.001$ and $F_{5,20} = 10.837$, $p < 0.001$ respectively). This data demonstrates clearly that expression of *MOB1*-specific antisense RNA in *T. brucei* results in a reduction in population growth rate, indicating that MOB1-1 and/or MOB1-2 protein is required for normal growth of procyclic *T. brucei*. (Statistical analysis courtesy of S. Humphries).

The phenotype observed in this experiment was not as pronounced as had been hoped, taking 4-6 days to reach levels where it was readily detected. Consequently, it was decided that inducible expression of *MOB1-1* double-stranded RNA in *T. brucei* would be attempted.

5.2.3 Production of a construct for expression of *MOB1-1* double-stranded RNA in *T. brucei*

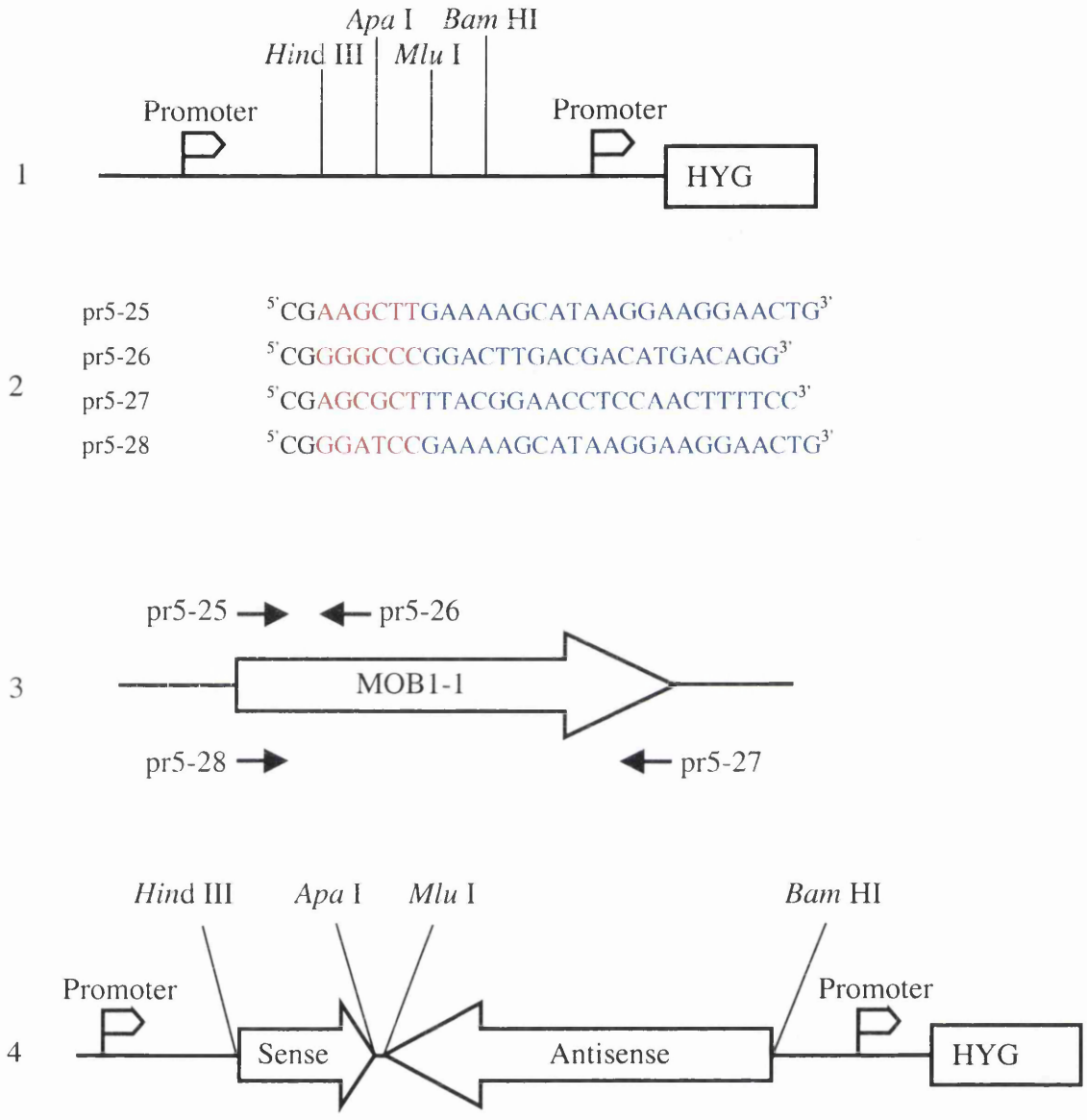
The action of double-stranded RNA (dsRNA) has been demonstrated to be a gene-specific phenomenon, resulting in silencing of expression in *C. elegans* (Fire *et al.*, 1998), *T. brucei* (Ngo *et al.*, 1998) and plants (Waterhouse *et al.*, 1998). Furthermore, in both *C. elegans* and plants the effect of dsRNA, termed RNA interference (RNAi), has been shown to be a far more potent inhibitor of gene expression than its antisense RNA counterpart (Fire *et al.*, 1998; Waterhouse *et al.*, 1998). The mode of action of dsRNA remains a subject of conjecture, although current evidence points away from non-reversible alteration in cellular DNA (Montgomery *et al.*, 1998) and towards targeted mRNA degradation (Ngo *et al.*, 1998; Waterhouse *et al.*, 1998).

Ngo *et al.* (1998) describe plasmid pGFPFAT which contains, amongst other things, two complete copies of the *T. brucei* α-tubulin 5' UTR (113 bp each) in a head-to-head configuration, separated by a 700 bp sequence unrelated to α-tubulin. Transient expression of this plasmid in *T. brucei* resulted in cells becoming rounded, possessing multiple nuclei and kinetoplasts and displaying pronounced ruffling on the cell surface. It was proposed that expression of plasmid pGFTFAT produced a transcript that formed a dsRNA stem consisting of the complementary α-tubulin UTRs, and a single-stranded RNA loop corresponding to the 700 bp insert. Subsequent analysis involving transfection of cells with *in vitro* synthesised α-tubulin-specific dsRNA revealed that the aforementioned morphology was related to specific degradation of α-tubulin mRNA.

In order to inducibly express a *MOB1-1* dsRNA construct in *T. brucei*, primers pr5-25 and pr5-26 were designed to amplify bases 4-227, and primers pr5-27 and pr5-28 to amplify bases 4-624, of the *MOB1-1* open reading frame. Oligonucleotide sequences are detailed in Figure 5.12. PCR amplification of the *MOB1* fragments was as described previously, using *Pfu* DNA polymerase together with pPCR23Mob1 as template. PCR products of the correct size were isolated and cloned into pGEM-T vector to produce plasmids p240dsMobGEM and p640dsMobGEM respectively. The identity of the inserts was confirmed by sequence analysis.

The *Hin*d III/*Apa* I insert from p240dsMobGEM was sub-cloned into the *Hin*d III/*Apa* I sites of pHD675, as shown in Figure 5.12, to produce plasmid p240dsHD675. The Mlu I/Bam HI insert from p640dsMobGEM was sub-cloned into the Mlu I/Bam HI sites of p240dsHD675, as shown in Figure 5.12, to give plasmid pRNAiMobHD675.

Figure 5.12 Schematic representation of subunits constituting pRNAiMobHD675



Schematic representation of (1) the multiple cloning site of plasmid pHD675 with respect to the PARP promoter and HYG resistance gene. (2) Oligonucleotide sequences based upon analysis of *MOB1-1* gene. Regions in red denote restriction enzyme sites [*Hind* III, *Apa* I, *Mlu* I and *Bam* HI respectively], and regions in blue denote sequence matching the open reading frame of the gene. (3) *MOB1-1* gene with PCR primers aligned. (4) 220 and 620 bp *MOB1-1* fragments within the multiple cloning site of pHD675 in a head-to head orientation (pRNAiMobHD675).

Introduction and inducible expression of plasmid pRNAiMobHD675 in *T. brucei*

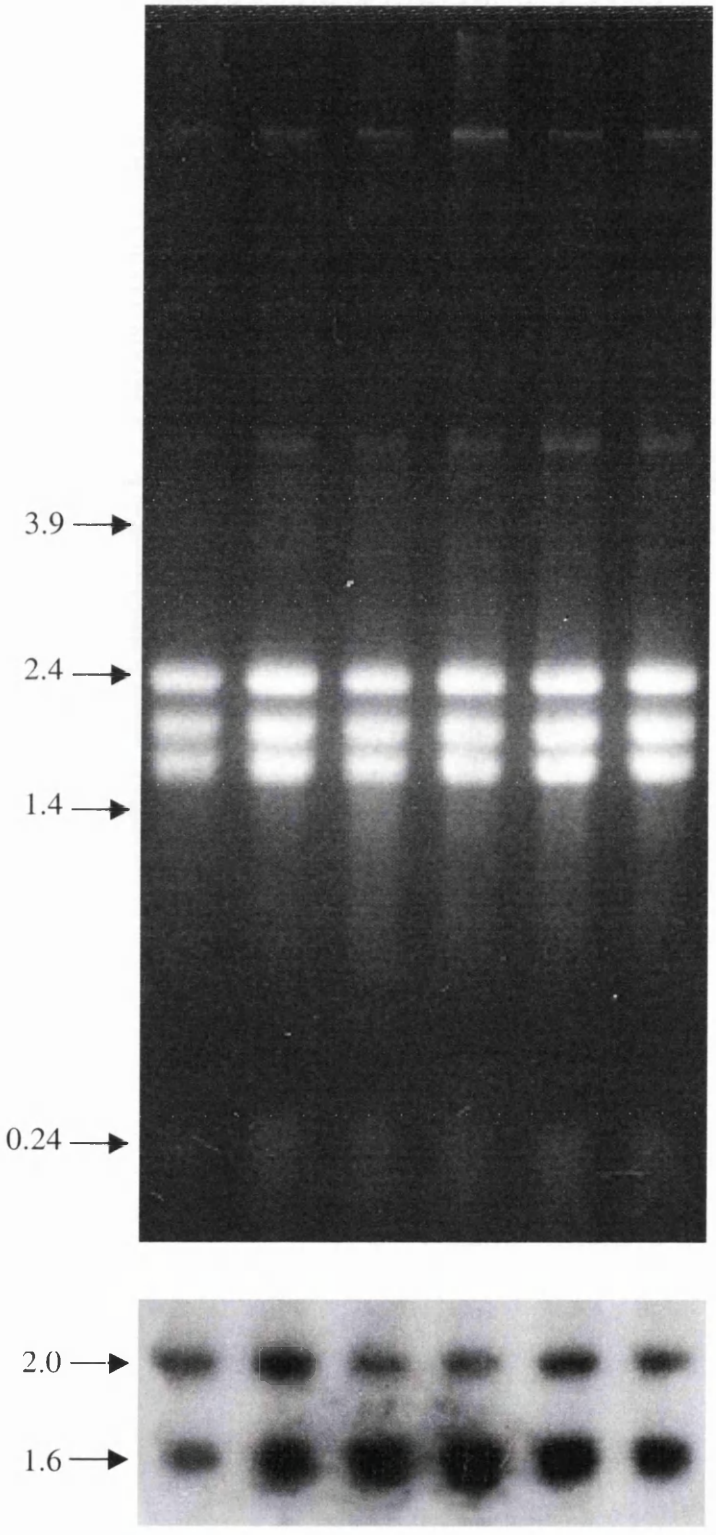
Plasmid pRNAiMobHD675 was linearised by digestion with *Not* I, extracted twice with phenol/chloroform, ethanol precipitated and resuspended in sterile dH_2O at 2 mg/ml. 30 μg linear pRNAiMobHD675 was then used to transfect *T. b. brucei* stock EATRO 795 containing construct pHD449. Following overnight recovery populations were cloned by limiting dilution with appropriate antibiotic selection.

Two clonal populations survived selection, and were named tRNAiMob clones 1 and 2. Cells from each population, together with untransfected controls, were induced overnight by addition of 20 ng/ml tetracycline to medium. Phase contrast and fluorescence microscopy were carried out on cells as describes previously for tAntiMob clones 1 and 2. No differences were detected between controls and induced cells under these conditions.

Total RNA was prepared from 1 x 10^9 cells from populations of tRNAiMob clones 1 and 2 and untransfected controls +/- tetracycline induction. RNA was quantified spectophotometrically and 10 μg subjected to agarose gel electrophoresis. RNA was blotted onto Hybond N+ membrane, UV cross-linked then hybridised with a ^{32}P-labelled probed derived from the insert of clone pTyMobGEM1 and representing the open reading frame of *MOB1-1*. Signal was detected with autoradiograph film. Results are displayed in Figure 5.13.

Levels of hybridisation were relatively constant between both experimental and control groups, the exception being a reduction in signal detected from control cells without tetracycline (Figure 5.13, lane 1). It is thought that this was a result of slightly lower

Figure 5.13 Northern blot analysis of tRNAiMob clones 1 and 2 +/- tetracycline



Total RNA was prepared from tRNAiMob clones 1 and 2 following overnight culture in the presence or absence of 20 ng/ml tetracycline induction. Control cells of the parental cell line contained construct pHD449, and were also subjected to culture +/- tetracycline. 10 μg total RNA from each sample was subjected to agarose gel electrophoresis (upper image), then blotted and hybridised with a ^{32}P-labelled probe derived from the insert of clone pTyMobGEM and containing the open reading frame of *MOB1-1*. Signal was detected autoradiographically (lower image). Lanes were (1) control, (2) control + tetracycline, (3) tRNAiMob clone 1, (4) tRNAiMob clone 1 + tetracycline, (5) tRNAiMob clone 2 and (6) tRNAiMob clone 2 + tetracycline. Numbers on the left denote sizes in kp.

loading of RNA in this lane, as indicated by reduced intensity of rRNA bands detected by EtBr staining. The marginal differences observed in hybridisation between experimental lanes indicated that either the RNAi had not been expressed, or that expression had occurred without effecting levels of *MOB1-1* or *MOB1-2* mRNA. One observation of note was the increase in relative intensity of smaller to larger transcript when compared to previous analysis (Figure 3.8, Plate C). The reason for this shift in relative transcript abundance in these cells is not known, but could be related to cell lineage.

5.2.4 Production and inducible expression of a Ty-tagged MOB1-1 protein in *T. brucei*

Sequence encoding a Ty-1 epitope tag (Bastin *et al.*, 1996) was added into the 3' terminus of the *T. brucei MOB1-1* gene (Figure 5.14). Oligonucleotides for this purpose were designed and PCR amplification of the *MOB1-1* open reading frame carried out using *Pfu* proof reading DNA polymerase in order to minimise the probability of introduced errors, together with genomic DNA as template and primers pr5-29 and pr5-30. The single PCR product of 670 bp was cloned into pGEM®-T Easy vector to give plasmid pTyMobGEM.

Plasmid pTyMobGEM was digested with *Hind* III and *Mlu* I in order to release the 670 bp insert for subcloning into *Hind* III/*Mlu* I digested plasmid pHD675, producing plasmid pTyMobHD675.

Plasmid pTyMobGEM was sequenced using commercial T7 and SP6 RNA polymerase primers [Promega]. Sequence data derived from the T7 primer was of good quality, corresponded to the 3' end of the gene, and indicated that no errors had been introduced into the sequence during PCR amplification. Sequence data derived from the SP6 primer was of insufficient quality to determine with certainty whether errors had been introduced into the 5' end of the gene. Due to time constraints it was decided to proceed with work on plasmid pTyMobHD675 in the absence of sequence confirmation.

Figure 5.14 Oligonucleotide sequences for introduction of Ty-1 epitope tag into *T. brucei* MOB1-1

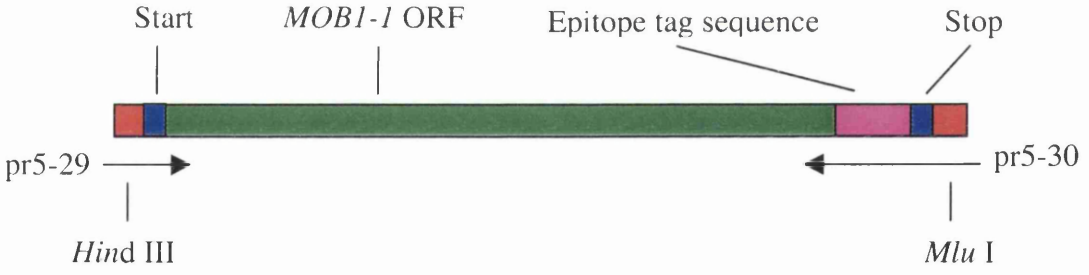
Forward primer (pr5-29)

$^{5'}$CGAAGCTTATGGAAAAGCATAAGGAAGGAACTG$^{3'}$

Reverse primer (pr5-30)

$^{5'}$CGACGCGTTTAGTCAAGTGGATCCTGGTTAGTATGGACCTCCGGAA CCTCCAACTTTTCCTTCG$^{3'}$

PCR product



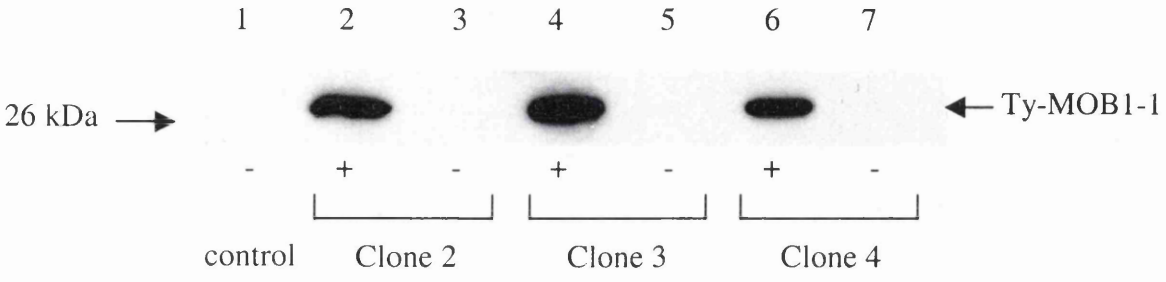
Oligonucleotides for the introduction of sequence encoding a Ty-1 epitope tag into the *T. brucei MOB1–1* was based upon analysis of the open reading frame of the gene (Section 5.2.1) and the published sequence of the Ty-1 epitope (Bastin *et al.*, 1996). Regions in red denote restriction enzyme sites [*Hind* III in forward and *Mlu* I in reverse], regions in blue denote the start and stop codon, the region in pink denotes sequence encoding the Ty-1 epitope tag and regions in green denote the terminal 5' and 3' bases of the *MOB1-1* open reading frame respectively.

Introduction and expression of construct pTyMobHD675 in *T. brucei*

Plasmid pTyMobHD675 was linearised by digestion with *Not* I, extracted twice with phenol/chloroform, ethanol precipitated and resuspended at 2 mg/ml in sterile dH_2O. 20 μg linear pTyMobHD675 was used to transfect a clone of *T. b. brucei* stock EATRO 795 containing a copy of construct pHD 449 (Biebinger *et al.*, 1997) stably integrated into its genome. Following overnight recovery populations were immediately cloned by limiting dilution with appropriate antibiotic selection.

Phenotypic analysis was carried out on three clonal populations (from a total of five). Following overnight induction with tetracycline 1 x 10^8 cells were harvested. Protein equivalent to 2 x 10^5 trypanosomes was subjected to glycine SDS-PAGE and Western blotting, then immunodetection using the mouse monoclonal BB2 α-Ty as the primary antibody (Bastin *et al.*, 1996) and α-mouse IgG/HRP conjugate [Promega] as secondary antibody. Detection of antibody was through use of the SuperSignal® system [Pierce]. Results are in Figure 5.15 below.

Figure 5.15 Immunoblot analysis of clonal procyclic populations transfected with construct pTyMobHD675



Immunoblot of total protein from 2 x 10^5 procyclic trypanosomes / lane probed with monoclonal antibody BB2. Cells were (1) uninduced EATRO 795 containing construct pHD 449, (2) induced clone 2, (3) uninduced clone 2, (4) induced clone 3, (5) uninduced clone 3, (6) induced clone 4 and (7) uninduced clone 4.

The predicted size of the TyMOB1 protein was 26 kDa. As can be seen in Figure 5.15 each induced population gave a band of the correct size, while uninduced cells did not produce detectable levels of the protein. As described previously the BB2 antibody did not cross-react with proteins from untransfected control cells (lane 1). Clonal cell lines 2, 3 and 4 will henceforth be referred to as tTyMob clones 2, 3 and 4 respectively.

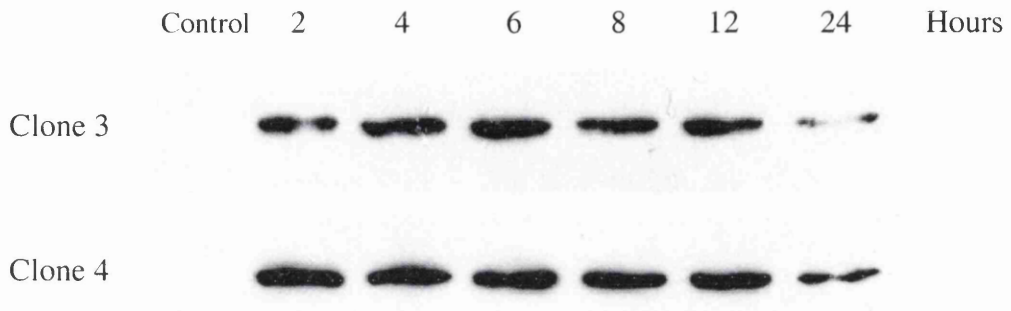
Stability of TyMOB1-1 *in vivo*

In order to determine the stability of the TyMOB1-1 protein within procyclic trypanosomes, tTyMob clones 3 and 4 were induced to produce protein overnight. Cells were harvested, washed once with fresh SDM 79 to remove tetracycline then resuspended at 1 x 10^6 cells/ml in fresh complete SDM 79. At timepoints 0, 2, 4, 6, 12

and 24 hours after removal of tetracycline 1 x 10^8 cells were harvested and frozen at –20°C for temporary storage. Control cells were of the same cell line as experimental cells but were not exposed to tetracycline induction. Protein equivalent to 4 x 10^5 cells was subjected to glycine SDS-PAGE, Western blotting and immunodetection as described previously. Results are presented in Figure 5.16.

The stability of the TyMOB1-1 protein expressed by tTyMob clones 3 and 4 was comparable to that reported in the previous chapter for TyQM (Section 4.2.3). No detectable reduction in protein levels was observed in either clone during the first 12 hours post induction, and the decrease observed thereafter is likely to be due in part to the increase in cell numbers relative to tagged protein. The unknown factor in this experiment is of course the stability of the mRNA encoding the tagged protein - a stable transcript could result in persistence of translation for several hours after removal of induction, thus interfering with determination of protein stability.

Figure 5.16 Turnover of TyMOB1-1 protein within procyclic cells over time

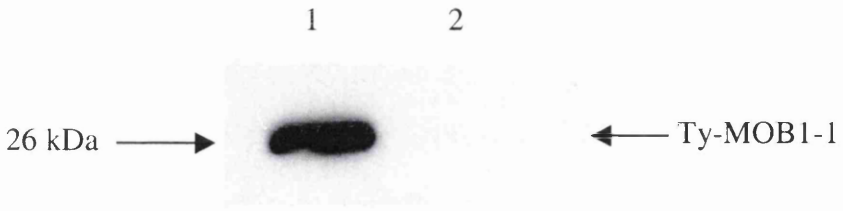


Procyclic tTyMob clones 3 and 4 were induced overnight, harvested, washed and resuspended at 1 x 10^6 cells/ml in fresh complete SDM 79. 1 x 10^8 cells were harvested at time-points 0, 2, 4, 6, 12 and 24 hours after removal of tetracycline. Protein equivalent to 4 x 10^5 trypanosomes was subjected to immunoblot analysis with BB2 monoclonal antibody. Controls were from the same stock as experimental cells.

Cell fractionation analysis of TyMOB1-1 protein

Procyclic tTyMob clone 4 cells were induced to express protein overnight. 2 x 10^8 cells were harvested as described previously, pelleted then resuspended in 1 ml LSGI (Section 2.21) for 30 minutes on ice. Lysate was centrifuged at 100 000 x g for 45 minutes at 4°C in a Beckman Optima™ TL Ultracentrifuge. Supernatant was aspirated and stored on ice, and the pellet washed 4 times with 1 ml LSGI before being resuspended in 75 µl 0.25% Triton-X 100 and 25 µl 4 x Laemmli buffer. Protein from the soluble fraction and insoluble fractions equivalent to 5 x 10^5 cells was subjected to glycine SDS-PAGE, Western blotting and immunodetection. Results are presented in Figure 5.17.

Figure 5.17 Cell fractionation analysis of TyMOB1-1



tTyMob clone 4 cells were lysed in LSGI then fractionated by ultracentrifugation at 100 000 x g. Protein equivalent to 5 x 10^5 trypanosomes from (1) soluble fraction and (2) insoluble fraction was subjected to immunoblot analysis using BB2 α-Ty.

Following disruption of cells in LSGI buffer and ultracentrifugation at 100000 x g the TyMOB1-1 protein was found to be present in the soluble fraction alone (lane 1). The soluble nature of this protein suggests that it is either not part of a large complex, or that it is peripherally associated with such a complex and that this interaction is disrupted by LSGI which contains 1% Triton X-100.

Indirect immunofluorescence microscopic analysis to determine subcellular location of the TyMOB1-1 protein *in vivo*

Due to the fact that all tTyMob populations analysed produced similar levels of tagged protein coupled with undetectable non-induced expression, immunofluorescence analysis was carried out on a single cell line, tTyMob clone 4. Cells were induced to express protein overnight by incubation with 20 ng/ml tetracycline, following which slides were made as described in Section 2.18. Immunofluorescence microscopy was carried out using the mouse BB2 (α-Ty) as primary antibody, and goat α-mouse IgG/FITC conjugate [Sigma] as secondary antibody.

The level of fluorescence detected within cells was low, requiring relatively long exposure times to be detected. As a consequence, fluorescence with relation to the cell cycle was not thoroughly investigated. At the subcellular level the TyMOB1-1 protein appeared to display a homogenous distribution, with the possibility of slightly elevated levels proximal to the nucleus (Figure 5.18). This lack of localisation within the cell led to 3 hypotheses. Firstly, that there was localisation of the tagged protein within the cell, but that overexpression resulted in "swamping" of the signal. Secondly, that introduction of the Ty-1 epitope tag into the C-terminus of the protein resulted in disruption or loss of a localisation signal, causing the tagged protein to localise differently to its native counterpart. Finally, that the native MOB1-1 protein also exhibited a generalised distribution throughout the cell.

In order to investigate the first of these hypotheses, immunofluorescence microscopy was carried out as described above, using 5 ng/ml tetracycline to induce cells instead of the customary 20 ng/ml. It was hoped that by lowering the level of the induction signal, lower levels of protein would be produced, thus allowing any localisation to be more easily detected. As can be seen in Figure 5.19, reduced levels of protein were observed within cells under this induction regimen, as compared to the previous study (Figure 5.18). However, while a more punctate pattern was observed, distribution indicative of organellar localisation was not detected. Washing tetracycline away from cells and performing microscopy at later time-points also failed to detect any distribution other than cytoplasmic. If localisation of TyMOB1-1 did occur within cells, it was not detectable under the conditions employed in this study.

Figure 5.18 Immunofluorescence microscopic analysis of procyclic *T. brucei* induced to overexpress TyMOB1-1 with 20 ng/ml tetracycline

tTyMob clone 4 cells were cultured overnight in the presence of 20 ng/ml tetracycline to induce overproduction of Ty-Mob1-1 protein. Cells were fixed, permeabilised and treated with the mouse monoclonal BB2 α-Ty as primary antibody, then goat α-mouse/FITC conjugate as secondary antibody. Control cells were from the same parental cell line and contained construct pHD449 but not pTyMobHD675. DAPI was added to cells during incubation with secondary antibody. Tiles are (1) phase contrast, (2) DAPI, (3) α-Ty and (4) α-Ty merged with DAPI. Tiles (5) and (6) are phase contrast and α-Ty with control cells. The bar at the top of each tile denotes 5 um.

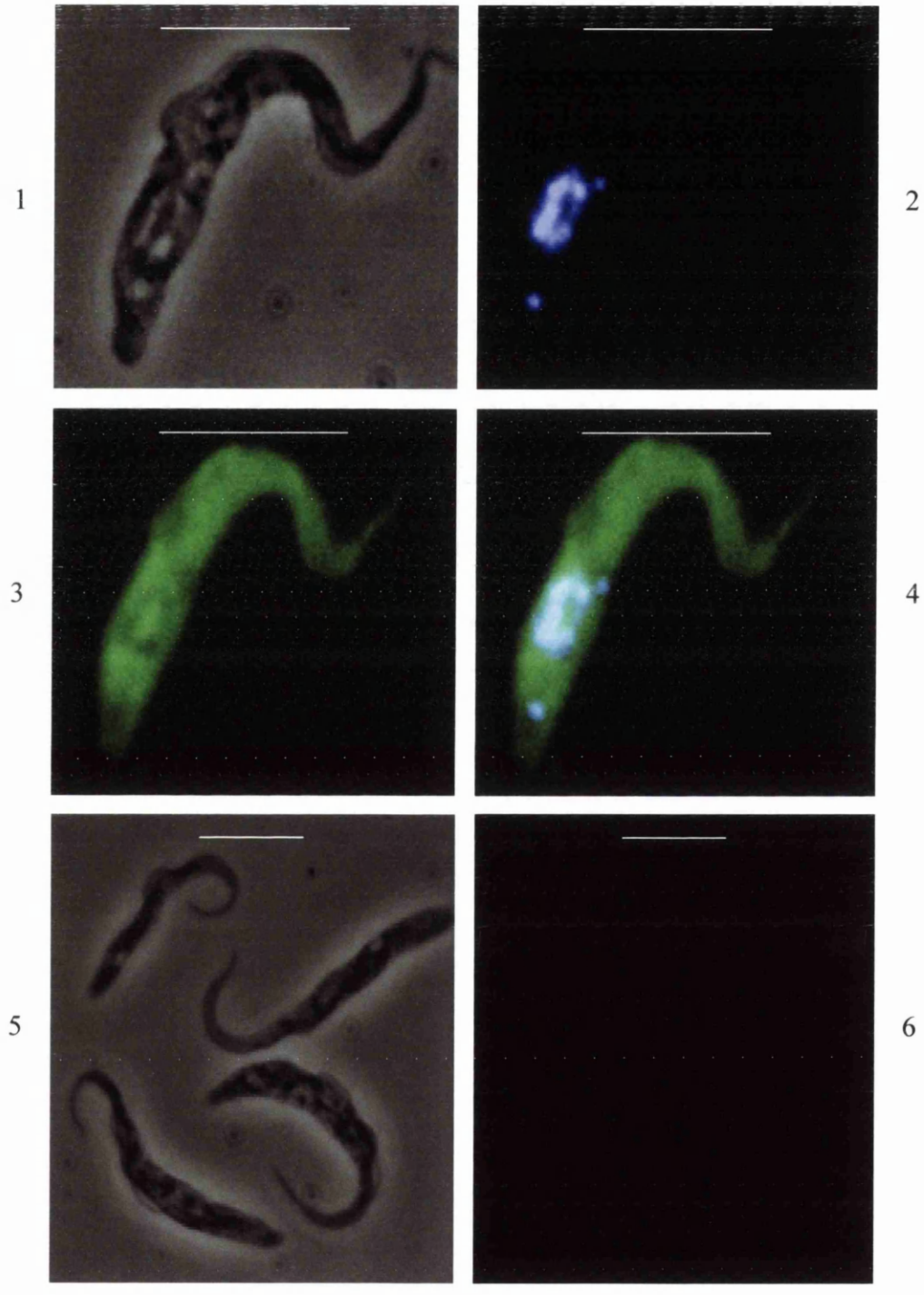
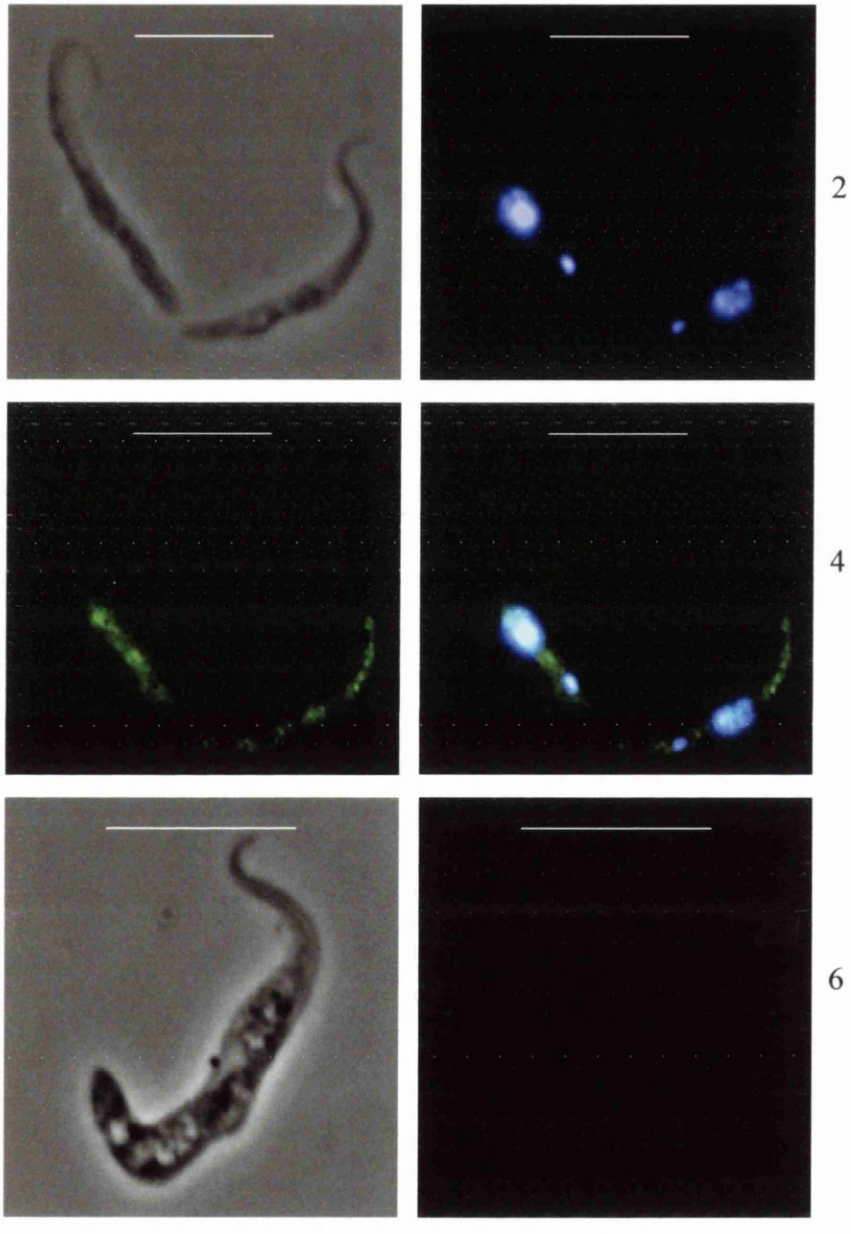

1
2
3
4
5
6

Figure 5.19 Immunofluorescence microscopic analysis of procyclic *T. brucei* induced to overexpress TyMOB1-1 with 5 ng/ml tetracycline

Procyclic cells of tTyMob clone 4 were cultured overnight in the complete SDM79 supplemented with 5 ng/ml tetracycline. Cells were fixed, permeabilised and treated with mouse monoclonal BB2 α-Ty as primary antibody then goat α-mouse/FITC conjugate as secondary antibody. Control cells were from the same parental cell line as experimental cells, and contained construct pHD449 but not construct pTyMobHD675. DAPI was added to cells during incubation with secondary antibody. Tiles are (1) phase contrast, (2) DAPI, (3) α-Ty and (4) α-Ty merged with DAPI. Tiles (5) and (6) are phase contract and α-Ty with control cells. The bar at the top of each tile denotes 5 um.



In order to answer the question of whether the subcellular distribution of TyMOB1-1 was similar to that of its native MOB1-1 counterpart, an antibody specific to the native protein was required. Production and purification of a recombinant protein for this purpose is described in the Section 5.2.5.

5.2.5 Production of a MOB1-maltose binding protein fusion protein

In order to produce an antibody to the *T. brucei* MOB1, it was necessary to first produce and purify a recombinant MOB1-1 protein for injection into rabbits. The system used to achieve this was the pMAL protein fusion and purification system [New England Biolabs]. The principle behind this system is that the gene of interest (in this case *MOB1-1*) is cloned into the pMAL vector immediately downstream of the malE gene, which encodes maltose binding protein (MBP). Following induction of expression with IPTG *Escherischia coli* could produce up to 100 mg of the MBP-MOB1 fusion protein per litre of culture. This can be affinity purified from other cellular constituents by passing soluble cell lysate through a column containing amylose resin, washing thoroughly then eluting by addition of 10 mM maltose. The MBP-MOB1 fusion protein contains a factor Xa recognition site (I-E/D-G-R) in the linker region between the MBP and the protein of interest, allowing cleavage and subsequent purification of protein by one of several possible methods.

At this stage of the project sequence data pertaining to the 5' terminus of the MOB1-1 ORF was not available. Consequently primers pr5-31 and pr5-32 were designed to amplify the 3' terminal 408 bp of the *MOB1-1* gene (Figure 5.20). This *MOB1-1* sequence encodes a predicted MOB1-1 peptide of 17 kDa. PCR amplification was performed with *Pfu* DNA polymerase, primers pr5-31 and pr5-32 and pMOB1 as

template. A single fragment of the predicted size of 438 bp cloned into pGEM®-T Easy vector to give plasmid pMobMBPGEM. Plasmid DNA was digested sequentially *Bam* HI and *Hind* III then sub-cloned into Bam HI/Hind III cut plasmid pMAL™-c2 to give plasmid pMobMBPMAL. The identity of the insert within plasmid pMobMBPGEM was confirmed by sequence analysis.

Figure 5.20 Primers for amplification of the 3' terminus of the *T. brucei MOB1* gene

Forward primer (pr5-31)

5'GGATCCTCGTCAGGTCCACGCTATGAG3'

Reverse primer (pr5-32)

5'AAGCTTGCGCACACAAATTGTTTACGG3'

Primer sequences for amplification of the 3' terminal 408 bp of the *T. brucei MOB1-1* gene were based upon sequence analysis of the open reading frame of the gene (Section 5.2.1). Regions in red denote restriction enzyme sites [*Bam* HI in forward and *Hind* III in reverse], region in blue denotes the stop codon, regions in green denote bases corresponding to the *MOB1-1* open reading frame and the region in black denotes the *MOB1* 3' flank.

MBP-MOB1 fusion protein was prepared as described in Section 2.8.6. A trial factor Xa cleavage was carried out in order to determine that the MBP-MOB1 fusion protein would indeed cleave, and that the factor Xa did not cleave at non-canonical sites within the MOB1 protein fragment. Trial cleavage was as suggested in the pMAL protein fusion and purification system instruction manual (NEB). Briefly, 0.2 μg factor Xa was

added to 20 μg affinity purified fusion protein in a final volume of 21 μl. 5 μl aliquots were placed in each of 4 tubes, which were then incubated at room temperature for 2, 4, 8 or 24 hours. At the indicated time points 5 μl 2 x Laemmli sample buffer was added to the appropriate tube, which was then transferred to 4°C for temporary storage. Once all time points had been collected samples were subjected to glycine SDS-PAGE, together with an un-cleaved control and appropriate standards. The gel was coomassie stained and is displayed in Figure 5.21.

Cleavage of the MBP-MOB1 fusion protein by factor Xa was successful under the conditions employed, converting the 60 kDa fusion protein into its respective 42 kDa MBP and 17 kDa MOB1 fragments as predicted. The level of cleavage observed within 24 hours, while not complete, was deemed acceptable for preparation of protein on a larger scale. Consequently, 100 μg factor Xa was added to 10 mg affinity purified MBP-MOB1 fusion in a final volume of 5 ml and incubated at room temperature for 24 hours. Glycine SDS-PAGE and coomassie staining of a 5 μl aliquot determined the percentage cleavage at this time to be comparable to that demonstrated in Figure 5.21 (lane 5). A fine precipitate was observed subsequent to cleavage. In order to determine the nature of this material 100 μl cleaved protein was centrifuged at 14000 rpm for 1 hour at 4°C in a micro-centrifuge. Supernatant was collected and stored on ice, and the pellet was washed twice in 100 μl 20 mM Tris-HCl (pH 8.0), before being resuspended in 50 μl of the same buffer. Supernatant, washes and pellet material were subjected to tricene SDS-PAGE and coomassie staining. Results can be seen in Figure 5.22.

Figure 5.21 Trial cleavage of MBP-fusion protein with factor Xa

MBP-fusion protein at 1 mg/ml was cleaved by addition of 1% w/w factor Xa. Following incubation at room temperature samples were collected at 2, 4, 8 and 24 hours. Samples were subjected to glycine SDS-PAGE, together with an un-cleaved control and appropriate standards, and coomassie stained. Lanes are (1) uncleaved, (2) 2 hours, (3) 4 hours, (4) 8 hours and (5) 24 hours incubation. Numbers in the left margin denote the size of protein standards in kDa.

Figure 5.22 Investigation of precipitate produced during MBP-fusion protein cleavage

Following cleavage of MBP-fusion protein with factor Xa, precipitate from 100 µl sample was pelleted, washed twice and resuspended in 50 µl 20 mM Tris-HCl (pH 8.0). Lanes are (1) soluble material, (2) wash 1, (3) wash 2 and (4) insoluble material. Numbers in the left margin denote the size of protein standards in kDa.

Figure 5.21

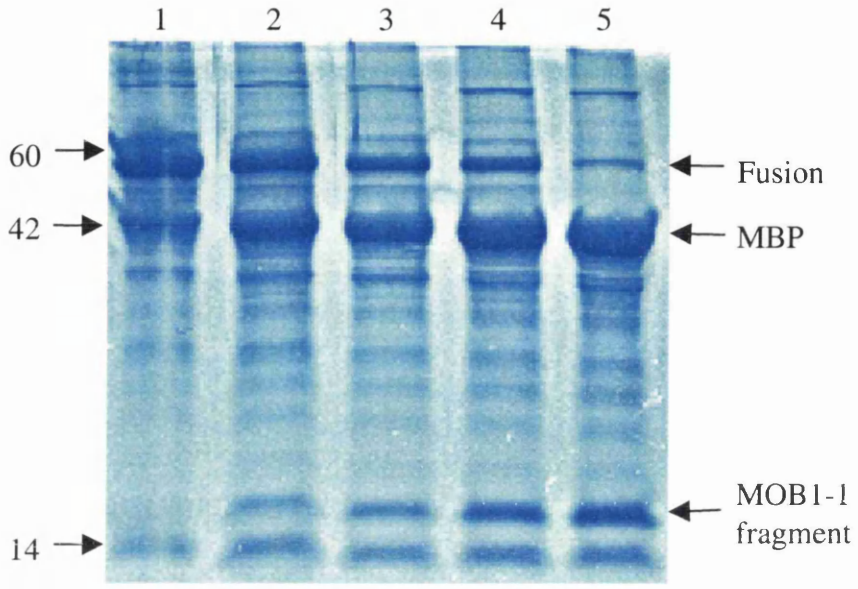
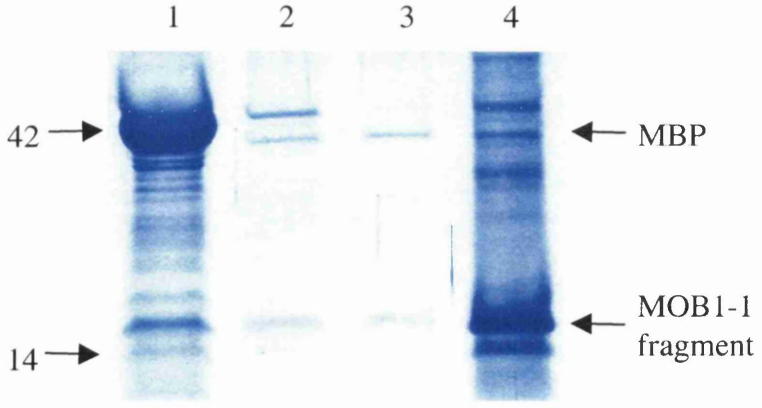


Figure 5.22



Following cleavage from its MBP partner the MOB1-1 fragment, together with several contaminant proteins, became insoluble. As affinity purification following cleavage was not feasible without first making the MOB1-1 fragment soluble, it was decided that gel purification of protein was a more practical option. Consequently, 600 μg cleaved protein in a final volume of 300 μl was subjected to tricine SDS-PAGE together with appropriate pre-stained standards. Protein was detected by immersion of the gel in 100 ml 4 M sodium acetate for 30 minutes. This treatment caused SDS within the gel to precipitate, highlighting protein as clear bands on a cloudy background. The appropriate region was excised from the gel and washed in 3 changes of 100 ml dH_20 for 15 minutes each. The gel slice was frozen and ground to a fine powder under liquid nitrogen using a mortar and pestle. Powdered acrylamide/protein was collected and the volume made to 1 ml with dH_2O. This suspension was passaged through a 25-gauge needle until it flowed freely, then divided into 4 x 250 μl aliquots and frozen. Acrylamide/protein was sent to the Scottish Antibody Production Unit (Law Hospital, Carluke, Lanarkshire) for inoculation into rabbits. The above procedure was repeated a second time in order to produce sufficient protein to inoculate 2 rabbits 4 times each (8 x 250 μl).

Rabbits R753 and R754 were injected with acrylamide/protein at 28 day intervals following the initial immunisation, and were bled on the seventh day following each injection. Serum resulting from bleeds was analysed by Western immunoblot analysis for its ability to detect both MOB1-1 fusion protein and native MOB1-1 of *T. brucei*. Serum resulting from the first bleed of each rabbit (35 days after initial immunisation) was able to detect the MOB1-1 fusion protein, but did not detect a band of the size predicted for *T. brucei* MOB1-1 in trypanosome lysates. Furthermore, extremely high

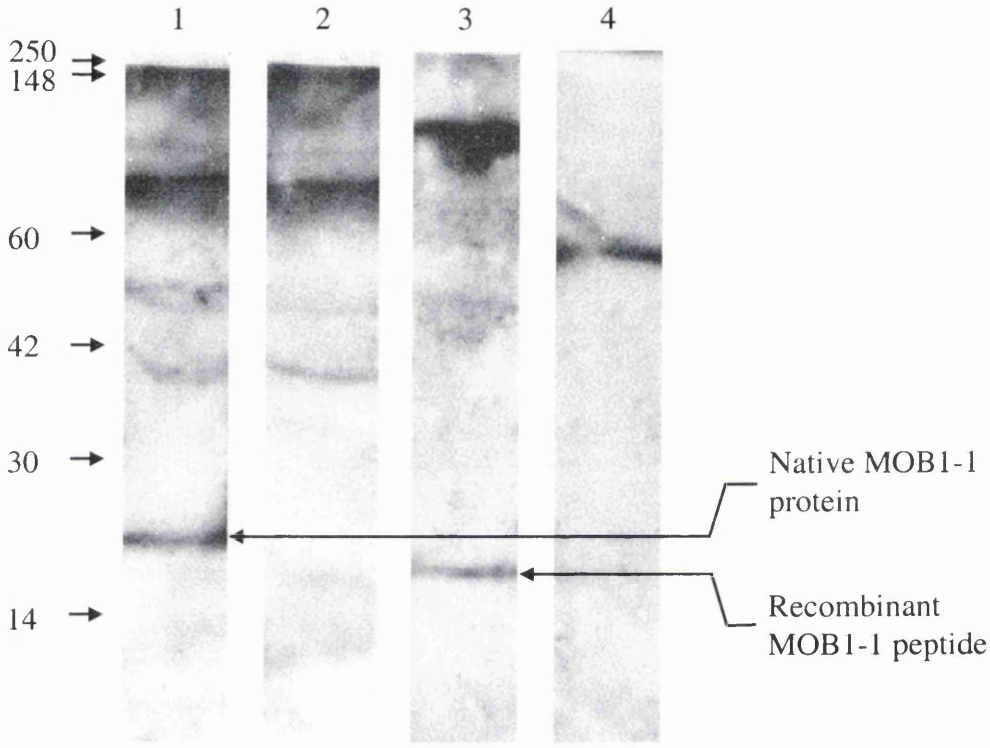
background signal was encountered when using the blotting and immunodetection protocol detailed in Section 2.17.3.

Serum resulting from the second bleed of R754 contained evidence of a significant amount of haemolysis and was not analysed further. Serum from the second bleed of rabbit R753 was subjected to immunoblot analysis based on the method outlined in Section 2.17.3 with modifications. Hybond-C Super membrane was substituted with PolyScreen® PVDF Transfer Membrane (NEN™ Life Science Products, Inc., Boston, USA). TBST was substituted with PBST. The remainder of the protocol was as previously described. A glycine SDS-PAGE gel was run with procyclic *T. brucei* extracts, and factor Xa-cleaved MBP-MOB1 fusion protein. Results are in Figure 5.23.

When the factor Xa-cleaved MBP-MOB1 fusion protein was subjected to immunoblot analysis with serum from R753 as primary antibody (Figure 5.23, lane 3), protein was detected at both 17 kDa and 120 kDa. The protein at 17 kDa coincided with the predicted position of the recombinant MOB1 fragment. The identity of the band of approximately 120 kDa is unknown. The 17 kDa and 120 kDa proteins were not detected following pre-incubation of the primary antibody with fusion protein (lane 4), demonstrating that the reaction was specific.

Immunoblot analysis of procyclic *T. brucei* cell extracts with serum from R753 resulted in detection of major proteins at 20 kDa and 80 kDa, and minor proteins at 40 kDa and 50 kDa (lane 1). Only the 20 kDa protein was specifically blocked by pre-incubation with the fusion protein (lane 2). As the predicted size of the *T. brucei* MOB1-1 is 24.6

Figure 5.23 Western immunoblot analysis of polyclonal antiserum against both trypanosome lysate and MBP-MOB1 fusion protein



Lanes 1 and 2 each contained protein equivalent to 1 x 10^7 mid-log phase procyclic *T. brucei*. Lanes 3 and 4 each contained 2 μg MBP-MOB1 fusion protein cleaved with factor Xa. Lanes 1 and 3 were incubated with R753 α-MOB1-1 as primary antibody. R753 α-MOB1-1 was pre-incubated with 10 μg/ml MBP-MOB1 fusion protein (uncleaved) prior to incubation with lanes 2 and 4. Secondary antibody was goat α-mouse/HRP conjugate. Numbers on the left denote size in kDa.

kDa, the protein estimated at 20 kDa that was present in lane 1 but not in lane 2 is likely to represent this protein.

Due to the presence of antibodies in the serum of R753 that cross-reacted with *T. brucei* proteins other than MOB1-1, it was considered that affinity purification of the α-MOB1-1 antibody should be carried out prior to subsequent immunofluorescence microscopy. Due to time constraints this work could not be carried out within the current study and is being persued by colleagues.

5.3 Discussion

MOB1 is an essential gene of the budding yeast *S. cerevisiae*, participating in completion of mitosis and implicated in maintenance of ploidy (Luca and Winey, 1998). It was first identified in 1998 as a protein that interacted with MPS-1, a dual specificity protein kinase involved in both spindle pole body duplication and mitotic checkpoint (Luca and Winey, 1998). Subsequent work demonstrated that MOB1 also interacts with DBF2, a protein kinase with a role in late mitosis (Komarnitsky *et al.*, 1998).

Both Southern blot and sequence analysis revealed that the *T. brucei* genome contains 2 copies of the *MOB1* gene, which for the purposes of this study were referred to as *MOB1-1* and *MOB1-2*. These 2 genes are likely to have arisen as the result of a duplicative event involving a region of the genome containing at least one other putative gene, represented as a conserved region approximately 200-300 bp 5' of each *MOB1*. The immediate flanks of the *MOB1* genes are not conserved, indicating that duplication was not a recent event. Furthermore, MOB1-2 possesses at least 17 (and possibly as many as 82) residues in its N-terminus that are not encoded by the *MOB1-1* gene. The implications of this finding are currently not clear. Sequence analysis of the *MOB1-1* gene was carried out completely on both strands. However, due to time constraints the ORF of the *MOB1-2* gene was only sequenced partially in both directions, with the 5' and 3' termini having only single read sequence. Two ambiguous bases in the 3' region resulted in the translation of 2 ambiguous residues in the C-terminus of MOB1-2. Both genes shared a common stop codon. The identity of the start codon for *MOB1-2* is currently unknown, with three candidates. It is suggested that the most logical means of determining which of these is correct would be to identify the splice acceptor site of

MOB1-2 by means of PCR using a *MOB1-2* specific primer in conjunction with the spliced leader primer (detailed in Appendix II) and cDNA template. Cloning and sequencing of the PCR product would not only provide data clarifying the identity of the start codon, but would also provide valuable sequence confirmation for this region.

Southern blot analysis of genomic DNA revealed that *MOB1-1* and *MOB1-2* were situated within 12 kb of each other. This result was substantiated by identification of both genes within a single LambdaGEM-12 clone. Sequence analysis revealed a single *Bcl* I recognition site in the 3' flank of *MOB1-2*, indicating that *MOB1-1* was located in a region 5' of *MOB1-2*. As polycistronic transcription units are a common feature of kinetoplastid genome organisation (Graham, 1995; Myler *et al.*, 1999), it is suspected that the *MOB1-1* and *MOB1-2* genes are arranged in a head-to-tail orientation, probably as part of a single polycistronic unit. However, this is currently speculation. In order to determine the relative orientation and spacing of the *MOB1* genes further restriction mapping of the λ clone would be required. Additional sequence analysis of the *MOB1* locus would also be of value prior to any knockout attempt, as related genes in tandem array have previously been reported as being interspersed with unrelated genes in *Leishmania mexicana* (Wiese, 1998).

Northern blot analysis of procyclic RNA identified *MOB1* transcripts of approximately 1.6 and 2 kb. A high level on sequence identity at the nucleotide level between *MOB1-1* and *MOB1-2* suggests that the *MOB1-1* probe employed should detect both genes. Northern blotting with a ^{32}P-labelled probe derived from the 5' sequence unique to *MOB1-2* could be used to determine the identity of each transcript. It would also be of interest to determine whether the production of stable *MOB1* transcripts is related to *T.*

brucei lifecycle by investigating expression in both long slender and short stumpy bloodstream-form cells.

Analysis of MOB1 peptide data from diverse organisms revealed a putative casein kinase II (CK2) phosphorylation site conserved in all species examined, yet absent from the *Saccharomyces cerevisiae* MOB2. Both MOB1-1 and MOB1-2 possess this motif. CK2 is a ubiquitous enzyme that phosphorylates the serine/threonine residue of the sequence S/T-x-x-D/E (Pinna, 1990). CK2 is a heterotetramer, composed of two catalytic α and two regulatory β subunits, that phosphorylates proteins involved in signal transduction, gene expression, protein synthesis, metabolism and cell cycle progression (Pinna and Meggio, 1997; Bhatia et al., 1998). The degenerate nature of the CK2 recognition motif means that it is likely to be detected in a multitude of proteins, but may not be functional in all. While it is known that the *S. cerevisiae* MOB1 is a phosphoprotein *in vivo*, and that it is a substrate for MPS1 *in vitro* (Luca and Winey, 1998), the kinase(s) responsible for phosphorylation of MOB1 *in vivo* is currently unknown. Consequently, the significance of a conserved motif in MOB1 putatively recognised by CK2 is currently unknown.

In order to characterise the *T. brucei* MOB1-1, sequence encoding a Ty-1 epitope tag (Bastin *et al.*, 1996) was inserted into the 3' terminus of the *MOB1-1* ORF and the recombinant protein overexpressed under inducible control in procyclic *T. brucei*. Three clonal populations were analysed by Western blotting, and were determined to produce comparable levels of a 26 kDa protein, the size predicted for the Ty-tagged MOB1-1. Stability of the TyMOB1-1 protein *in vivo* was investigated by overexpression followed by removal of tetracycline. Western blotting of samples

collected at subsequent time-points revealed the TyMOB1-1 to be a stable protein in both clones investigated (as was the case for TyQM), displaying little or no reduction for the first 12 hours post induction, and remaining detectable for at least 24 hours. As discussed in the previous chapter such stability is not a direct consequence of the Ty-tag, as TyCyc2 is turned over relatively rapidly in procyclic *T. brucei* under the same conditions employed in the current study (J. Van Hellemond, personal communication). The stability of the mRNA transcript encoding TyMOB1-1 is currently unknown, and could undoubtedly influence apparent stability of the protein. Consequently, Northern blot analysis of induced cells over the time course could be used to monitor both *MOB1* and *TyMOB1-1* transcript levels throughout this experiment. This approach would also be valid for the study carried out on TyQM. The Western blot analysis carried out in the present study of TyMOB1-1was not quantitative. The pulse-chase approach described in the previous chapter could also be implemented to quantify stability of TyMOB1-1.

In order to determine whether TyMOB1-1 demonstrated subcellular compartmentalisation, indirect immunofluorescence microscopy was conducted on fixed procyclic cells of tTyMob clone 4. Overexpressed TyMOB1-1 had a homogenous distribution, with no organellar localisation detected. Subcellular fractionation analysis of the TyMOB1-1 suggested that this protein is not part of a large protein complex *in vivo*. The subcellular distribution of MOB1 in yeast has not yet been determined. However, due to its association with completion of mitosis (Luca and Winey, 1998), coupled with physical interaction with subunits of the CCR4 transcription regulatory complex (Komarnitsky *et al*, 1998), the predicted outcome of the current study had been the detection of nuclear localisation. While this was clearly not the case it is recognised

that the results of any epitope tag study must be interpreted with caution due to the potential for the tag to interfere with protein function/targeting. In order to demonstrate conservation of function of tagged proteins *in vivo* inducible expression in the null mutant may be attempted.

In order to address the question of localisation of native protein within procyclic *T. brucei* it was necessary to generate antibody to the MOB1 protein. At the time that this work was initiated data pertaining to the 5' terminus of the *MOB1-1* gene was not available, and no sequence data was available for *MOB1-2*. Consequently, a fusion protein was produced consisting of the C-terminus of MOB1-1 and maltose-binding protein. The choice of MBP for this fusion was due to a combination of factors such as its superior ability to promote the solubility of polypeptides to which it is fused (Kapust and Waugh, 1999), yield, ease of purification with amylose resin and cleavage with Factor Xa. Yield of fusion protein by this system was indeed high, and the vast majority proved soluble. One problem encountered during the current work was the poor level of purity of fusion protein that could be achieved by passing soluble bacterial extract through an amylose column. High levels of contaminating protein were encountered on all occasions, despite alteration of flow rates during loading and excessive washing of bound fusion. Cleavage of the MOB1-MBP fusion was successful, but the resultant MOB1 polypeptide was insoluble, which made it difficult to purify further. Consequently, cleaved protein was electrophoresed on an SDS-PAGE gel and protein/acrylamide used to inoculate rabbits.

Serum from the second bleed of rabbit R753 identified a *T. brucei* protein of 20 kDa, approximating the predicted size of MOB1-1. The specificity of this interaction was

confirmed by competitive binding of serum with MBP-MOB1 fusion protein prior to application to Western blots. It is recognised that this approach did not rule out cross-reaction between a *T. brucei* protein and an antibody raised to MBP. Other *T. brucei* proteins detected by the serum from R753 demonstrated no such inhibition, indicating that they represented non-specific cross-reaction with antibodies innate to the rabbit. Due to the high level of amino acid identity between the region of MOB1-1 used in production of the fusion protein and the comparable region of MOB1-2, it had been predicted that α-MOB1-1 antiserum would detect MOB1-2. The predicted molecular weight of MOB1-2 is 26.9, 31.3 or 34.3 kDa, depending on the identity of the start codon, but specific hybridisation to a protein of this size was not detected by Western blotting with serum from rabbit R753. This result indicated either that translation of MOB1-2 did not occur or that it was not detected by antibody under the hybridisation conditions employed. It should be reiterated that this was the second bleed from R753, terminal exsanguination having been carried out subsequent to a fourth protein/acrylamide inoculation. In theory the titre of antibody specific to MOB1-1 (and MOB1-2) should be higher within this terminal sample than was the case in previous bleeds. MOB1-2 may therefore be detected in future Western blots. Due to the level of cross-reaction observed between serum from the second bleed of R753 and *T. brucei* proteins, affinity purification is recommended prior to use in immunofluorescence microscopy.

Endogenous expression of antisense RNA has been successfully utilised in the study of gene function in a variety of systems, including plants (Bhalla *et al.*, 1999), mammals (Barden *et al.*, 1997) and the protozoan parasites *Toxoplasma gondii* (Nakaar *et al.*, 1997) and *Entamoeba* spp. (Alon *et al.*, 1997; Ankri *et al.*, 1999). In order to gain

insight into the function of *MOB1-1* and/or *MOB1-2*, an inducible antisense RNA approach was adopted. At the time that this study was initiated the 5' terminus of the *MOB1-1* gene had not been sequenced, as was the case for the entire *MOB1-2* gene. Consequently, bases 32-544 of the *MOB1-1* ORF were amplified by PCR and cloned into the inducible expression vector pHD675 in an antisense orientation relative to the promoter. The nucleotide identity between this region of *MOB1-1* and the corresponding region of *MOB1-2* is 98%, there being 10 base substitutions of which 2 are ambiguous bases in *MOB1-2*. Antisense inhibition of *MOB1-1* expression incurred by induction of construct pAntiMobHD675 was as therefore also likely to affect *MOB1-2*. Following induction of the construct in two independent clonal populations, alterations in phenotype with relation to uninduced controls were sought using a combination of phase contrast microscopy on both living and fixed cells, and fluorescence microscopy on fixed cells stained with DAPI. Since the MOB1 of *Saccharomyces cerevisiae* is associated with completion of mitosis and maintenance of ploidy, cell cycle related deficits such as arrest, altered morphology or an increase in ploidy might have been observed. Unfortunately, no such defects were detected. This led to three hypotheses: that the phenotypic changes occurring in trypanosomes were more subtle than could be detected under this regimen, that MOB1 was not important for cell cycle progression, or that levels of MOB1 remained unchanged during this experiment. In order to address the first of these hypotheses the effect of induction upon growth rate of cells was observed over time, revealing that induced cells containing the pAntiMobHD675 construct grew at a slower rate than non-induced counterparts. While the phenotype observed was not spectacular, it was proven to be statistically significant. This result suggested that expression of the *T. brucei* MOB1-1

and/or MOB1-2 was necessary for normal cell cycle progression, but did not give any indication as to what role may be played by these proteins.

There are several possible reasons why the reduction in growth rate observed during this study was so small. Firstly, it is possible that only a small quantity of *MOB1-1* antisense RNA was actually transcribed upon induction, resulting in a limited antisense effect. It has been noted both in this study and in previous work that levels of inducible expression using pHD675 vary between both clonal populations (Section 4.2.4; J. Van Hellemond, personal communication) and genes of interest (QM vs. MOB1). Secondly, that antisense *MOB1-1* RNA was transcribed at a relatively high rate, but that the transcript was unstable, being degraded by the cell relatively rapidly. Northern blot analysis of tAntiMob clones could be used to investigate both the second and third hypotheses. A third possibility is that either the antisense *MOB1-1* RNA or *MOB1-1/MOB1-2* mRNAs possess secondary structure that prevents efficient duplex formation between the partners, thus reducing any phenotype that would result from a reduction in protein expression. Fourthly, it is possible is that the antisense *MOB1-1* concentrates in a different subcellular compartment than either *MOB1-1* or *MOB1-2* mRNAs. This would reduce the likelihood of physical interaction between the prospective partners and thus of an antisense effect. Such a scenario could be investigated by *in situ* hybridisation of induced cells with both sense and antisense *MOB1-1* probes. Finally, it is possible that that the transcription of antisense RNA was successful, and that expression of either or both of the *MOB1* genes was successfully inhibited. The lack of a major phenotypic change would therefore indicate that *MOB1* was not an essential gene in *T. brucei*, its function(s) being covered by other genes (such as a *MOB2*). Based on the importance of *MOB1* in *Saccharomyces cerevisiae* and its degree of

conservation between species this last possibility is currently thought to be the least likely. Observation of the level of native transcripts by Northern blot analysis, coupled with Western blotting for native protein, could be used to address this possibility.

Based on the fact that the reduction in growth rate following expression of antisense *MOB1-1* was slight, further studies on tAntiMob clones were not carried out. Rather, a switch was made from an antisense RNA to an RNA interference (RNAi) approach to disruption of *MOB1* expression. RNAi has been demonstrated to be more a more potent inhibitor of gene function than antisense RNA in both the nematode *C. elegans* (Fire *et al.*, 1998) and plants (Waterhouse *et al.*, 1998). Furthermore, *in vivo* expression of gene specific RNAi in *T. brucei* was able to halt expression of α-tubulin, causing a cytokinesis block in cells (Ngô *et al.*, 1998). While the mechanism by which RNAi induces gene silencing remains uncertain, it appears to be post-transcriptional (Montgomery *et al*, 1998) and to involve specific mRNA degradation (Ngô *et al.*, 1998; Waterhouse *et al.*, 1998).

The *MOB1* RNAi construct produced in the inducible vector pHD675 consisted of two copies of the 5' terminus of *MOB1-1* (223 bp each) in a head-to-head configuration, separated by 397 bases from the centre of the *MOB1-1* ORF in an antisense orientation with relation to the promoter. Two independent trypanosome clones were obtained following selection, but induction of these failed to result in any change in phenotype, as determined by phase contrast microscopy on both cultured and fixed cells, and fluorescence microscopy on fixed cells stained with DAPI. Northern blot analysis of both experimental cells and controls revealed that degradation of *MOB1* mRNA had not occurred in this system. Furthermore, no additional product representing the double-

stranded RNA was detected by Northern blotting. It is thus suggested that expression of the construct failed on this occasion, possibly as a result of loss of all or part of its MOB1-specific portion during the antibiotic selection process. This possibility could be investigated by Southern blotting of restriction digested genomic DNA.

In summary, the current work has demonstrated the presence of two *MOB1* genes in the *T. brucei* genome that are hypothesised to be the result of a duplicative event. Evidence derived from overexpression of a Ty-1-tagged MOB1-1 suggested it is a soluble protein with a cytoplasmic distribution. No evidence indicating a function was gained, although a phenotype associated with cell cycle progression was detected through inducible expression of antisense RNA. Polyclonal antiserum was raised to a recombinant MOB1-1 protein, and specifically detected a single *T. brucei* protein following Western blotting. Once purified, this antibody will facilitate future experimentation on the MOB1 protein and its role in the trypanosome.

Chapter 6

Discussion

6.1 Validity of the use of RADES-PCR

The aim of the current project was to identify genes involved in the ConA-induced cell death of *T. brucei*. To this end a differential display PCR technique was employed, aimed at identifying transcripts displaying altered levels of expression throughout the time-course of death. This approach was chosen for two main reasons, namely that:

1. Differential display reverse transcription-PCR (including RADES) is probably the least involved method currently available that allows comparison of expressed transcripts from multiple samples. Furthermore, this technique has less of a bias towards high copy number transcripts than is the case for subtractive hybridisation, and does not have a prerequisite for complete sequence information of the organisms genome, as is required for serial analysis of gene expression (SAGE).
2. Due to the utilisation of random primers for DDRT-PCR, many novel transcripts may be identified from organisms whose genomes are incompletely characterised. Of the 29 transcripts identified during the current study, 16 had not been previously identified in *T. brucei*. However, with the progress currently being made by the *T. brucei* genome project the probability of detecting novel transcripts is rapidly diminishing, raising the likelihood that such "gene fishing" exercises will become obsolete in the not to distant future.

There also are a number of arguments against the use of DDRT-PCR for the study of trypanosome cell death, which run as follows:

1. DDRT-PCR is renowned for being prone to artefacts, requiring confirmation of each result either by Northern blot or semi-quantitative RT-PCR. However, in *T. brucei* cells that are dying there is no marker that is known to be constitutively expressed that may be used as a control for such experiments, rendering any results open to interpretation. This issue has yet to be resolved.

2. A common obstacle faced by researchers investigating apoptosis in metazoan cells is the asynchronous nature of the cultures with which they work. As many of the events occurring during the apoptotic process are transient, detection and quantitation of them is often problematic (Willingham, 1999). As no satisfactory method has been described to synchronise trypanosome cultures (Mutomba and Wang, 1996) no attempt was made to do so in the current study. Consequently, transient changes in gene expression were unlikely to be detected, potentially skewing the data set towards genes displaying alterations over a prolonged period and of greater magnitude.
3. The premise for the current work was that death of *T. brucei* induced by ConA was programmed, possessing apoptotic-like biochemical and morphological features (Welburn *et al.*, 1996). Apoptotic cell death in many metazoan cells relies heavily on a proteolytic cascade of cellular constituents, rather than on *de novo* gene expression. This is not to say that *de novo* gene expression does not play a role in apoptotic cells, but rather that that role may be relatively minor and unlikely to involve many of the key components. Consequently, any attempt to investigate cellular processes involved in such a system should ideally involve proteomics, rather than the detection of differentially expressed mRNA transcripts. Such an approach is currently not feasible for any but the most specialised laboratories, but is likely to become more accessible for future studies.
4. Successful DDRT-PCR requires high quality template material in order to minimise the production of both artefactual bands and background smear. If an asynchronous cell population is induced to die there will come a transitional period towards the end of the process whereby some of the cells are living while others have died. The mRNA of dead cells will be partially degraded, resulting in reduced quality of

cDNA produced from such cultures. Differential display PCR carried out on these samples will theoretically produce banding patterns that are less reproducible. Such a phenomenon was noted on occasion (48 and 72 hours post-treatment) in the current study.

Taken as a whole these arguments point to the fact that the application of DDRT-PCR to ConA-treated *T. brucei*, with the aim of gaining insight into the cellular processes occurring during death, was not completely satisfactory. However, having considered the alternative approaches that could have been utilised, it is concluded that at the time this project was initiated differential display PCR was the most appropriate techinque to adopt. It is clear that further experimentation in this field should not be initiated until a suitable constitutively expressed marker for Northern blots is established that correlates with the DDRT-PCR data during death. α-tubulin is one possible candidate, although there is no reason to assume that this will be any more reliable than the β-tubulin employed in the current study. The use of actin has also been suggested (S. Graham, personal communication), and has previously been utilised as a control during a differential display screen of apoptotic cells (Chen *et al.*, 1998).

6.2 The genes identified

The transcripts identified during the course of this study were drawn from a variety of cellular processes including protein synthesis (ribosomal proteins and peptide chain release factor), energy metabolism (arginine kinase and cytochrome c1), cell signalling (receptor for activated protein kinase C), cell cycle (MOB1) and intracellular transport (mitochondrial transporter, pteridine transporter). 14 of the transcripts identified are

currently classified as "unknown", either because they did not match any database entries, or because the database entries that they matched had unknown function.

6.3 Characterisation of *QM*

At the time of its isolation in the current study, available evidence suggested that QM was likely to be a transcription factor, with putative links to tumour suppression. Based on the obvious link between tumour suppression and programmed cell death (Kaelin, 1999) it was decided that *QM* would make a prime candidate for further characterisation. While the current work was in progress, several groups provided evidence that QM was unlikely to act as a transcription factor due to its nuclear exclusion (Tron *et al.*, 1995; Mills *et al.*, 1999), and was in fact associated peripherally with the 60S ribosomal subunit (Dick *et al.*, 1997). Analysis of the *T. brucei QM* gene and derived protein sequence revealed approximately 60% amino acid identity with QM homologues from highly diverged eukaryotes. A conserved putative protein kinase C phosphorylation site was identified. Indirect immunofluorescence microscopy provided evidence of a ribosomal subcellular location for the epitope-tagged QM, and distinct nuclear exclusion of the protein was noted. A ribosomal association was substantiated by the insoluble nature of the TyQM protein from trypanosome lysate under Triton X-100 fractionation conditions. Taken together these results strongly suggest that the *T. brucei* QM is likely to be a functional homologue of the QM proteins of other eukaryotes.

6.4 Characterisation of *MOB1*

When the RADES product encoding the *MOB1* cDNA fragment was first isolated it had homology to sequences from several organisms lodged on various internet databases, but the function of these proteins was at that time unknown. In 1998 the yeast *MOB1* was identified as a gene whose product interacted with both MPS1 and DBF2, protein kinases associated with the yeast cell cycle (Luca and Winey, 1998; Komarnitsky *et al.*, 1998). Characterisation of MOB1 in *Saccharomyces cerevisiae* revealed a role for this protein in completion of mitosis and maintenance of ploidy (Luca and Winey, 1998). During the current study extensive Southern blot and sequence analysis was carried out, revealing the presence of two non-identical *MOB1* genes in *T. brucei*. Identity between MOB1 amino acid sequences from diverse eukaryotes ranged from approximately 30-50% when comparing the entire deduced protein sequences, and between 40-60% when comparing only the central consensus region. A conserved putative casein kinase II phosphorylation site was identified in all MOB1 sequences analysed.

In order to gain insight into gene function, disruption was attempted by the inducible expression of either antisense RNA or RNAi. Based on the essential nature of the *S. cerevisiae* gene and the mitotic arrest associated with its temperature sensitive alleles at the restrictive temperature (Luca and Winey, 1998), it was hypothesised that disruption of *T. brucei* MOB1 expression may result in arrest of the cell cycle. The antisense approach resulted in a reduction in cellular proliferation, indicating a role for MOB1 in progression of the cell cycle. While this result was shown to be statistically significant, the phenotype was relatively minor, indicating either that the *T. brucei MOB1* is not an essential gene, or that expression of antisense *MOB1* was less successful than had been hoped with respect to disruption of MOB1 expression. Analysis of MOB1 levels in

induced/control cells by Western blot analysis using serum from R753 could be used to evaluate these possibilities. Inducible overexpression of *MOB1*-specific RNAi failed to produce a detectable phenotype, and Northern blot analysis revealed that *MOB1* transcript levels did not vary between induced, non-induced or untransfected cells. Furthermore, the RNAi transcript was not detected by Northern blot, leading to the conclusion that either the construct was not expressed, or that expression was at a level too low to be detected by analysis of total RNA. While it is known that the portion of construct pRNAiMobHD675 encoding the hygromycin resistance gene became stably integrated within the clones examined (i.e. they were resistant to hygromycin B), Southern blot analysis would be necessary in order to determine if this was the case for the *MOB1*-RNAi encoding portion. A recombination event resulting in the loss of this region would explain the observed results.

Indirect immunofluorescence microscopy to an epitope tagged MOB1-1 was employed with the aim of detecting subcellular localisation of the protein. Distribution throughout the cell was shown to be homogenous, a result negating the possibility of commenting on potential function at this stage. The subcellular location of MOB1 in yeast has yet to be established, but based on its involvement in completion of mitosis and maintenance of ploidy (Luca and Winey, 1998), coupled with two hybrid interactions with the mitotic kinases MPS1 (Luca and Winey, 1998) and DBF2 (Komarnitsky *et al.*, 1998), and with elements of the CCR4 transcription regulatory complex (Komarnitsky *et al.*, 1998), a nuclear localisation could be hypothesised. Such localisation was not detected in the current study, possibly resulting from the fact that the overexpressed MOB1-1 protein bore a C-terminal epitope tag which could potentially interfere with targeting to

the nucleus. Antiserum from rabbit R753, raised to recombinant MOB1-1 polypeptide, could be used to investigate localisation of native MOB1-1/MOB1-2 protein.

6.5 Future experiments

The subcellular fractionation of TyQM carried out in Section 4.2.4 indicated only an interaction with a large protein complex which was assumed to be the ribosome. Consequently, ribosomes could be purified using a sucrose gradient, and the fractions analysed by Western blot as described by Dick *et al.* (1997) using the BB2 α-Ty antibody to detect tagged QM. A literature search failed to find reference to antibodies specific to known *T. brucei* ribosomal proteins that could be used as markers for this experiment. Antibodies to ribosomal proteins from other eukaryotes, such as the *Saccharomyces cerevisiae* 60S ribosomal protein L3 (Dick *et al.*, 1997) or the human QM (Loftus *et al.*, 1997) have been published and could be utilised to co-localise signal from TyQM.

MOB1 is an essential gene in *S. cerevisiae*, but its status in *T. brucei* is currently unknown. It is therefore suggested that complete sequencing of the trypanosome *MOB1* locus be carried out, then *MOB1-1* and *MOB1-2* knocked out both individually and in combination using homologous recombination and selectable markers to establish whether the genes are essential. Successful replacement of the genes can be confirmed by Southern blot analysis, and any phenotypic alterations in cell populations can be analysed.

During the current study a recombinant MOB1-1 polypeptide was used to raise antiserum. This antiserum specifically detected a *T. brucei* protein approximating the

size of MOB1-1 on a Western blot. As non-specific cross-reaction to *T. brucei* proteins was also detected, affinity purification of this antiserum using immobilised MBP-MOB1 should be carried out prior to use in downstream applications. As mentioned previously, there is a possibility that the Ty-1 epitope tag of TyMOB1-1 interfered with subcellular localisation of this protein. One potential use of an α-MOB1-1 antibody would be determination of the subcellular localisation of native MOB1-1 by immunofluorescence microscopy. Another application for this antibody would be immunoprecipitation of native MOB1-1, together with any proteins associated with it *in vivo*. Micro-sequencing could then be used to identify interacting partners.

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Appendix I

Sequence data derived from RADES-PCR products

101(1)T7

CGAGCACAATGCAGAAGTGATGTCAGAGTCACCAACATCCGTAATGGAAAGTACGCAAGCA
CGGAAATGCCTCCCGCATGCCGTTCCAAGGTCAAGGTTGTTGCCGCTGTAGCGGTGAATTGG
CGTCTTGCTTAAAGTGCAGTAGTACTCAATCTCCGCCTTGCGGATCGGCGGGCAGTTAGCGG
AAATGACNACNAGTTTACTGCGGCCCTGACNAAGTGTCTTGAGTGTCTGCTGCGTCCCGAGA
ACGTATTTGCCGGATTTCATCACCAGTTGGATCTTGGTGTTGATGGTGTCCACCTTCGACTTG
ACC

101(1)SP6

GGTCAAGTCGAAGGTGGACACCATCAACACCAAGATCCAACTGGTGATGAAATCCGGCAAA
TACGTTCTCGGGACGCAGCAGACACTCAAGACACTTCGTCAGGGCCGCAGTAAACTCGTCGT
CATTTCCGCTAACTGCCCGCCGATCCGCAAGGCGGAGATTGAGTACTACTGCACTTTAAGCA
AGACGCCAATTCACCGCTACAGCGGCAACAACCTTGACCTTGGAACGGCATGCGGGAGGCA
TTTCCGTGCTTGCGTACTTTCCATTACGGATGTTGGTGACTCTGACATCACTTCTGCATTGTG
CTCG

101(2)T7

TCGAGCACAATGGGCAGACGTACCGGTCTATTTACAGAGCTGTGGGTGTGATATGGAAGGA
AGAGGGATTGCGTGCTCTCTTTCGTGGATGTCACGTTGCGGTATTGGGGGCTGTTGTAGCGT
GGGGTGTGTACATGTTCGTATATCACGCGCTGTGCGACCTGTACATCCCAACAAGTAACAAA
AGAGCGGGGGACGATTTCTTGTTTCGCACTGTTCTTTCCAGTATTGCCAGTTGCAGTTGCGCA
GTCGTGGGAAACCCTATTTGGCTCCTAAAAACTCGGATGCAGATCGAGGAAAATCGCCTCAC
GTGAGGCGGCAGTGGCTGGAGCTTCTATCTTTCGAAACAGCAAAAATTACACGTCATTCTTT
GGTGGGTTAAAGATACGCTATTCAAACTGACGGTGTTTTGTTCNCTGTGGCGTTGGTGTCTCC
CGCACAGGTCCTGCTAGGTTTACCGAACGCACTTAACTTTCCCGCTTATGANGCACTGAAAT
CGTTCTGGTTGCAGCGCAATGACCGAANAACCCCTTTACAGTTATGAAGCGTGCATTTGTTC
AACAGCCAGCAAAACTGCCGTTTCNATTATCNGGTTTCCCCTACACNTTTTTTNANACAAGA
ATGCCAGATCNGANA

101(2)SP6

GGCGACTTCTCTGATCTTGCATTCTTGTCTTAACAACGTGTAGGGGATACCCGATAATCGAA
ACCGCAGTTTTGCTTGGCTGTTGAACAAATGCACGCCTCATAACTGTAAAGGGGTTCTCGGT
CACTGCGCTGCAACCAGAACGACTTCAGTGCCTCATAAGCGGGAAAGTTAAGTGCGTTCGGT
AAACCTAGCAGGACCTGTGCGGAGACACCACGCCACAGCGACAAAACACCGTCAGTTTGAA
TAGCGTATCTTAACCCACCAAAGAATGACGTGTAATTTTTGCTGTTTCGAAAGATAGAAGCT
CCAGCCACTGCCGCCTCACGT

508(1)T7

GCTTTTGAAGCGCCTGCGTGAGGCGAANAAGAACGTGAAGGCCGGTGAGAAACCAAAAGCT
GTGAAGACACACCTCCGTGATGTTGTGATCACGCCTGAGATGGTGGGTTCTGTTGTGGGAAT
CTACAATGGTCGCCAGTTTAATGCGGTGGAGATCAAGGGTGAGATGATTGGCCATTATCTTG
GAGAGTTCTCGCTGACGTACAAACCTGTCGCTCACGGCCGCCCCGGTTTTGGTGCGACTCAC
TCTTCTCGTTTCATTCCCCACAAGTAGTGAAGCATCGAGAGTTGAATGGGATGCAATCCATG
ACTTGTTTGGTCTTGTTTTTATTTTCATTTCTGTGACAGGGGCCG

508(1)SP6

TCGGCCCCTGTCACAGAAAATGAAAATAAAAACAAGACCAAACAAGTCATGGATTGCATCC
CATTCAACTCTCGATGCTTCACTACTTGTGGGGAATGAAACNAGAAGAGTGAGTCGCACCAA
AACCGGGGCGGCCGTGAGCGACAGGTTTGTACGTCAGCGAGAACTCTCCAAGATAATGGCC
AATCATCTCACCCTTGATCTCCACCGCATTAAACTGGCGACCATTGTAGATTCCCACAACAG
AACCCACCATCTCAGGCGTGATCACAACATCACGGAGGTGTGTCTTCACAGCTTTTGGTTTCT
CACCGGCCTTCACGTTCTTCTTCGCCTCACGCAGGCGCTTCAAAAGCAATCCCGCGGCCAT

526(1)T7

GATTGCCGTCCGAGTAGTTGTTTGCCTGTTTTTAAAAGTGCGTTGTTCAATAGAAAGTACTTT
TTCAGGGAGGCAGATCTAACGGAAGATAGTACGCTCGGTCGTGGTCCTgGGGGGCAGACAA
CGAACAGGCGGAAACAGACGGCTATTGTGAAGCATGTGCCAACTGGAATTACCGTGAAGTT
CAGCAAGTTTCCCTCTTACTGGCTTAACAGGAGGGCTGCACGGGATGTCCTCAACCTGCAGC
TGGAGGAACGTATGCTCGGG

527(1)T7

TGCGCGCAGCGTCACAACTGCTACCGTAACGAGCGCCGCTTCACGGTGTTGGGTAACATGTG
CAAACACGTTGGATGGAAGTACAGCGACGTTGTGGAGAAACTTGAGGCAGCCCGTATTGAA
AAGTCCGGGCGTCACCACAAGAAGATGGAAAAGGTTCGCGTCGCTTGGAAGAATGCCCGCA
AGGAGGCCCTCAAGAAGATGCCCCAGAAGAATGTGGAGGTTCTCAAGAAGTTTGGTCTTGC
GTAATTCACATGGGATTCACTGAGAAGTCTCTGCCGTANAAGCGTGGCTGAGGTGCCGTCTG
AAAGGTCTAATCTCTCTCTAATTTTGTTCATTTACTACTCATTTCTTGTTTCANAAAGNAAAA
AAAAAGAGACGATATTTTTGCTTGTACGGTTGCTTCACCCAGCAACTGTGGCACCATCGTTG
GTTGCGGGTCCTGCACTGATGATTCGCTGCGCGC

527(1)SP6

GCGCGCAGCGAATCATCAGTGCAGGACCCGCAACCAACGATGGTGCCACAGTTGCTGGGTG
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NTAAATGAACAAAATTAGAGAGAGATTAGACCTTTCAGACGGCACCTCAGCCACGCTTCTAC
GGCAGANACTTCTCAGTGAATCCCATGTGAATTACGCAAGACCAAACTTCTTGAGAACCTCC
ACATTCTTCTGGGGCATCTTCTTGAGGGCCTCCTTGCGGGCATTCTTCCAAGCGACGCGAACC
TTTTCCATCTTCTTGTGGTGACGCCCGGACTTTTCAATACGGGCTGCCTCAAGTTTCTCCACA

ACGTCGCTGTACTTCCATCCAACGTGTTTGCACATGTTACCCAACACCGTGAAGCGGCGCTC
GTTACGGTAGCAGTTGTGACGCTGCGCGCA

541(1)T7

CCAACCTCAGCCCCTGCCGGTTACCCCACGAGCCATTAATAACCCGCCCACTTAAACCAACC
GCCGATCCGACCAGCGGAACCCCATTTGAGTCAACAGTAGTTGAGTCAACCCCACGCGCCTT
CCTCATGAGCTGACAAAACTGGTTGTAGAGTAACTCCCCTTCACTTTTCCCCACCAATGCATG
AAACTGCACATTCCGACCCTTCAATTTTCCCCCAAGTGACGCCTGTGGTACGATAAGCACAT
CAGCTGAGGGGAAGCTGCTTAAAGAGTCGGAAGATCGTGGCAGCCGC

541(1)SP6

GCGGCTGCCACGATCTTCCGACTCTTTAAGCAGCTTCCCCTCAGCTGATGTGCTTATCGTACC
ACAGGCGTCACTTGGGGGAAAATTGAAGGGTCGGAATGTGCAGTTTCATGCATTGGTGGGG
AAAAGTGAAGGGGAGTTACTCTACAACCAGTTTTGTCAGCTCATGAGGAAGGCGCGTGGGG
TTGACTCAACTACTGTTGACTCAAATGGGGTTCCGCTGGTCGGATCGGCGGTTGGTTTAAGT
GGGCGGGTTATTAATGGCTCGTGGGGTAACCGGCAGGGGCTGAGGTTGG

875(3)T7

CAAAAAGAAAAAAGGAAGTAAAACGTTCCTACTTCCCTACATAAGAAAAAAAAATTGCTCC
ATAAACATCTAAGATGCTTTCTTTTTGCGACCAGAAAGCATCGGAAACGCATGTGAGGGTTC
CTATCGGGACGCTCGTTTGTGTAGATGCCTCTGCAATAGTCAGCATTGTCTCAAAAAAGAGG
CACAAAAATAAACAGGAGAAAAGGGTAAAAAGAATGGATGAAGGTAAAAGAACGAGATCC
ATATTTTTTCTGCGGCACGGATAATAGAATCAAGCTGGCAAATCCTGCGGTTCCCAATGAAC
AGCCATCATATAATTGCTCACGGAACAATCACTAATTGCAGATAGAAGCTCCAGC

875(3)SP6

AATTATATGATGGCTGTCATTGGAACCGCAGGATTTGCCAGCTTGATTCTATTATCCGTGCCG
CAgAAAAAATATGGATCTCGTTCTTTTACCTTCATCCATTCTTTTTACCCTTTTCTCCTGTTTAT
TTTTGTGCCTCTTTTTTGAGACAATGCTGACTATTGCAGAGGCATCTACACAAACGAGCGTCC
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1209(2)T7

GCGGCCGCGAATTCWTTWTTGATTTGGCCCCGCWTCATTGGAWATAAGGTWWGAGTGCCA
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1209(3)T7

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GTGGTTCCTCAAGCGACGAAAACWTCGTTCATCCACTGTCTTCGGATGGTWACACTGTGAC
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1209(4)T7

GCGGTCGCGAATTCACTAGTGATTGGTCCCCGCTGGATCTGATGGACAATTCTTTGAATCAG
CTTTGGACGGCTTGTCCATGGGTTTTGAYGATCCGAAGCCCATWTCTTTTTTGTTTCGTGTGC
TCTGGTAGATGTTGTGAGTGCTTACTCAATACAAAAATTGCTGTCGGTAGTATGGTTTCTTCT
GGTTGTAAAACTGTYGCCTTTTTTCTGTTCGCTAGTGGTGGTTGC

1209(4)SP6

GGAGGTCTCCCATAGGGTCGACCYGCAGGCGGNCGCGAATTCAGTATTGATTTGGTTCCCGC
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CGAAATTTTTGTCMTTACTGATGAAACTCATAMGAAWGATGTTAAGCTGYMAGCWGTCTCG
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GACWCAAGTCGCATTCACCACCG

1365(1)T7

TCCTCAACCTGCAGCTGGAGGAAACGTATGCTCGGGTCGAAATCGGAGCTCGGCCGTATACA
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1365(1)SP6

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1365(2)T7

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CTT

1365(2)SP6

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1365(3)T7

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CGAAGGCTTTCGAAAGCAACGGCCACCACTGCCCACACT

1499(1)T7

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1499(1)T7

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1499(2)SP6

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CTT

1499(2)T7

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TCGCTT

1499(3)T7

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1499(3)SP6

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CGGCGTGACCTCAGTTCCGTACCTCCTAATCGGCATCATATTCTTCGGTCTCTGCTCAACGGT
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1499(3a)T7

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1499(3a)SP6

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1499(4)T7

AAGCGAGCCGCGTCAGGACCTCTTGGGCGCGACCCCGTTGCCCTCCGCATTGGGTGTCACAT
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1499(5)T7

TGGTCGTTTAGGCCATGATGTTGTATGGGGTGATCTTACCCTTTGGTGTGATCAGCTTCACGT
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1499(5)SP6

ACAAGGAATGTTTCCACATGCGTATCCGCGCCCATCCGATCCACGTACTTCGCATCAACAAA
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1501(1)T7

CCCGCAACTTGCACAAAGCTATGGCGCCTTCTTTGTCAAGTTTCCGGTCCGCTTTGTATCTGT
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ATGCAAAGGGCGTTGTATGACATAGTGGCTTATATATCAACGCCAAGGGATACTTCCTTTTT
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1501(1)SP6

CGGCCGGTCAAAGCTCTAATAGTAAACGGCTCAACAAAAAAGGAAGTATCCCTTGGCGTTG
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1501(2)T7

CGGCCGGTCACATTTTTTCACTTGCGCGATACCCATCGAGTCACGAAGTTGCATGCCAATAC
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1501(2)SP6

TTCAGGTTGACTGAGTGCTGCAAGCGCCGCGAAGAACGTTTACAGGAAGAGCGAGAGTACA
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1501(3)T7

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1501(3)SP6

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1501(4)T7

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CAAACCCCTGAAACCGCAACGAAGGTGGTATCCACGTCCCGGGACAAGACCCTTCTTTCATG
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1501(4)SP6

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1501(5)T7

AACAGTTCCTGGGAACGCATTGCCATGCCAAAGACCGGTGCAAGATTTTCTGCAAACTTGGC
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1501(5)SP6

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1504(1)T7

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1504(1)SP6

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AC

1504(3)T7

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1504(3)SP6

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1504(4)T7

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GGCGTCTCCA

1504(4)SP6

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1504(4a)T7

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1504(4a)SP6

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AACGGCGCATCTTTGTGGAGTGCACTACGCCACGGAGGATACGGCCACGAATGGACACATT
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1504(7)T7

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1504(7)SP6

GGAGACGCCCGAATATTACGGGTCGGTGAGTTTACCATAATAAAGGGTAGGCAGTTTTTGTA
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GCAGTAGGGGCCTTGAGAGTAGGGAACGCACAACCCGATCGTATGTGAGTTGCGAAATGTA
CTATCATAAGTGGAGAGGGATGCTGCAGGGGATTGGTTGGCAGCCGCGGCACGGGATCAGT
TGGGCAATCGCCGCAGATTGGTGGCGGGGGGTGATGGTCCTCCAgACACCGGGTGCCAACCC
TTAAACTGCATTCT

Appendix II PCR primer sequences

Spliced leader	TAGGCGCGCCTAGAACAGTTTCTGTACTATATTG
pr4-1	ACTCATCCACTGGCGCACGG
pr4-2	TTAAGTTGGGTAACGCCAGG
pr4-3	GGTTTGCCGTACGACTGCCGC
pr4-4	CCGTGTGTGTCCATGTTGTG
pr4-5	TACCCTTTGGTGTGATCAGC
pr4-6	AATGAACGCTCAATCGAGC
pr4-7	CTGAGGCATTGGAAGCTGCC
pr4-8	TAGTACGACCACCTTCCGCC
pr4-9	AGTTGCAACAACGCGGCCTCCACG
pr4-10	TTCAATTTAACATACCGACC
pr4-11	CGATGCTGTCCGTCATCAGG
pr4-12	AGAAAAGCTTATGGCTCGCCGTCCCGCACG
pr4-13	GTAGAGGATCCTTAGTCAAGTGGATCCTGGTTA-
	-GTATGGACCTCGGCCTAGATGTTGTATGGGGTG
pr4-14	GATCCACTTGACTAACTCGAGG
pr4-15	GTGAACTGATTGAGCTCCCTAG
pr4-16	CCGTGCGCCAGTGGATGAGTT
pr5-1	T3
pr5.2	AGATAAGCTAGCTAGCTGCC
pr5-3	GGATTTATGAATCATTGTGGC
pr5-4	GGCAAACTTGTGAGGTTGTAACG
pr5-5	CAGATCTGTGCCTTGCGGTAGC
pr5-6	Spliced leader (see above)
pr5-7	TTGATCTGCCGTTCGATCCACTCC
pr5-8	AAATAAATTCCACAAGCGGC
pr5-9	TACCAGCAATTCACGCAGCGG
pr5-10	TCATGTCGTCGGGTCCACGC
pr5-11	GTTGGGACGAAAGTGAAGGC

pr5-12	TAAACGACTCCTCACCTTCC
pr5-13	T7
pr5-14	T3
pr5-15	CACTTGAGGCTCCTGCGACC
pr5-16	CTTTGGTTACGCTTTTACACGC
pr5-17	CCATCTTGTTGGCATCACGC
pr5-18	AATCACCAACACAAGGGCG
pr5-19	CCACACACGCCATTGTTAACG
pr5-20	CGTAGCTATCAATATCCTCGG
pr5-21	CACCTAAACCAAGTTCCGCG
pr5-22	T7
pr5-23	GGATCCACAACCTCCACAAGTTTGCC
pr5-24	GGGCCCTTACATAGGAATCAGATCAAACTCCC
pr5-25	CGAAGCTTGAAAAGCATAAGGAAGGAACTG
pr5-26	CGGGGCCCGGACTTGACGACATGACAGG
pr5-27	CGAGCGCTTTACGGAACCTCCAACTTTTCC
pr5-28	CGGGATCCGAAAAGCATAAGGAAGGAACTG
pr5-29	CGAAGCTTATGGAAAAGCATAAGGAAGGAACTG
pr5-30	CGACGCGTTTAGTCAAGTGGATCCTGGTTAGTA- -TGGACCTCCGGAACCTCCAACTTTTCCTTCG
pr5-31	GGATCCTCGTCAGGTCCACGCTATGAG
pr5-32	AAGCTTGCGCACACAAATTGTTTACGG
pr5-33	CAGATCTGTGCCTTGCGGTAGC
pr5-34	GTTGGGACGAAAGTGAAGGC
Leish1	GACCTCGGCCTTTGCATGCG
Leish2	CGTCAAGTCTGGCGACCTGCG
Leish3	AGGCGACGAGGGTGTCGGCG
T7	TAATACGACTCACTATAGGG
SP6	ATTTAGGTGACACTATAG

Appendix III SDS-polyacrylamide gel electrophoresis conditions

Glycine SDS-PAGE

Resolving Gel			
	12%	10%	7.5%
dH_2O	3.35 ml	4.00 ml	4.85 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	2.5 ml	2.5 ml
10% (w/v) SDS	100 µl	100 µl	100 µl
Acrylamide/Bis (30%)	4.0 ml	3.3 ml	2.5 ml
10% ammonium persulfate	50 µl	50 µl	50 µl
TEMED	10 µl	10 µl	10 µl

Stacking Gel (4%)	
dH_2O	6.1 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml
10% (w/v) SDS	100 µl
Acrylamide/Bis (30%)	1.3 ml
10% ammonium persulfate	75 µl
TEMED	10 µl

10 x running buffer (1l)

Glycine 144 g
SDS 10 g
Dissolve in 1 litre dH_2O then pH to 6.3 with solid Tris

Glycine SDS-PAGE gels were run at 10 mA.

Tricene SDS-PAGE

	Resolving Gel	Stacking Gel
Acrylamide/Bis (30%)	4.2 ml	1.2 ml
Gel buffer	3.3 ml	2.0 ml
Glycerol	1.0 ml	-
dH_2O	1.5 ml	6.8 ml
10% ammonium persulfate	75 µl	80 µl
TEMED	7.5 µl	15 µl

5 x Anode buffer (bottom reservoir)

1 M Tris-HCl, pH 8.9

5 x Cathode buffer (top reservoir)

Tris 500 mM
Tricene 500 mM
SDS 0.5%

Gel buffer

Tris-HCl 3 M, pH 8.45
SDS 0.3%

Tricene SDS-PAGE gels were run at 80 V.

Appendix IV Sequence of 2.8 kb *MOB1-2* clone

CTGTTCACGCCTGAACAAACCAGCAACCGAGTAATTGTTGTGGTTGATTGAAGGTTTGCGAA
GCAAATCTACCACCTTCAGTTGTGTCCTTGTGCACCGAACCTCTCGGTTTCAGTGAATGTTGT
GTGGTGACTGTTTTCTTTCCTTTTCTTTGTTCTTTTCGTTCTTTCCGTGTCATATCTCCTCCCTT
TTCCTCGGACAGATGATGTTTATCGATTTCGAGACTTACTCACACTTGAGGCTCCTGCGACCA
GGACCCTCTTTTCTGTTTTTTCTGGATGGTGGAGGATTTATGAATCATTGTGGCTGTGCAACA
TATCTGTTTAACACCTCTACTGCACTCATCGTCCCTCCACAATTATGTTCCGTTGTTTCCTTAA
TTTGCTTTTAGAAAGGTAAGCTCTGGTATTCGTTTGCTCTTCACAATATGTCGGGGAAACATA
TTTTCACTTGTTATGACGATATTATAGGGAGCTGCATGTGAAGGACTGCCGTTGTGGGTCCTC
GAGCGGCTGTGTGGATACAATAATCTTTCGTTACGCTTTTACACGCACTTGCGCGCATATTTC
CTTCCATCTTCTTCTTGCCATGTGCCCGTCTTTTGCGGGTGCTACGCTAACTTGTCGGAAGGT
RTAGAATGTTGGCGACACAACRTACMTGTTTGGGGAGGTGGTTGATCCTGGATATTGCTGCT
CGGAGAWATAACTCAGTTAATATGGTATTGCGCCCTGGTACAATTACACACCGGAGAGATG
CCTGTTACCCATCTTGTTGGCATCACGCACTACTCACTCACCCCACCTTCACCAATCCGTCTA
TTCTAACAAGCCCTATGAAGCGTTTTAAGGCAAAGCTCTTCGACTCCGACAGAACATACAAA
CCTAAGAAAAAGCATAAGGAAGGAACTGAACGTTACAACCTCCACAAGTTTGCCAAATCAC
TTGtGCGCTCAGGTGACCTCAGTGCTGCTGTAAAGCTACCGCAAGGTGCCGATCTGAATCATT
GGCTTtCCGTACACACTGTAGATTTCTACAACATCACTAATGTTTTGTACGGCTCTCTAACAG
AGTTTTGCACGAACAGCAGTTGTCCTGTCATGTCGTCAGGTCCACGCTATGAGTATTTATGGC
GTGACCCGCCGGAATATCCAAAAGCAACGAAGGTGAGCGCACCGGAGTATGTGAGATTGTT
GATGGAGTGGATCGAACGGCAGATCAATGATGAACGTGTGTTTCCGTCTGAGGATCGTAATC
CCTATCCACCARATTTCGCGGATAGGGTGAAGGCGTGTTTCAAGCGACTGTTCCGCGTTTAT
GCACACGTTTACTATTYCCACTTTGCGAAGATTCGTGAGTTGCAGGAGGAATTTCACATCAA
CCAcCCCGCACTGAAACATTTTATGTATTTTGTGTGGGAGTTTGATCTGATTCCTCGTGAGGA
GGTGTCTCCGCTGCGTGAATTGCTGGTAAACTTAATGGGTCAGCGCGCGAAGGAAAAGTTGG
AGGTTCCGTAAACAATTTGTGTGCGCGCCCTTGTGTTGGTGATTTTATTTCAATCTGCAGTGC
TTCTCGAGTGCGCTCCGAAGGTTGTTTACATCATCGAAGAGGGTCTCTGTGTTTTCGTTCCCT
GTATTTCATGTTTATTCTGTGGTGATATAGGTTAAACATAGTGCTGGGCATTATTCTAAGAGG
ACGAGCTGTAGGTAACGTATATTGTAGCGGTTCCCGTGTGATGTTTGGTGAGAAGGCAGTTC
CGATACATGCATACTATATGCATACATGTATTGGACTGCTGGCCGTCGTTGACGGGATCCGT
ACCGGTTCCTTGTAACGATTACTTGAAGTGACTTAAATTCTCGTTAACAATTGGCGTGTGTGG
AGGAAGAGGGGGGAACGATGATATGCAGTGAGCCCGTGGCCCGTTCCGTTTGTGTGGTGGC
AGGACCTGTGCGTGTGCGTGTGTGTTGTATTCGGTGGACAGTAAAAAGATGTTTTGCAGGTG
TGTAGGACGGCGTATTGCACCTCAGGCATGTTATTGTCTTCGCCGTATACTTCCCACCAACAG
TGTGTCTTTTCTCCCTTTACTTTTTTTCTTGAAAAAAAAAATTACAAAGTGCATTTGTCGGATT
GGAGCACTGCCTACATGGCTGATCATGAAGGTGTAATTCGTCCCATCCGTAGAGGACCCAAT
GTTTTATCGACCGAGGATATTGATAGCTACGTGAAGGAGCTACTGTATAGTGCAGGGCGAAA
AAGGAACGCTGGGCAAAACTCTTTTAAAGGGAATGTGCAGCAGAAATCCCTGACAGTTGAG
TATATACTTGAGAAAGTTTACGGGAGATTTTTCAATCAATCGGGGCAATCTCCTTTTCCTGAT
GGTTTACCCTTTGCTTTGAAGCAGATTCCATCAATTGCCAGAATTGCCGAACTACAACTGAG
AGCTACAGCACTAGTTCAATCTGCAGTTGCCACACGGCCCGTCGTCACTCTTCATGAATTAG
AGTTGGAGGTTTGCAGTGCTGAAGGTGTTTCAATTTTCGCGGAACTTGGTTTAGGTGATTCAC
TGACCGCGTTGCCGTTTGTGCAGCACGCGTTCAATCTGCGGAGCTCAACAGGAGTTGTGGCC
CCTATTACCAGCGACGATTTTTTACAATTTTTGTTGTTTCATGCTGACGCTGAAATAATGTTG
ACAAGCGGTGGGGACGCGGGGGATGCTGTACGTGCCTTTGCACAGTTTTACAAAAATGGCT
ACTACACCCCTCTGCAG