Characterisation of cdc2-related kinases from *Trypanosoma brucei*

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by

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Dedicated to Kieran,

thanks for keeping me up enough to finish this.

"Basically, Gareth, your cells are ******", J. Mottram.

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

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Abbreviations.

ATP	Adenosine triphosphate.
BSA	Bovine serum albumin.
DAPI	4, 6-diamino-2-phenylindole.
DTT	Dithiothreitol.
EDTA	Ethylenediaminetetraacetate (disodium salt).
EtBr	Ethidium bromide.
EtOH	Ethanol.
FCS	Fetal calf serum.
IPTG	Isopropylthio-beta-D-galactoside.
PBS	Phosphate buffered saline.
PCR	Polymerase chain reaction.
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate).
Tris	Tris(hydroxymethyl)aminomethane.
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside.
ZPFM	Zimmerman post-fusion medium.

Measurements.

bp	base pairs.
kb	kilobases.
Mb	megabases.
kDa	kilo Dalton.
s	seconds.
min	minutes.
hr	hours.
mm	milimeters.
µg, mg and g	micrograms, miligrams and grams.
µl, ml and l	microlitres, mililitres and litres.
µM, mM and M	micromolar, milimolar and molar.
V	Volts.
A	Amperes.
µF	microfarads.
^{o}C	degrees centigrade.
rpm	revolutions per minute.

Acknowledgements

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The football team kept me (relatively) sane through the later years, you have my gratitude for not kicking me too hard during the five-a-side games. Thank you for everything you've done for me Paul, it's not everyday some-one travels 400 miles to pop in for a few drinks and a curry.

To Martin and Tracey, my God some of those films were bad, but the hospitality, beyond the call of duty, was the best. Jamie and Lorraine may have taken years off my liver's life expectancy but those evenings will be remembered for a long time (if my memory returns).

I wouldn't have got this far without the love and support of Leslie. I suppose that now I've finished I'm going to have to do some tidying up. Finally, all this is for Kieran, you're pretty amazing considering you share some of my genes.

Summary.

Trypanosoma brucei is a kinetoplastid protozoan parasite of man and other mammals in Africa, that is transmitted by the bite of the tsetse fly. *T. brucei* has a complicated life cycle involving both dividing and non-dividing stages. The non-dividing stages are to some extent pre-adapted to the environment of the next host, and as such are essential for efficient transmission from both tsetse fly to mammal and mammal to tsetse fly. It was the aim of this project to initiate the analysis of cell cycle controlling genes from *T. brucei*, with a view to assessing their involvement in the regulation of the division status of the various life cycle stages.

The Cyclin Dependant Kinases (CDKs) are a highly conserved family of serine/threonine protein kinases, many members of which are vital for the regulation of the cell cycle in eukaryotes. The archetypal CDK is cdc2, a protein that regulates both S phase and mitosis in yeast; the homologous protein in higher eukaryotes regulates mitosis, while a closely related protein, CDK2, is involved in S phase control. The kinase activity of the CDK proteins is tightly regulated by phosphorylation (both negative and positive) and by the association of a regulatory subunit (a cyclin).

Two cdc2-Related Kinase genes (*crks*) from *T. brucei* had already been cloned and sequenced at the start of the Ph.D. (*tbcrk1* and *tbcrk2*) and another gene fragment (*tbcrk3*) had been isolated by PCR. The genomic locus of the tbcrk3 gene was subcloned and sequenced. Analysis of the predicted protein sequences for the TbCRK proteins revealed no obvious candidate for the role of cdc2 homologue.

The three TbCRK proteins were expressed in *E. coli*, and used to raise antisera, as well as to assess the cross reactivity of the antisera available in the laboratory. These antibodies were then used to analyse the expression of the TbCRK proteins in three of the parasite's life cycle stages. No important differences in expression were observed between dividing and non-dividing stages. TbCRK3 was found to be in the insoluble fraction of the protein extracts made, implying an association with either DNA or the

cytoskeleton.

Another component of the cdc2/cyclin complex in fission yeast, $p13^{suc1}$, has previously been used to purify cdc2 related kinases from a variety of organisms. This approach was followed using both $p13^{suc1}$ and a homologue ($p12^{LmmCKS1}$) from the kinetoplastid parasite, *Leishmania mexicana*. Kinase activity could be bound to the $p12^{LmmCKS1}$ beads, and both TbCRK1 and TbCRK2 were shown by Western blotting to be part of the protein fraction purified on the beads.

An attempt was made to analyse TbCRK3 function by reverse genetics. Null mutants were created by homologous recombination and their phenotype assayed. The mutant cultures did not survive longer than three months. Microscopy using DNA binding dyes show a phenotype of aberrant cytokinesis, morphological abnormalities and a breakdown in cell polarity. The relevance of this phenotype to the insoluble nature of TbCRK3 is discussed.

CHAPTER ONE
INTRODUCTION

1.1 *T. brucei* and the kinetoplastids.

1.1.1 Trypanosome basic biology.

Trypanosomes are unicellular, flagellated, protozoan parasites of the order Kinetoplastida. The kinetoplastids form an extremely early branch of the eukaryotic kingdom as shown in Figure 1.1.1 (adapted from Sogin *et al.*, 1986), and therefore the degree of divergence between them and other members of the eukaryotes is large.

Trypanosomes are responsible for a number of diseases that cause human fatalities, as well as severe economic damage. In Africa, *Trypanosoma brucei* causes sleeping sickness in humans and Nagana in domestic animals. There are three sub-species of *T. brucei*; *T. b. gambiense* and *T. b. rhodesiense* cause the chronic and acute forms of sleeping sickness respectively, while *T. b. brucei* causes Nagana (Hoare, 1972). Both *T. b. gambiense* and *T. b. rhodesiense* are resistant to lysis by human serum (Takayanagi *et al*, 1992) but the genetic basis for the differences between the sub-species is still unclear. *T. congolense* also causes serious disease in farmed animals in Africa (Hoare, 1972). All these trypanosomes are spread by insect vectors, and so are restricted to the vectors' habitats. Both *T. congolense* and *T. brucei* are transmitted by the tsetse fly (*Glossina*). Within the region of sub-Saharan Africa where the tsetse fly is common, the farming of domestic livestock is extremely difficult. Due to variation of the major surface protein of *T. brucei*, the infection avoids clearance by the immune system , and infected animals which do recover are not fully protected against further challenges by *T. brucei*. The mode of transmission of trypanosomes varies, but both *T. brucei* and *T. congolense* are salivarian trypanosomes, infecting the host via the bite of the fly, when the parasites are injected into the mammalian bloodstream. Some trypanosomes have eliminated the need for a specific vector, and in one case, any vector. *T. evansi* is spread by mechanical means, without replication in the biting insect. *T. equiperdum* has no vector, and is spread as a venereal disease of horses.

Figure 1.1.1 Evolutionary relationship of *T. brucei* and other Eukaryotes

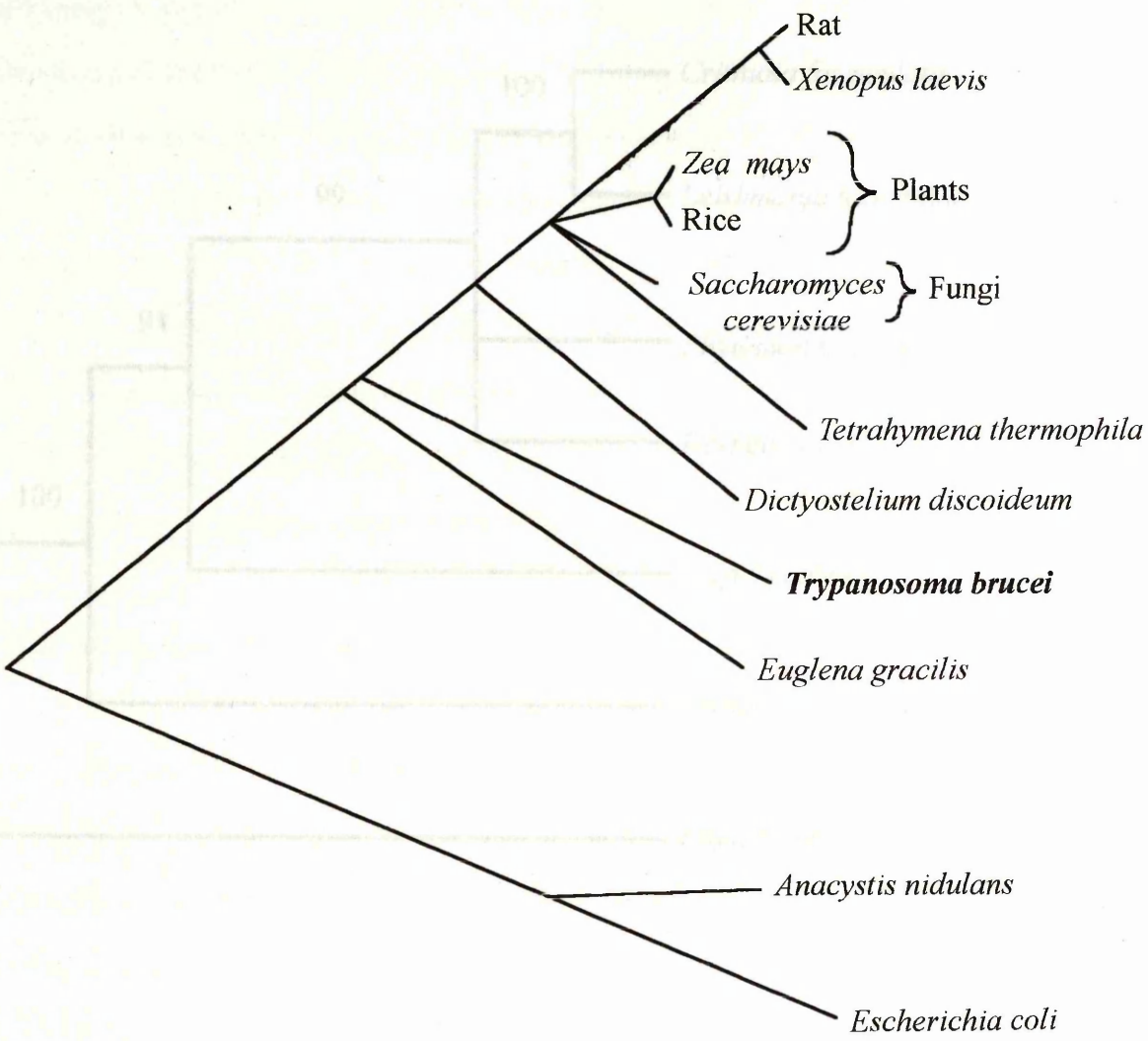


Figure 1.1.1 The phylogenetic tree is adapted from Sogin *et al.*, 1986. It was calculated using nuclear small subunit ribosomal RNAs. The degree of evolutionary divergence is represented by the horizontal distance between any two points.

Figure 1.1.2 Predicted evolutionary relationships of the *Kinetoplastida*

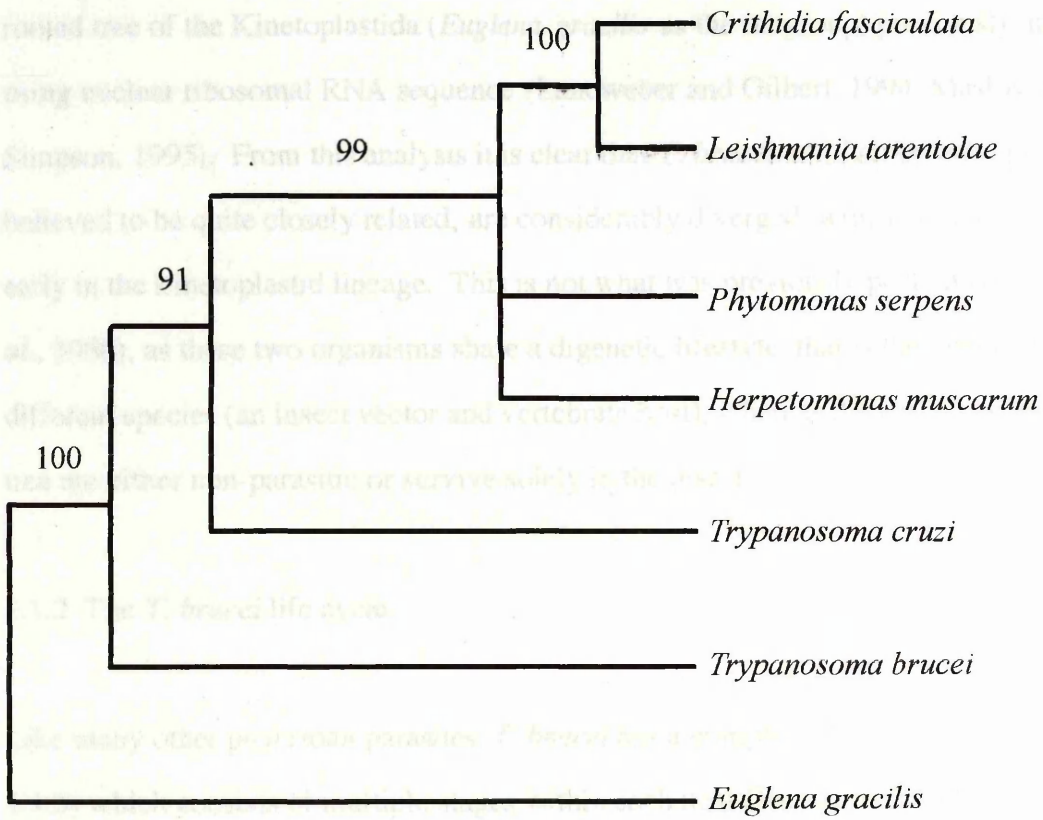


Figure 1.1.2 The consensus tree for the *Kinetoplastida* (adapted from Maslov and Simpson, 1995; Landweber and Gilbert, 1994) was derived using nuclear 18S rRNA sequence. The numbers shown are the bootstrap support for each branch. The early branching of *T. brucei* is well supported, as is the late divergence of the *Leishmania* and *Crithidia* branches.

Within the Kinetoplastida, the relationships of different species is now becoming clearer with the increase in available molecular data. Figure 1.1.2 shows a rooted tree of the Kinetoplastida (*Euglena gracilis* as the outgroup) previously analysed using nuclear ribosomal RNA sequence (Landweber and Gilbert, 1994; Maslov and Simpson, 1995). From this analysis it is clear that *T. brucei* and *Leishmania*, previously believed to be quite closely related, are considerably diverged, with *T. brucei* branching early in the kinetoplastid lineage. This is not what was previously postulated (Lake *et al.*, 1988), as these two organisms share a digenetic lifestyle, that is they parasitise two different species (an insect vector and vertebrate host), whereas the other species on the tree are either non-parasitic or survive solely in the insect.

1.1.2 The *T. brucei* life cycle.

Like many other protozoan parasites, *T. brucei* has a complex life cycle (see Figure 1.1.3) which consists of multiple stages within each host (Vickerman, 1985). The mammalian bloodstream forms are of two main types. There are dividing cells, the long slender form, and non-dividing cells, called the short stumpy form. Within the tsetse fly vector there are three major forms (Vickerman *et al.*, 1988). The procyclic form is found in the fly midgut, and is rapidly dividing, as is the epimastigote form which is seen in the tsetse fly salivary glands. The metacyclic form is also found in the salivary glands of the fly, but is non replicating.

In the early infection, the parasitemia in the bloodstream of the mammalian host is primarily of the rapidly replicating long, slender form. After the infection is established a low level of the short, stumpy form occurs which increases as each infection cycle progresses (Vickerman, 1985). These are non-dividing and primed to adapt to the conditions within the Tsetse fly midgut. The bloodstream forms all have the Variant Surface Glycoprotein (VSG) coat, and are complement resistant (Takayanagi *et al.*, 1992). The trypanosomes have been found to change the VSG expressed on the parasite surface, which is thought to shield the other extracellular

Figure 1.1.3 The life cycle of *Trypanosoma brucei*.

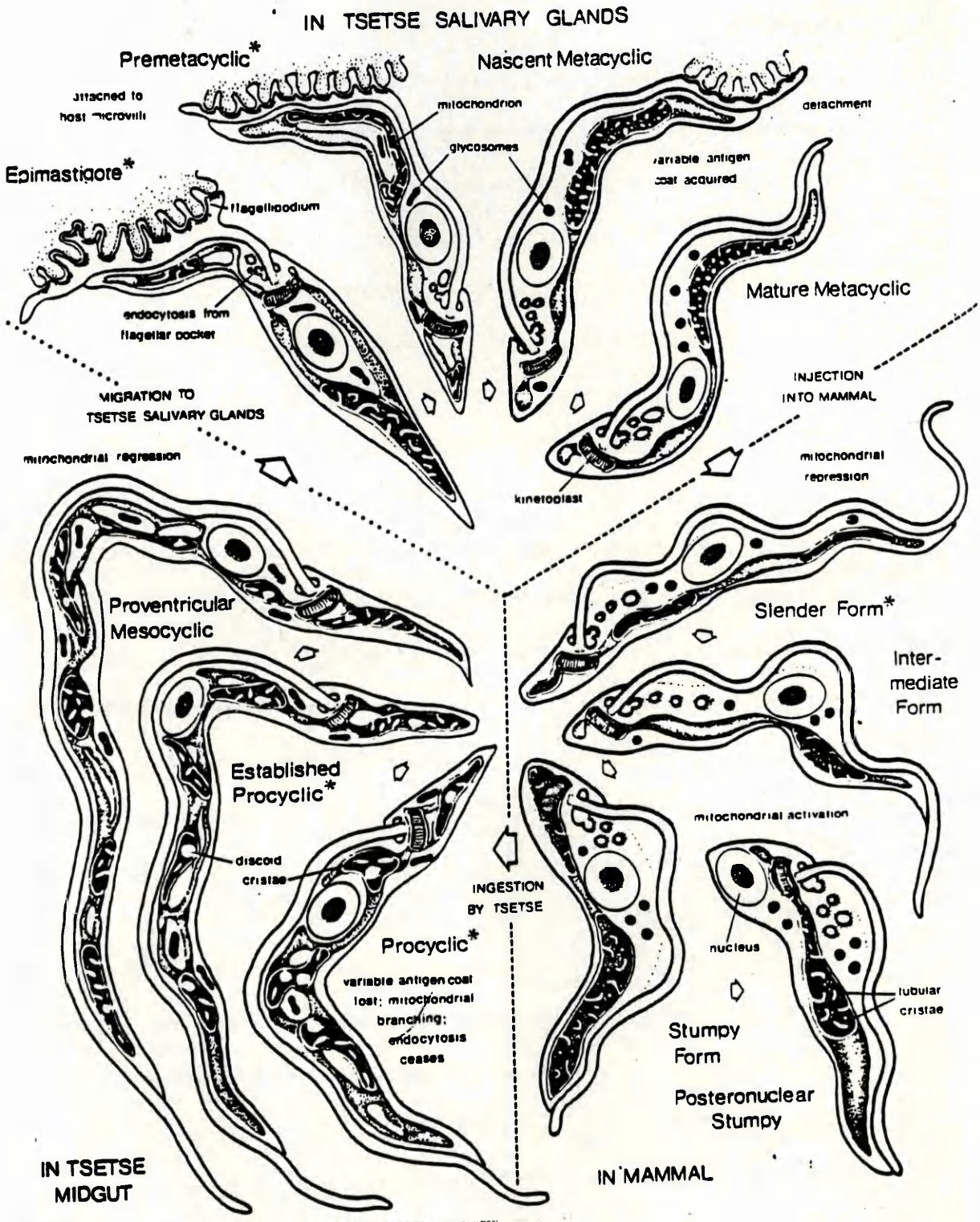


Figure 1.1.3 The life cycle of *T. brucei* consists of several morphologically distinct forms in both the mammalian and insect vector. Following initial infection of the mammalian host by a metacyclic trypanosome, the parasite differentiates into a long slender form trypanosome which is rapidly dividing. These parasites can differentiate through an intermediate stage to the non dividing short stumpy form which is infective to the tsetse fly. When taken up by the fly the short stumpy form differentiates to the dividing procyclic form. These differentiate into non-dividing proventricular mesocyclic forms which migrate from the midgut to the salivary glands, transforming into the epimastigote form which further divides. This form then differentiates to the non dividing metacyclic form, which is adapted to survive in the mammalian host. After injection by the tsetse fly the metacyclic form begins to undergo division and becomes long slender form trypanosomes, completing the cycle (From Vickerman, 1985).

components from the host immune system, up to once every 100 divisions (Barry, 1989; Barry and Turner, 1991). These switches, to VSGs that the immune system has not yet been exposed to, allow the parasitemia to last for a long period with repeated peaks of infection. The mitochondrial citric acid cycle is non-functioning in the bloodstream form, although the mitochondrion of the short stumpy form is partially developed as a step towards differentiation into the procyclic form (Williams *et al.*, 1991; Bienen *et al.*, 1991; Durieux *et al.*, 1991). The bloodstream trypanosomes' energy needs are provided predominantly by glycolysis, which takes place in organelles specific to the trypanosomes, called glycosomes (Aman and Wang, 1986; Aman *et al.*, 1985; Opperdoes, 1985; Opperdoes, 1990). These organelles catalyse the metabolism of glucose to pyruvate with an extremely high throughput rate, but low efficiency of ATP synthesis. This lack of efficiency is not important to a parasite living in the nutrient rich environment of the mammalian bloodstream. When taken up in a bloodmeal by the feeding fly, the short stumpy forms differentiate to the procyclic form trypanosomes which divide and set up the infection within the midgut (Vickerman, 1985). In *in vitro* experiments, it is possible for long slender form *T. brucei* to differentiate into procyclic form trypanosomes in a non-synchronous fashion (Simpson *et al.*, 1985; Matthews and Gull, 1994a and 1994b). It appears that the long slender form is only responsive to the extracellular signals for differentiation during a short period in the G1 phase of the cell cycle, and in the Tsetse fly midgut, if differentiation does not occur quickly the cell dies (Matthews and Gull, 1994b). During the differentiation process, the trypanosomes lose the VSG coat, synthesise a new glycoprotein (called PARP or Procylin) coat, and the mitochondria start producing ATP, with proline apparently used as the main source of energy (Durieux *et al.*, 1991; Brown *et al.*, 1973; Priest and Hajduk, 1994a and 1994b; Matthews and Gull, 1994b) The procyclic form trypanosomes migrate to the salivary glands of the Tsetse fly and differentiate into the other dividing stage, the epimastigote. In the salivary glands the trypanosomes stop dividing and re-express VSG (Lenardo *et al.*, 1986; Hirumi *et al.*, 1992). The metacyclic form is pre-adapted to the environment of the mammalian

bloodstream. It is resistant to complement (this is possibly due to the expression of VSG), has a mitochondrion without functional cytochromes, and is infective to the mammal when injected by the fly (Vickerman, 1985). After infection of the mammal, the block on replication is released, and the metacyclic form divide and transform into morphologically long slender form trypanosomes (Brun *et al.*, 1984).

Procyclic *T. brucei* can be grown in axenic culture in high numbers (>$1x10^7$ / ml) and this form is apparently identical to those isolated from tsetse fly midgut infections in all tests performed (Pearson *et al.*, 1987; Brun and Schonenberger, 1979). The bloodstream forms of some strains can be grown in culture, but at present only to very low concentrations i.e. $1x10^6$ / ml. Because of this, laboratory strains adapted to grow in mice and rats are usually used if large quantities of bloodstream trypanosomes are required. These adapted pleomorphic lines, when grown in irradiated animals can result in populations of *T. brucei* containing >90 % of the short stumpy form. After serial passage several of the laboratory adapted strains have lost the ability to differentiate to the short stumpy form, and these monomorphic lines can be used to isolate pure long slender form cells.

1.1.3 The molecular biology of *T. brucei.*

(i) Genomes and genes.

The method of Pulse Field Gel Electrophoresis (PFGE) has enabled the visualisation of the chromosomes of *T. brucei* (Van der Ploeg *et al.*, 1989). This was not previously possible as the chromosomes do not condense during the cell cycle. Used with Southern blotting techniques this has shown the diploid nature of most of the genome. The total (diploid) genome size is approximately $6x10^7$bp, and consists of chromosomes of sizes varying from 50 kb to 6 Mb (Van der Ploeg *et al.*, 1989; Gottesdiener *et al.*, 1990). They consist of three main types:

1) the minichromosomes, of 50-150 kb which apparently consist of repetitive sequences and VSG genes. It is therefore presumed that these chromosomes, which are predominantly haploid (Weiden *et al.*, 1991; Chung *et al.*, 1990), act as a resevoir of VSG genes to increase the VSG repertoire.

2) intermediate chromosomes of 200-450 kb.

3) the megabase chromosomes of 700 kb to 6 Mb.

Both of these larger types of chromosomes carry housekeeping genes (as well as VSGs), and are typically diploid. Variation in chromosome number, e.g. triploidy, can be seen in some stocks, typically those which have undergone genetic crosses (Gibson *et al.*, 1992; Wells *et al.*, 1987), and therefore this may be an artefact of these experiments. Southern blotting and hybridisation to specific gene sequences has shown that homologous chromosomes can be considerably different sizes (up to 200-300 kb difference), possibly due to variations in the copy number of some of the large tandem arrays of genes seen in the Kinetoplastida (see below). However, the large variations seen in *T. brucei* imply that there are other causes as well, probably changes in the sub-telomeric repeats (Myler *et al.*, 1988; Van der Ploeg *et al.*, 1984). These differences in size, and the rapid changes seen in passaged cells, show why there can be a high level of karyotype variation between different stocks of *T. brucei.*

Using these PFG and Southern blotting techniques, along with isoenzyme analysis and resistance markers integrated into the *T. brucei* genome (Gibson and Bailey, 1994), it has also been shown that some form of mating can take place during passage through the Tsetse fly (Turner *et al.*, 1990; Jenni *et al.*, 1986; Schweizer *et al.*, 1994). There is no evidence for, or against, a mating type comparable to that seen in yeast, or other unicellular organisms. In the published data there has been no combination of stocks tried for which hybrids have not been isolated, but there are extremely low levels of such hybrids, implying that the process is not obligatory for successful passage through the fly. Crosses within a heterozygous, clonal population have also been observed, but this does not preclude a mating type system, presuming such a system could switch between types (as in *Schizosaccharomyces pombe*).

Many of the genes isolated from trypanosomes have been found to be multicopy. In some cases they are found in tandem arrays of almost identical genes, e.g. the genes for actin, calmodulin, glyceraldehyde-3-phosphate dehydrogenase, hsp70, hsp83 and ubiquitin (Ben Amar *et al.*, 1988; Tschudi *et al*, 1985; Michels *et al.*, 1986; Lee and Van der Ploeg, 1990; Mottram *et al.*, 1989a; Chung and Swindle, 1990). In other cases, e.g. the tubulin locus, the genes alternate between copies of alpha and beta tubulin, and there can be over 13 copies of each of the 2 tubulin genes per haploid genome (Thomashow *et al.*, 1983). Of the more recently isolated genes from *T. brucei*, many are single copy, e.g. those for the Glycosyl-phosphatidylinositol specific Phospholipase C, the RNA polymerase I large subunit, the Ef-hand 5 protein (Mensa-Wilmot *et al.*, 1990, Jess *et al.*, 1989, Wong *et al.*, 1993). There may be a crude link between the number of gene copies and the level of transcription of those genes transcribed by RNA polymerase II in *T. brucei*, although experiments in a related kinetoplastid (*Leishmania enriettii*) show that loss of genes in a tandem array can be partially compensated for by an increased level of transcription and/or post transcriptional controls, e.g. mRNA stability (de Lafaille and Wirth, 1992).

(ii) Homologous recombination and reverse genetics in the Kinetoplastida.

The diploid nature of the Kinetoplastida and their lack of, or difficulty in using, a sexual cycle have made the use of genetic techniques to analyse gene function problematical. Recent advances in DNA transfection techniques have allowed expression of plasmid encoded genes in a variety of different kinetoplastids, including *T. brucei* (Clayton *et al.*, 1990; Rudenko *et al.*, 1990; Zomerdjk *et al.*, 1990;Coburn *et al.*, 1991; Ryan *et al.*, 1993). These experiments initially showed transient transfection to be possible, this was followed by the development of stable episomal vectors (Metzenberg and Agabian, 1994). Further experiments showed evidence of efficient pathways of homologous recombination (Tobin *et al.*, 1991; Lee and Van der Ploeg, 1990). This mechanism has been used to insert heterologous DNA into specific sites in

the nuclear genome (Cruz and Beverley, 1990; Eid and Sollner-Webb, 1991; Ten Ashbroek *et al.*, 1990). Relatively small regions of homologous DNA (200bp) are enough for precise insertion (Tobin and Wirth, 1992). Linear DNA has been found to integrate with higher efficiency than circular, but it is not necessary for both of the termini of the DNA molecule to be homologous to the genomic sequence (Cooper and Cross, 1993). These techniques have allowed the use of reverse genetics to assess gene function. Cloned regions of DNA can be precisely targeted for deletion (de Lafaille and Wirth, 1992; Cooper *et al.*, 1993; Cruz and Beverley, 1990; Cruz *et al.*, 1993), replacing them with a selectable marker. There are now several such independent markers conveying resistance to different antibiotics, including the genes *neo* (neomycin phosphotransferase) (Ten Ashbroek *et al.*, 1990), *hyg* (hygromycin phosphotransferase) (Lee and Van der Ploeg, 1991), *pac* (puromycin resistance) (Freedman and Beverly, 1993) and *phleo* (phleomycin binding protein) (Jefferies *et al.*, 1993). Antibiotic resistance markers are necessary as auxotrophs have been difficult to produce in the diploid Kinetoplastida.

By repeating the insertion event with two different selectable markers it is theoretically possible to create null mutants, and thereby assess mutant phenotypes (Cooper *et al.*, 1993). This is easier if the gene of interest is single copy within each haploid genome, but tandem arrays can be targeted, and replacing large amounts of genomic DNA (>44 kb) with a relatively small selectable marker (1 kb) does not seriously compromise the efficiency of the replacement (de Lafaille and Wirth, 1992).

If the gene targeted is essential to parasite *in vitro* viability, then null mutants cannot be isolated. Instead, organisms which show tetraploidy or aneuploidy (Cruz *et al.*, 1993), the amplification of the targeted gene, or the creation of extra-chromosomal elements containing either copies of the targeted gene (de Lafaille and Wirth, 1992), or the selectable marker (J. Mottram, unpublished data), are isolated.

(iii) Transcription.

All alpha-amanitin sensitive transcription in *T. brucei* is polycistronic, and no well characterised promoters for RNApolII protein coding genes have been isolated (Wong *et al.*, 1993; Ben Amar *et al.*, 1991; Muhich and Boothroyd, 1988; Tschudi and Ullu, 1988). The promoters for the highly expressed PARP/Procyclin and VSG genes have been isolated, but they are not typical (Sherman *et al.*, 1991; Zomerdijk *et al.*, 1990; Zomerdijk *et al.*, 1993). Transcription of PARP and VSG is performed by an alpha-amanitin resistant RNA polymerase, either Pol I or a modified Pol II, unlike the other protein coding genes isolated whose transcription is as sensitive to alpha-amanitin as that seen in other organisms (Konig *et al.*, 1989; Kooter and Borst, 1984; Grondal *et al.*, 1989). Although transcription is polycistronic, the mature mRNA is monocistronic. This processing occurs rapidly, initially by the *trans*-splicing of a 39 nucleotide mini-exon from the 140bp Spliced Leader RNA (SLRNA) to a region upstream of each gene, called the splice acceptor site (Murphy *et al.*, 1986; Sutton and Boothroyd, 1986; Ullu *et al.*, 1993). This spliced leader contains the functional equivalent of the 5' cap seen in other eukaryotes, and is necessary for expression of the protein. The *trans*-splicing event is also, apparently, one factor involved in directing the polyadenylation of the upstream region of the transcript, which may also rely on some extremely degenerate sequence motifs (Schurch *et al.*, 1994) e.g. a polypyrimidine tract. Genes transcribed in the same polycistronic precursor RNA have been found to have widely varying steady state mRNA levels, and regulation at the post transcriptional stage appears to be important for the control of gene expression (Gibson *et al.*, 1988). No *cis*-splicing has been described in the Kinetoplastida, except for an intron in a tRNA (Schneider *et al.*, 1993), and to date no protein coding genes containing sequences that look like introns have been described.

1.1.4 The Mitochondrion of *T. brucei,* editing of RNA and mitochondrial translation.

Unlike other eukaryotes, the kinetoplastids have only one mitochondrion per cell. The mitochondrial genome, the kinetoplast, is a huge concatenated network of two classes of circular molecules (Simpson, 1986). The maxicircles, which are the equivalent of the mitochondrial genome in other cells, are approximately 20 kb in length in *T. brucei*, and there are numerous (~35) identical copies. These are joined by ~$1x10^4$ interconnected minicircles of about 1 kb in size, which are very diverse in sequence (>400 different classes) with multiple copies of each sequence type. Notably, *T. evansi* and *T. equiperdum*, which do not pass through an insect vector, only have a very restricted variety of minicircle types (Barrois *et al.*, 1981; Ou *et al.*, 1991). Transcripts from the maxicircle consist of tRNAs, rRNAs and the mRNAs which encode proteins presumably translated in the mitochondrion (Simpson and Shaw, 1989). Many of the genes in the maxicircle, however, require editing before translation of the correct Open Reading Frame is possible. This editing is guided by short (~70bp), complementary (allowing for G:U wobble pairing) RNAs (gRNAs) transcribed from the minicircles, and proceeds from the 3' to the 5' of the mRNA, by inserting, or occasionally removing, uridine residues in the precursor RNA. The editing takes place in a large protein/RNA complex which has been called the editosome (Goringer *et al.*, 1994, Koller *et al.*, 1994). Each type of minicircle encodes 3 gRNAs which gives enough coding capacity for editing all the known transcripts even given the high levels of overlapping in the gRNA sequences, which may ensure processivity and be a proof reading mechanism (Corell *et al.*, 1993). Not all of the mRNAs are edited, and the amount of editing is variable. In some cases the editing process forms the starting AUG codon, which gives in built regulation of translation of the message. Although there is no stage specific regulation of maxicircle transcription, there is stage specific regulation of the editing of a number of transcripts, with COII and CYb completely edited in the procyclic stage only (Feagin *et al.*, 1985), while ND7 has its 3' domain edited only in the bloodstream forms (Corell *et al.*, 1994). It is not clear how this occurs, but is

presumably due to control of gRNA function or presence. The processive nature of editing, with one gRNA often editing the region to which another will anneal, means that the control of one gRNA can cause the editing of the whole transcript to be abortive.

As mentioned above, the bloodstream form *T. brucei* do not have a functional citric acid cycle, and it is possible to isolate strains in bloodstream culture that have lost the kinetoplast (Agbe and Yielding, 1994), similar to the trypanosome species that have lost the ability to multiply in insect vectors with a commensurate loss of minicircle variation. The mitochondrion may still, however, carry out tasks encoded by the nucleus, and the retention of the maxicircles in the kinetoplast in *T. evansi* and *T. equiperdum* is evidence that some unedited genes in the mitochondrion are needed for optimum viability *in vivo* even in bloodstream form *T. brucei.* The reduction in growth rate of akinetoplastic *T. evansi* compared to the wild type (Silvatahat *et al.*, 1995) points to the fact that the mitochondrial DNA is still important in the bloodstream form *T. brucei*. If this is true, then translation of mitochondrial transcripts may be important in both bloodstream and procyclic stages.

The translation machinery in other eukaryote mitochondria is apparently related to that seen in eubacteria, with similar rRNAs and protein factors. One of the protein factors, for which a gene has been isolated from eukaryotes, is mitochondrial Elongation Factor G (mEF-G). EF-G is a GTP binding protein that is involved in controlling the tri-nucleotide translocation of the eubacterial ribosome during translation. This gene is nuclear encoded in *Saccharomyces cerevisiae* and rat (Vanbutas *et al.*, 1991; Barker *et al.*, 1993), the two eukaryotes from which the gene has been isolated, and the protein has been shown to be imported into the mitochondria of *S. cerevisiae* and cows (Vanbutas *et al.*, 1991; Chung and Spremulli, 1990). The gene responsible for the same activity in the cytoplasm of eukaryotes, and in archaebacteria, Elongation Factor 2, is related, but distinct (see Chapter 3.3.1).

1.2 The Eukaryotic Cell Cycle.

1.2.1 Introduction - the typical eukaryotic cell cycle.

The typical mitotic eukaryotic cell cycle consists of 4 stages (Figure 1.2.1). There are three major control points during the cell cycle. During G1 (Gap 1) there are a series of decision points at each of which the possible development options are reduced. At some point the cell becomes committed to another division cycle; this is called START in yeast, or the Restriction Point in higher eukaryotes. The other control points are at the entry and exit of mitosis, at which DNA replication and chromosome separation, respectively, must be complete.

After division the cell enters G1 phase, during which the cell synthesises proteins necessary for cell growth (e.g. enzymes required for DNA synthesis) and division. Prior to START the cell is responsive to outside factors, such as mating pheromones (Obara-Ishihara and Okayama, 1994; Fujimara, 1994; Peter and Herskowitz, 1994) in yeast, and growth factors in higher eukaryotes (Kato *et al.*, 1993; Duronio and O'Farrell, 1994). These different factors can cause the cell to stop its progression through the cell cycle, and force it onto an alternate pathway, i.e. into conjugation/mating in yeast, cellular differentiation or apoptosis in other organisms. In multicellular organisms the cells can also exist in a state called G0. Cells in this state are not growing, and have not passed through the restriction point. However they are neither senile nor terminally differentiated as they can be stimulated to re-enter the cell cycle and undergo division. G0 can be considered a separate stage of the cell cycle, or as a sub-state of G1. At some point after START, nuclear DNA synthesis begins. This is the defining characteristic of S phase. The synthesis of mitochondrial DNA in all eukaryotes examined, apart from the kinetoplastida (see Chapter 1.2.3), is less tightly regulated, with replication being continuous throughout the cell cycle (Bogenhagen and Clayton, 1977), but possibly predominantly during S phase and G2 (Clayton, 1991;

Figure 1.2.1 The eukaryotic cell cycle

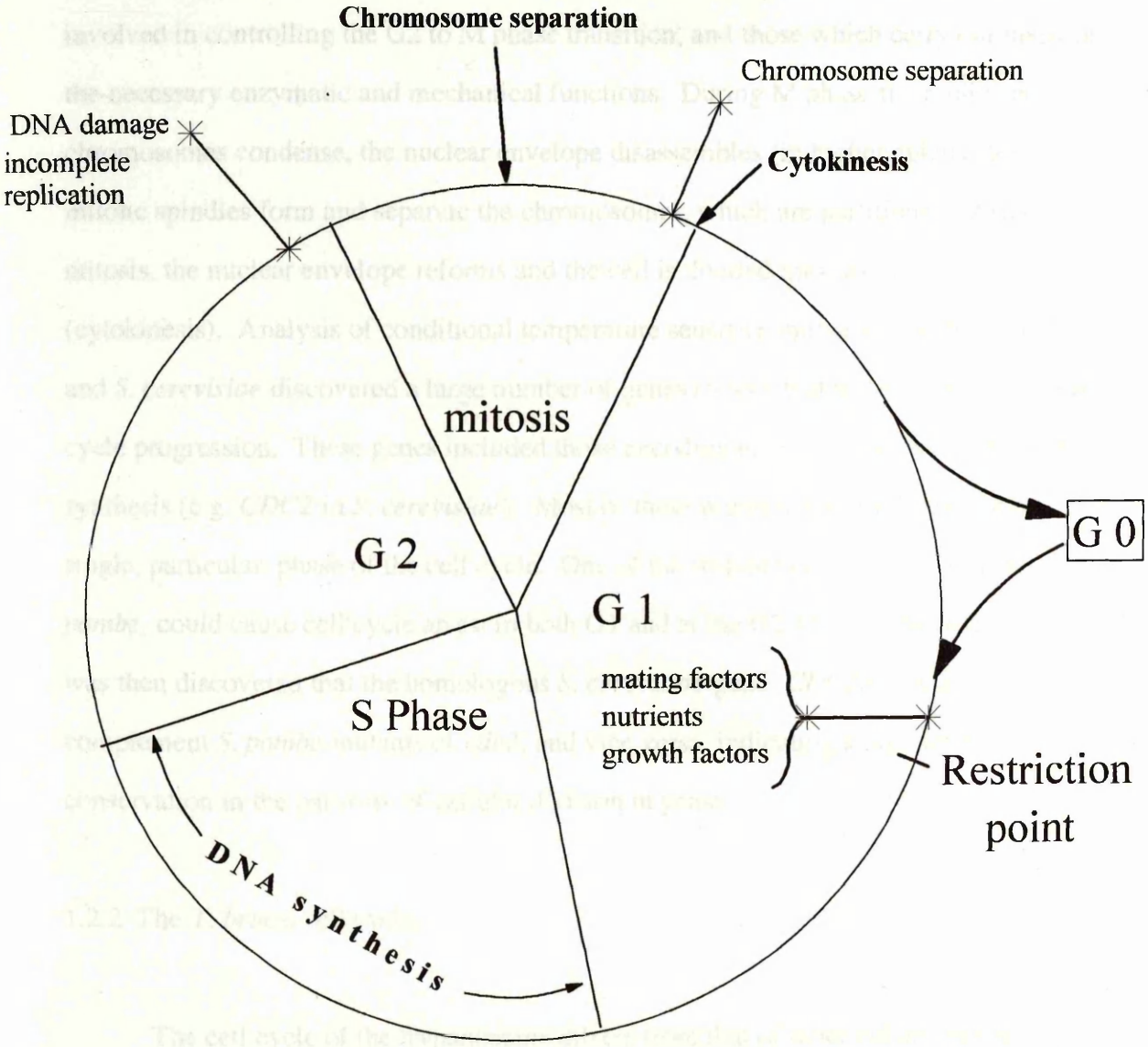


Figure 1.2.1 The four stages of the typical eukaryotic cell cycle, plus G0.

Arrows with **bold text** show occurances during the cell cycle.

Starred lines show checkpoint controls during the cell cycle.

Attardi, 1988). An increase in mitochondrial DNA synthesis during this time may be simply to do with the availability of DNA precursors. When nuclear DNA synthesis stops the cell continues to grow, and in this G2 phase the cell synthesises the proteins involved in controlling the G2 to M phase transition, and those which carry out many of the necessary enzymatic and mechanical functions. During M phase the replicated chromosomes condense, the nuclear envelope disassembles (in higher eukaryotes), the mitotic spindles form and separate the chromosomes, which are partitioned. After mitosis, the nuclear envelope reforms and the cell is divided into two daughter cells (cytokinesis). Analysis of conditional temperature sensitive mutants in both *S. pombe* and *S. cerevisiae* discovered a large number of genes (>100) that were essential for cell cycle progression. These genes included those encoding enzymes responsible for DNA synthesis (e.g. *CDC2* in *S. cerevisiae*). Most of these mutations caused arrest in a single, particular, phase of the cell cycle. One of the isolated genes, *cdc2* from *S. pombe,* could cause cell cycle arrest in both G1 and at the G2/M phase boundary. It was then discovered that the homologous *S. cerevisiae* gene, *CDC28*, could complement *S. pombe* mutants of *cdc2*, and vice versa, indicating a high level of conservation in the pathway of cellular division in yeast.

1.2.2 The *T. brucei* cell cycle.

The cell cycle of the trypanosome differs from that of other eukaryotes in a number of respects (Sherwin and Gull, 1989; Gull *et al.*, 1990). The presence of a single mitochondrion and flagellum per cell means that the replication and division of the nucleus, kinetoplast, mitochondrion, basal bodies and flagellum must be tightly controlled. Figure 1.2.2 shows a schematic of the cell cycle of procyclic *T. brucei* grown in culture (Gull *et al.*, 1990). The early G1 cell contains one basal body attached to the flagellum, with a pro-basal body next to it. The first visible event in the *T. brucei* cell cycle, detectable with specific monoclonal antibodies, is the elongation of the pro-basal body and the formation of the basal plate. Next, the new flagellum begins to form

Figure 1.2.2 The cell cycle of procyclic form *T. brucei* .

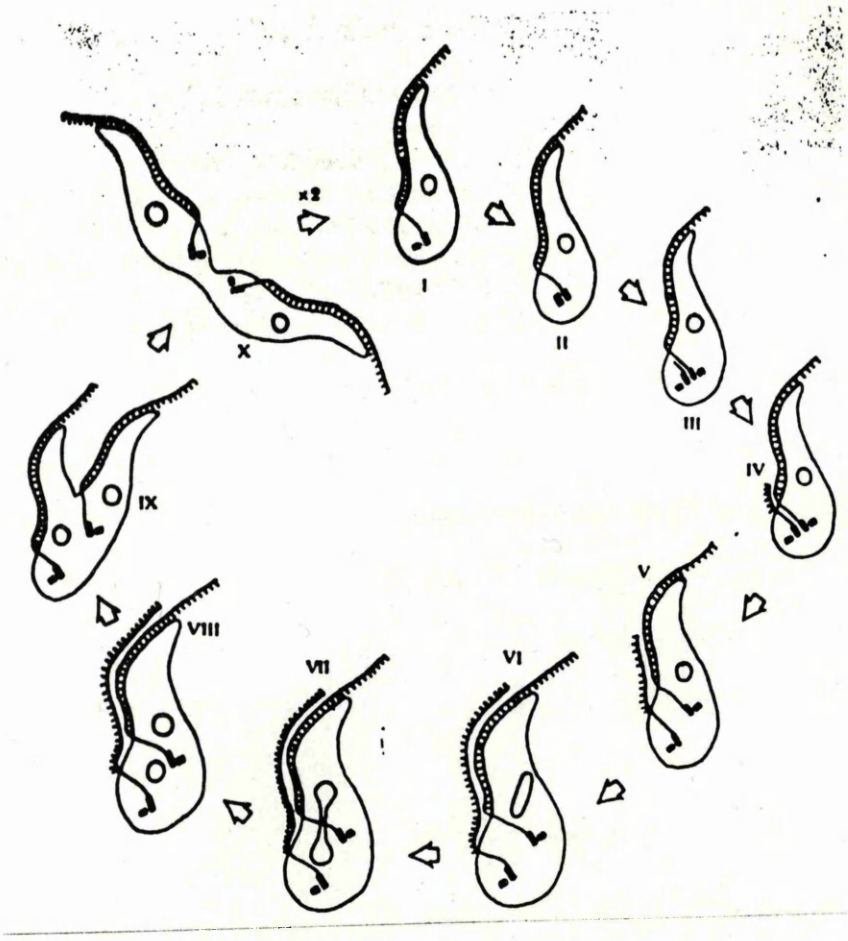


Figure 1.2.2 A schematic figure of the procyclic *T. brucei* cell cycle shows the changes in cell structure during division (taken from Gull *et al.*, 1990). The earliest stage of the cell cycle that is presently detectable is the growth of the second basal body from the pro basal body (II), which is followed by the formation of two new pro basal bodies (III). The new flagellum then starts to form (IV) and is always the posterior of the two flagella. As the new flagellum elongates the basal bodies separate, along with the attached kinetoplasts (V). By the time the flagella are of equal length the nucleus has replicated the DNA and nuclear division has started (VI). Once the two new nuclei are positioned correctly with respect to the basal body/kinetoplast (VIII), division of the cell begins at the anterior end (IX) and continues until the two cells are just connected by the posterior ends (X), when the cells separate.

on the new basal body. This is accompanied by the formation of two new pro-basal bodies. An example of the tight control over the cell cycle is the observation that the new flagellum is always the more posterior of the two. As it continues to elongate the basal bodies begin to separate. At this point the kinetoplasts have already divided, and are also observed to separate with the basal bodies. Experiments with drugs which inhibit protein microtubular motors (thought to be involved in organellar movement) or topoisomerase II (which allows the unlinking of concatenated circular DNA molecules), suggest that there is a physical linkage between the two organelles (Robinson and Gull, 1991). Purification of flagella from *T. brucei* after dissociation of the sub-pellicular microtubules with 1-3 mM Ca^{2+} has shown a stable linkage between the basal body and the kinetoplast DNA (Robinson and Gull, 1991). Electron microscopy of *T. brucei* cells has shown an electron dense region between the kinetoplast and the basal body, which implies a dense protein link (Robinson *et al.*, 1991). Labelling experiments have shown that unlike other eukaryotes, kDNA replication takes place in a well defined period of the cell cycle (Sk), starting at around the beginning of nuclear S phase (Sn) and finishing before nuclear replication ends (Woodward and Gull, 1990). In other organisms the evidence suggests that while some mitochondrial genomes may duplicate many times in one cell cycle, other mitochondrial genomes in the same cell will not replicate at all (Bogenhagen and Clayton, 1977). There is evidence that replication of each molecule of the kDNA, however, takes place only once in Sk (Hajduk *et al.*, 1984). This may be controlled by regulation of the final ligation reaction, which repairs the nick left by replication, during the G2 stage of the cell cycle. The division of the kinetoplast does not appear to carefully segregate the DNA content evenly, and the precise number of maxi and minicircles received by each cell may be uneven. This is presumably a reason for the multiple copy of the kDNA (e.g. 20-50 copies of the maxicircle), which ensures that each cell receives all the necessary DNA for viability. In this, the mitochondrion of *T. brucei* is similar to those found in other organisms. After basal body separation the next visible event is the start of nuclear mitosis. As in most lower eukaryotes, there is no nuclear envelope breakdown, and there is also no

visible condensation of the chromosomes in *T. brucei.* After nuclear division there is a delay, during which extensive rearrangement of the organelles occurs, so that each nucleus is paired with a kinetoplast/basal body prior to the start of cytokinesis. When this has been achieved, the cell divides longitudinally from the anterior end, with final separation occurring with the cells joined by the extreme posterior of each daughter cell.

Analysis of the *T. brucei* cell cycle has been held up by the present inability to synchronise cultures of trypanosomes. Procyclic cells in the same culture, at the same point in the cell cycle, can vary considerably in size, so making selection by elutriation difficult. Drugs affecting microtubule function which have been used in other organisms to synchronise cells in G2/M phase, cause trypanosome death. For those compounds which destabilise microtubules this is probably due to the complicated basket of sub-pellicular microtubules under the plasma membrane of *T. brucei* being essential for cell viability. Drugs which stabilise microtubules can also block cells within mitosis. In these cases, e.g. treating the procyclic form *T. brucei* cultures with rhidoxin (Robinson *et al.*, 1995), the cells do not undergo mitosis but do divide, creating anucleate fragments which contain a kinetoplast and a flagellum (zoids) and tetraploid cells. There is therefore a lack of checkpoints at this point in the cell cycle. Other reagents which usually block DNA synthesis, and so halt cells specifically in S phase, have been tried e.g. aphidicolin or hydroxyurea. The bloodstream form trypanosomes did stop dividing, but during the G2 phase of the cell cycle, DNA synthesis was not blocked. These experiments did result in a synchronised population of cells, but they did not undergo mitosis after release from the block, and the cells became polyploid and eventually died (Mutoba and Wang, 1996). Serum starvation has also been used with limited success (Morgan *et al.*, 1993, Gale *et al.*, 1994). The major problem being an apparent lack of a G0 stage in trypanosomes capable of division (Diffley and Mama, 1989), which results in a large number of cells that are not released from the block after starvation.

1.2.3 CDC2 - function and the family of related genes within the eukaryotes.

In *S. pombe* the *cdc2* gene encodes a 34 kDa protein kinase often called $p34^{cdc2}$. Different alleles of *cdc2* can cause temperature sensitive cell cycle arrest during G1 and/or at the G2/M phase transition when at the restrictive temperature. This indicated an important role for *cdc2* in the cell cycle, as functional $p34^{cdc2}$ was necessary at both of these transition points. Different mutations in *cdc2* can result in different phenotypes, with the cell cycle block being predominantly in G1, at the G2/M boundary, or sometimes both. This implies that $p34^{cdc2}$ interacts with specific factors at each of these control points.

S. cerevisiae has two genes closely related to *cdc2*. The protein encoded by *CDC28* has high sequence identity to $p34^{cdc2}$ (65 %) and the fact that it can complement, and be complemented by the *S. pombe* homologue implies functional homology. The other such gene, *PHO85*, is less well conserved, and does not complement a *cdc2* mutant. Until recently the PHO85 protein was not thought to be directly involved in cell cycle control (but see below). As with *cdc2*, different temperature sensitive mutations in *CDC28* may cause arrest preferentially in either G1 or G2/M phase, and is therefore involved in control of both of these checkpoints.

Functional homologues of *cdc2*/*CDC28* genes have been isolated by complementation of the yeast temperature sensitive mutants from many organisms, ranging from plants to humans (Lee and Nurse, 1987, Jimenez *et al.*, 1990, Feiler and Jacobs, 1991, Hirayama *et al.*, 1991, Paris *et al.*, 1991, Hashimoto *et al.*, 1992, Michaelis and Weeks, 1992). These closely related genes (*CDC2*) which complement have predicted protein sequence identities of around 60-65 % (see Chapter 3.3.5), and initially, because of the complementation of both yeast transitions, were believed to be involved in both restriction point and mitosis control. It has now become clear that there is a large family of related kinases in the multicellular eukaryotes, many of which are involved in control of the cell cycle, and that the original *cdc2* genes from higher eukaryotes are involved principally in post-S phase control (Hamaguchi et al., 1992,

Fang and Newport, 1991). There are other *cdc2* related genes which complement the yeast mutants, e.g.human *cdk2* (*Cyclin Dependant Kinase 2*) (*eg1* in *X. laevis*) and *cdk3* (human) (Elledge and Spottswood, 1991; Meyerson *et al.*, 1992). The encoded proteins are also 60-65 % identical to the yeast cdc2/CDC28 kinases. Outside of this closely related group are many protein kinases from humans and mouse (isolated by PCR directed at conserved kinase domains) with significant, but lower, homology (45-55 %) to cdc2/CDC28. These genes (including *cdk4, cdk5, cdk6*, the PCTAIR subfamily and *cdk7*) do not complement yeast mutants of *cdc2/CDC28*, but in a number of cases the proteins have been shown to be involved in control of the mammalian cell cycle (Fesquet *et al.*, 1993, Meyerson *et al.*, 1992; Matsuoka *et al.*, 1994). The reasons for such a large number of kinase subunits is not clear, but may be due to the tight control needed in a multicellullar organism to stop the selective advantage of rapid division leading to carcinogenesis. Another possible reason is the large numbers of tissues which undergo differentiation in higher eukaryotes.

The kinase activity of CDC2, and the closely related CDK2, can be assayed *in vitro* by phosphorylation of histone H1 (Draetta and Beach, 1988). This may be a physiological substrate for CDC2, and possibly plays a role in chromosome condensation during M phase. The less conserved members of the CDK family, e.g. CDK4/5/6, have low activity when assayed with histone H1, but protein substrates have been found to which they have high activity e.g. the Retinoblastoma protein (Kato *et al.*, 1993; Grana *et al.*, 1994; Kitagawa *et al.*, 1994; Schnier *et al.*, 1994), neurofilament H (Hisanaga *et al.*, 1991, Pan and Hurwitz, 1993) and p107, a retinoblastoma-like protein (Pan *et al.*, 1993).

The kinase activity of the CDK family of proteins, as the name suggests, is usually reliant on the kinase subunit forming a dimer with a controlling protein called a cyclin (see CDK5 for exception). These were first discovered in oocytes of starfish and sea urchins, and named for their property of increasing in quantity through the cell cycle until the end of the M phase, when they were rapidly degraded. Several related genes have been isolated, and as with the CDC2 related proteins, the cyclins are

conserved between different organisms. However, the similarity is considerably lower than that seen in the CDK family. Typically the sequence identity is between 30-40 % within the region of the protein called the cyclin box, and very low outside this region of approximately 150 amino acids. This lower level of conservation is presumably due to there being less selective pressure on a regulatory subunit as opposed to the functional enzyme. The cyclin genes cloned from the higher eukaryotes are divided into several different families on the basis of conserved residues within the cyclin box, with Cyclins A to H so far isolated. Cyclin A (Pines and Hunter, 1989; Takahisa *et al.*, 1992) has been shown to bind both CDC2 and CDK2, and is involved in both sustaining DNA replication (with CDK2) in S phase (Cardoso *et al.*, 1993; Pagano *et al.*, 1992; Pines and Hunter, 1992), and in control of the G2/M transition (Minshull *et al.*, 1990, Pagano *et al.*, 1992, Rosenblatt *et al.*, 1992) with CDC2. The B type cyclins can consist of a gene family with up to 8 members in any given organism. They associate with CDC2 and, in the higher eukaryotes, are involved in controlling the G2/M transition (Minshull *et al.*, 1990, Hoffmann, 1993), as well the exit from mitosis. Expression of some mutated Cyclin B proteins results in a block in the final stages of M phase (Ghiara *et al.*, 1991; Luo *et al.*, 1994). This is due to the loss of a region (called the destruction box) involved in controlling the cyclin degradation pathway, showing that proteolysis of the Cyclin B molecules is a necessary step for exit from mitosis. Some of the large family of B type cyclins in *S. cerevisiae* are also involved in S phase control (Schwob and Nasmyth, 1993). A large number of different cyclins that are probably involved in G1 and S phase control have been cloned from mammalian cell lines (Lew *et al.*, 1991). The role of Cyclin C is not clear, but a related protein has been found in *Drosophila* (Leopold and O'Farrell, 1991), which implies that it will be conserved across the multicellular eukaryotes. Cyclins D and E have been shown to regulate passage through G1, and into S phase (Xiong *et al.*, 1992; Motokura *et al.*, 1992; Koff *et al.*, 1991; Richardson *et al.*, 1993). Cyclins F, G and H have also been isolated (Bai *et al.*, 1994; Okamoto and Beach 1994; Makela *et al.*, 1994) and Cyclin H has been shown to bind to CDK7 (Fisher and Morgan, 1994). The G1 cyclins from *S. cerevisiae* consist of

another class, called CLN1-3 (Rowley *et al.*, 1992), and a number of cyclin genes from the lower eukaryotes are not clearly members of any one particular category e.g. *mcs2* from *S. pombe* (Molz and Beach, 1993) and *CLG1* from *S. cerevisiae* (Matsumoto and Wickner, 1993).

1.2.4 Control of the cdc2 kinase activity in yeast.

To be functional as a kinase, *S. pombe* $p34^{cdc2}$ must bind a cyclin. For the G2/M phase transition the activating subunit is encoded by *cdc13* (Hagan *et al.*, 1988). The protein encoded by this gene is a member of the Cyclin B family. The B type cyclins, and until recently, also the A type cyclins found in metazoans, are sometimes called the mitotic cyclins. The activity of the kinase complex is further controlled by the phosphorylation of the cdc2 subunit. Phosphorylation of a tyrosine residue (Tyr-15) in the ATP binding site of cdc2 causes inhibition of kinase activity (Lundgren *et al.*, 1991). Replacement of this residue by non-phosphorylatable residues, e.g. phenylalanine, causes premature division of the cell, resulting in a lowering of viability due to divisions occurring when the cell has not completed DNA replication or nuclear separation, a phenotype described as mitotic catastrophe (Gould and Nurse, 1989). Mutations in another gene, *wee1*, cause a similar, though less severe, phenotype, with the cell dividing at a considerably smaller size than normal (Creanor and Mitchison, 1994). The protein encoded by *wee1* has been shown to phosphorylate cdc2 on the Tyr-15 residue (McGowan and Russell, 1993), as has the product of a related gene, *mik1*, which appears to co-operate in the phosphorylation of Tyr-15 (Lundgren *et al.*, 1991). If both of these genes are non-functional, the yeast cell viability becomes exceptionally low (approx. 4%), a phenotype described as mitotic lethality (Lundgren *et al.*, 1991). Mutations in another gene, *cdc25*, were found to alter the phenotype of *wee1* mutants. The encoded protein, cdc25, is a phosphatase, and has been shown to be responsible for the dephosphorylation of Tyr-15 (Millar *et al.*, 1991). For the cdc25 phosphatase to become highly active, the substrate cdc2 must be bound to Cyclin B. This is because

the Cyclin B molecule includes a region (the P box) important for phosphatase activation (Zheng and Ruderman, 1993). In addition to control of Tyr-15, it is necessary for threonine 167 of cdc2 to be phosphorylated for the cdc2/cyclin complex to be active (Gould *et al.*, 1991). The gene that encodes the kinase responsible for this is known as CAK, or CDK7, in higher eukaryotes (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993; Wu *et al.*, 1994). Another gene involved in G2/M phase control, implicated by genetic and biochemical studies, is *suc1* (Ducommun *et al.*; 1991, Brizuela *et al.*, 1987; Hayles *et al.*, 1986). This is an essential gene, mutations in which can suppress certain *cdc2* mutant phenotypes. Crystallographic studies of the $p13^{suc1}$ protein have shown a dimer (Endicott *et al.*, 1995), unlike the human homologue, CKS2Hs (Brizuela *et al.*, 1987), which forms hexamers (Parge *et al.*, 1993). It is postulated that the $p13^{suc1}$/CKSHs proteins may have a structural role in forming active CDK/cyclin complexes (Brizuela *et al.*, 1987; Booher *et al.*, 1989), bringing into close proximity different complexes. The difference in multimerisation may be related to the different numbers of both CDK and cyclin types in the respective organisms. The $p13^{suc1}$ protein binds cdc2 with high affinity and has been used to isolate cdc2 related kinase activity, and the proteins responsible, from many organisms (John *et al.*, 1991; Tang *et al.*, 1994).

Experiments have shown that some *cdc2* temperature sensitive mutants, which are blocked in G2 when raised to their restrictive temperature, undergo cdc2 degradation after a heat shock at 56 °C. In this case, the cells 'reset' to G1 phase even though they have already undergone DNA replication and have a DNA content of 2N. This results in cells that have a DNA content of 4N prior to division. It therefore appears that, in *S. pombe*, a modification of the cdc2 protein is one method by which the cell prevents itself from undergoing repeated replication (Broek *et al.*, 1991).

As with cdc2, the *S. cerevisiae* homologue CDC28, needs to bind a cyclin to be active. It also appears to be regulated by very similar phosphorylation events aswell as by the control of transcription (Nasmyth, 1993). Unlike *S. pombe*, the budding yeast cell cycle controls are well characterised at START, although *CDC28* mutants that

preferentially stop at the G2/M phase boundary have also been characterised (Amon *et al.*, 1993; Surana *et al.*, 1991). A large number of cyclins have been isolated from *S. cerevisiae*, including *CLB1-6* (Ghiara *et al.*, 1991; Surana *et al.*, 1991; Grandin and Reed, 1993; Schwob and Nasmyth, 1993), *CLN1-3* (Rowley *et al.*, 1992; Cvrckova and Nasmyth, 1993), *OrfD* (Measday *et al.*, 1994) and *HCS26* (Espinoza *et al.*, 1994). Genetic studies have shown that the CLB family, and the CLNs form complexes with CDC28 at different stages of the cell cycle. There is a high element of redundancy shown by the *S. cerevisiae* cyclins. A cell expressing any one of the *CLN* genes, the products of which are primarily involved in control of START [although CLN3 also affects cell size at mitosis (Vienot-Drebot *et al.*, 1991)], is viable, albeit possibly with imperfect cell cycle control e.g. in response to mating pheromones. A triple null mutation is however, lethal. Equally, either one of the *CLB5* and *6* genes, involved in CDC28 control during S phase (Schwob and Nasmyth, 1993), can apparently be dispensed with, and CLB1 and 2 have overlapping functions in M phase, allowing single mutants to be viable where double mutants are not. The cyclins encoded by *OrfD* and *HCS26* bind to PHO85. PHO85 was originally thought to be only involved in regulating phosphate metabolism, by its binding to PHO80, and phosphorylation of the transcription factor PHO4 (Kaffman *et al.*, 1994). The activating subunit, PHO80, was found to have a region of relatively high homology to regions found in OrfD and HCS26 and it was subsequently found that PHO85 does indeed bind to OrfD and HCS26. Either one of these kinase complexes is essential for passage through START in strains of *S. cerevisiae* that lack *CLN1* and *2* (Measday *et al.*, 1994; Espinoza *et al.*, 1994). This implies that the CDC28/CLN1, CDC28/CLN2, PHO85/OrfD, and PHO85/HCS26 complexes form four parallel pathways, any one of which is sufficient for traversing the START restriction point. The reason for *S. cerevisiae* having so many cyclins is not clear. It is possible that *S. pombe* has a similar number of cyclins but that these have not yet been isolated.

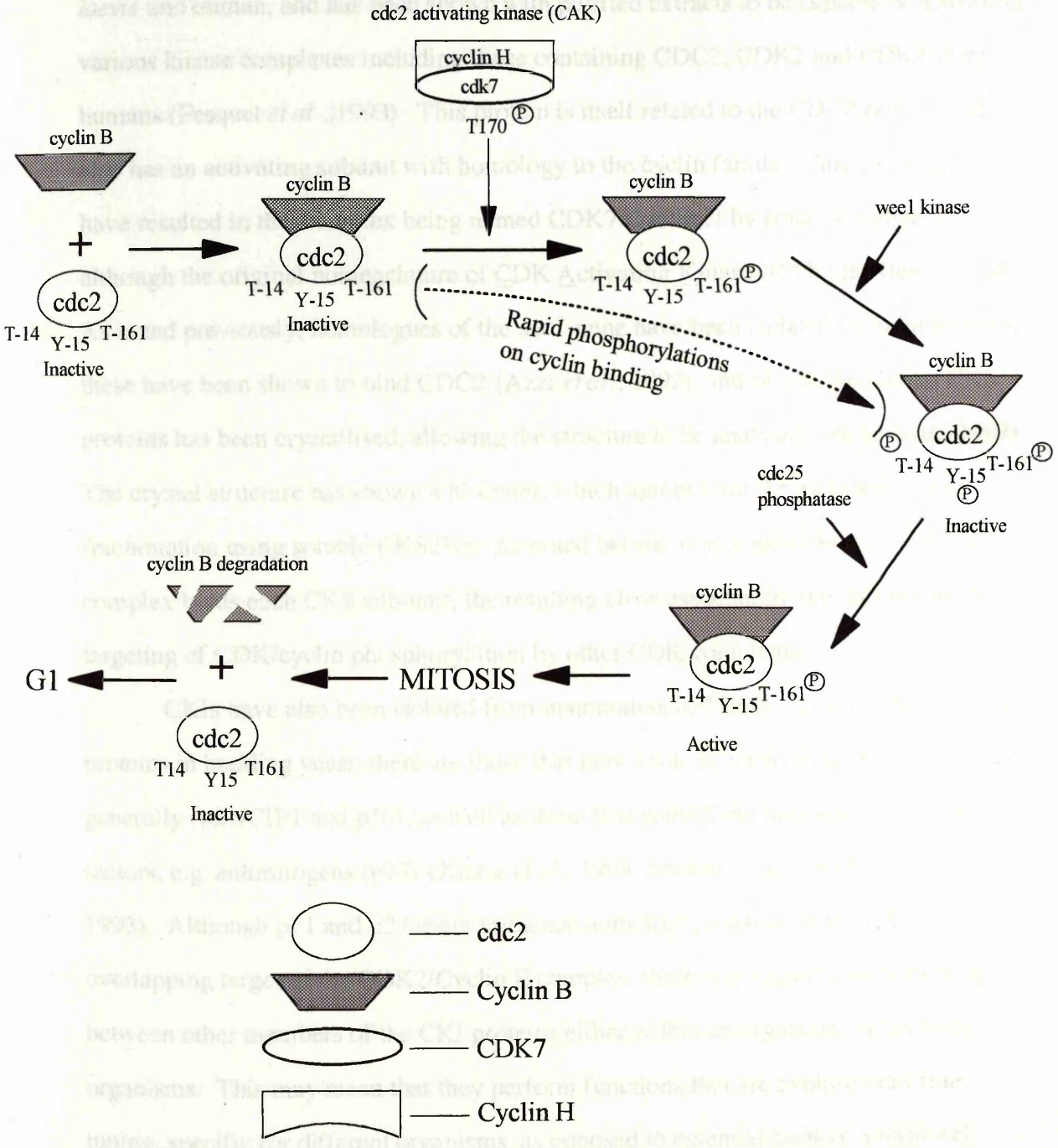
Other proteins involved in control of kinase activity have been isolated from *S. cerevisiae*, including a *suc1* homologue, CKS1 (Hadwiger *et al.*, 1989), and several

CDK Inhibitory proteins (CKIs). The CKI class of proteins has been characterised by their mode of inhibition. They bind to, and inactivate, the CDK or CDK/cyclin complex, but do not covalently modify the complex components, unlike the wee1 kinase. The CKI proteins cloned from *S. cerevisiae* do not share sequence similarities. Different CKIs are involved in controlling constitutive pathways and responses to extracellular signals. *SIC1*/p40 binds to complexes containing CDC28 with CLB5&6 (Schwob *et al.*, 1994), so regulating passage through S phase. FAR1 inhibits kinase complexes of CDC28 with CLN1-3 in response to mating pheromones (Peter and Herskowitz, 1994). This is responsible for the block in G1 which occurs under these conditions. PHO81 binds to the PHO85/PHO80 complex involved in regulating phosphate metabolism (Hirst *et al.*, 1994).

1.2.5 CDK control in higher eukaryotes.

The basic mechanisms controlling the kinase activity of the different CDK complexes seems to be well conserved between yeast and higher eukaryotes. Figure 1.2.3 shows the major controlling modifications and associations of HsCDC2 at the G2/M phase boundary. The CDC2 related kinases of higher organisms bind to a large family of cyclin subunits (Pines, 1993). CDC2 and CDK2 undergo the same inhibitory phosphorylation of the Tyr-15 tyrosine residue as in the yeast system, by wee1 related proteins but also are phosphorylated on the conserved threonine residue adjacent, Thr-14, a modification which has not been seen in yeast (Gu *et al.*, 1992; Igarashi *et al.*, 1991; Norbury *et al.*, 1991; Krek and Nigg, 1991). This threonine phosphorylation is not performed by wee1, but rather a membrane bound kinase, which in *Xenopus* is encoded by the *myt1* gene (Mueller *et al.*, 1995). There are also well conserved homologues of the *CDC25* gene, the products of which are involved in dephosphorylating the Tyr-15 of CDC2 and CDK2 (Jimenez *et al.*, 1990; Jinno *et al.*, 1994). The equivalent of Threonine-167 is also well conserved in the human family of CDC2 related kinases, and this residue, as in yeast, needs to be phosphorylated for the

Figure 1.2.3. Positive and negative regulation of cdc2/Cyclin B complexes during mitosis



kinase to be active (Gu *et al.*, 1992; Lorca *et al.*, 1992; Norbury *et al.*, 1991). The kinase responsible for this activity, or at least capable of it, has been cloned from *X. laevis* and human, and has been shown with purified extracts to be capable of activating various kinase complexes including those containing CDC2, CDK2 and CDK4 from humans (Fesquet *et al.*, 1993). This protein is itself related to the CDC2 family, and also has an activating subunit with homology to the cyclin family. These similarities have resulted in this complex being named CDK7/Cyclin H by some investigators, although the original nomenclature of CDK Activating Kinase (CAK) is often still used. As noted previously, homologues of the *suc1* gene have been isolated from humans and these have been shown to bind CDC2 (Azzi *et al.*, 1992), and one of the human CKS proteins has been crystallised, allowing the structure to be analysed (Parge *et al.*, 1993). The crystal structure has shown a hexamer, which agrees with the evidence of size fractionation using soluble CKS2Hs. As noted before, if as seems likely, one kinase complex binds each CKS sub-unit, the resulting close association may allow specific targeting of CDK/cyclin phosphorylation by other CDK complexes.

CKIs have also been isolated from mammalian cell lines. As with the inhibitory proteins in budding yeast, there are those that play a role in controlling the cell cycle generally (p21/CIP1 and p16), as well as those that control the response to outside factors, e.g. antimitogens (p27) (Xiong *et al.*, 1993; Serrano *et al.*, 1993; Gu *et al.*, 1993). Although p21 and p27 share sequence homology, possibly due to the overlapping target of the CDK2/Cyclin E complex, there is no significant homology between other members of the CKI proteins either within an organism, or between organisms. This may mean that they perform functions that are evolutionary fine tuning, specific for different organisms, as opposed to essential parts of a basic cell cycle control system. The *FAR1* gene from *S. cerevisiae* is non-essential, which gives credence to this idea, but the gene encoding p16 in humans has been implicated as a tumour suppresser gene (Okamoto *et al.*, 1994), and mutations have been found in familial cases of melanoma (Wainwright, 1994), suggesting that this gene may be important and conserved in multicellullar organisms.

1.2.6 CDK/Cyclin complexes in Metazoans, and control of the cell cycle.

Table 1.2.1 briefly shows the present published data as to which CDK binds to which cyclin in mammalian cells. In the case of CDK3, partners are not known, and there are some cyclins (F and G) for which kinase subunits have not been assigned.

The presence of CDK/cyclin complexes in early G1, immediately after cytokinesis, that play a role in cell cycle progression has not been clearly shown.

At START, the major complexes appear to be those containing D type cyclins, possibly complexed with any of 3 different CDK's (Xiong *et al.*, 1992). There is a consensus that CDK2, CDK4 and CDK6 form complexes with the Cyclin D family. The three D type cyclins are expressed at different levels in different cell types, and it is not clear how the different cyclin D's roles differ. Differences are apparent, D2 and D3 have been shown to bind to the Retinoblastoma gene product (Rb), whereas D1 does not (Hatakeyama *et al.*, 1994). All three can promote phosphorylation of Rb *in vitro* when co-expressed with CDK4, but in an *in vivo* assay, D1 did not cause Rb phosphorylation (Kato *et al.*, 1993). The hyperphosphorylation of Rb removes the block to cell cycle progression by interfering with the binding of Rb to the transcription factor E2F. The role of complexes containing Cyclin D1 is still unknown, but Cyclin D1 does form complexes which contain E2F (Zhang and Kumar, 1994; Schulze *et al.*, 1994), as well as those containing Proliferating Cell Nuclear Antigen, a protein involved in the control of nuclear DNA synthesis (Xiong *et al.*, 1992; Matsuoka *et al.*, 1994). Therefore it may be involved in controlling the start of DNA synthesis. The importance of the CDK4 complexes in control of cell cycle progression can be seen in experiments performed on a mink cell line, Mv1Lu. The antimitogen TGF-Beta-1, which arrests cells in G1 phase, was shown to be able to act by down regulation of CDK4 levels (Ewen *et al.*, 1993), which resulted in the accumulation of unphosphorylated Rb. Levels of Cyclins D1 and D2 (D3 not being expressed in Mv1Lu) were unaffected, as were levels of CDK2 and Cyclin E. Furthermore,

Table 1.2.1 CDK complexes and functions.

Kinase subunit	Cyclin subunits shown to bind	Function
CDC2 (CDK1)	Cyclins A, B1 and B2	G2/M transition, M phase exit
CDK2	Cyclins A, D1, D2, D3 and E	Initiation and regulation of S phase
CDK3	Not Known	G1
CDK4	Cyclins D1, D2, D3, and E	Transition of restriction point and initiation of S phase
CDK5	Cyclin D1 and D3 (Also p35, not a cyclin)	Not Known (Possible artefact) Control of neuronal cytoskeleton
CDK6	Cyclin D	Probable early G1 control
CDK7	Cyclin H	Control of CDK kinase activity
CDK8	Cyclin C	Phosphorylates RNApol II

unregulated expression of CDK4 resulted in the cell line showing unimpaired growth when exposed to TGF-β-1, while similar expression of D type cyclins or CDK2 had no effect.

The expression of CDK5 varies in different cell lines, and although it has been shown to bind to D type cyclins, and Proliferating Cell Nuclear Antigen, kinase activity in this case has not been shown (Xiong *et al.*, 1992). What has been proved is that CDK5 is highly expressed in terminally differentiated neurons, which do not express CDC2 (Hayes *et al.*, 1991), and it forms a complex with a 35 kDa protein that shares no homology to cyclins (Helmich *et al.*, 1992). This complex is active without further modification, unlike those containing other CDK molecules and can phosphorylate components of neurofilaments. Therefore, it seems to be involved in regulation of the cytoskeleton, specifically in the central nervous system. It is possible that the CDK5 association with cyclins D1 and D3 seen previously is an artefact of the system used, and CDK5 has no role in the regular cell cycle, or it may be that the physiological substrate has yet to be found.

CDK2 has been shown to be part of a complex with the D cyclins, and interacts with other components of the complexes, e.g. Rb, in a similar way to CDK4 *in vitro*. However, *in vivo* most of the CDK2 is associated with Cyclin E, and the evidence is that its interactions with the D type cyclins are limited and unlikely to be limiting factors in cell cycle progression (see above). CDK2 binds to Cyclin E, which is synthesised during G1, forming a complex that also contains p107 (which is related to Rb) and E2F. Levels of this complex, and the kinase activity associated with it, peak at the G1/S phase boundary, after which Cyclin E is degraded. As the levels of the CDK2/Cyclin E complex decrease, the levels of a complex containing CDK2/Cyclin A start to rise (Elledge *et al.*, 1992; Harper *et al.*, 1992). The different roles of the two complexes is unknown, although evidence suggests that the CDK2/Cyclin A complex is involved in maintaining DNA synthesis (Pagano *et al.*, 1992; Cardoso *et al.*, 1993). The control of kinase activity is apparently performed in a very similar way to the control of *S. pombe* cdc2, and a cdc25 homologue active during G1 and S phase has

been characterised from human cells (Jinno *et al.*, 1994). E2F has been shown to regulate the transcription of genes necessary for S phase, but the reason for different complexes in G1 and S phase with p107 is unclear. Also unclear is why, and how, the control of E2F by association with Rb and p107 overlaps during G1. During S phase, complexes containing CDK2 and Cyclin A have been co-localised to areas of DNA replication in the nucleus (Cardoso *et al.*, 1993). This co-localisation may have something to do with the binding seen in G1 between CDK2 and PCNA, which is an essential protein associated with DNA polymerase delta, and which also shows punctate staining of S phase nuclei.

By the end of S phase CDC2 is bound to A and B type cyclins (Draetta *et al.*, 1989), but the Cyclin B complexes are predominantly inactive due to the phosphorylation of Thr-14 and Tyr-15. It appears that the complexes containing Cyclin A have limited activity, but when this reaches a threshold activation of the CDC2/Cyclin B kinase activity occurs at the G2/M phase boundary extremely rapidly. This is achieved by a control system exhibiting positive feedback. Activated CDC2/Cyclin complexes phosphorylate CDC25 (Hoffmann, 1993; Izumi and Maller, 1993), which is then far more efficient at activating more CDC2/Cyclin. The negative regulators are less active in mitosis, and this may be regulated by the CDC2 kinase complex, probably one or more steps removed. In the case of wee1, phosphorylation causes the Tyr-15 kinase activity to be down-regulated. This combination results in a very rapid activation of all the available CDC2/Cyclin A and B complexes, which then phosphorylate the required substrates for M phase progression. Amongst these are the components of the nuclear lamina, which results in their disassembly (Nigg *et al.*, 1991; Peter *et al.*, 1990; Peter *et al.*, 1991). CDC2 complexes have also been shown to be associated with the mitotic spindle (Ookata *et al.*, 1993) and intermediate filament-associated proteins (Skalli *et al.*, 1992).

The degradation of the mitotic cyclins is another regulatory step necessary for the completion of mitosis. The Cyclin A molecules are degraded first, with the Cyclin B degradation occurring at the transition from M phase into G1 (Minshull *et al.*, 1990).

The degradation of the cyclins is signalled by their polyubiquitination, the control of which is to some degree regulated by CDC2 activity (Hershko *et al.*, 1994). The loss of the cyclins results in the loss of kinase activity (Draetta *et al.*, 1989). As mentioned previously, the expression of N terminal truncated mitotic cyclins, which lack a region targeting them for degradation (see Chapter 3.2.1), results in cell cycle arrest at the point that the proteins are usually broken down.

1.3 Project Aims.

The specific aims of the project were:

1) To attempt the isolation of cyclin gene(s) from *T. brucei*, and/or the related kinetoplastid *Leishmania mexicana*.

2) To isolate and sequence the genomic locus of *tbcrk3*, one of the *T. brucei cdc2*-related kinases, for which a gene fragment amplified by PCR was available.

3) To raise antisera to TbCRK3, and with other antisera available in the laboratory, analyse the expression, and if possible activity, of the members of the TbCRK family in the different *T. brucei* life cycle stages.

4) To analyse the function and activity of the TbCRK proteins, using biochemical and reverse genetic approaches.

1.4 Addendum-recent publications.

1.4.1 The Eukaryotic cell cycle; kinases, phosphatases and the cytoskeleton.

New research into CDK/cyclin complexes has shown more links between cell cycle control and transcription. The CDK7/Cyclin H complex has been found to form part of the Transcription Factor II H (TFIIH) complex, involved in the initiation of

RNA polymerase II transcription (Serizawa *et al.*, 1995; Sheikhattar *et al.*, 1995). It is thought that the CDK7/Cyclin H complex phosphorylates the C-terminal tail of the RNA polymerase II large subunit. It is thought that this complex is also the CDK Activating Kinase, but evidence from homologues of lower eukaryotes is contradictory. The CDK7 homologue of *S. pombe* (crk1 or mop1) can act as CAK *in vitro*, and is essential with mutant cells arresting in late M phase (Damagnez *et al.*, 1995). The homologue in *S. cerevisiae* (KIN28) is part of the TFIIH complex and necessary for transcription but shows no CAK activity (Cismowski *et al.*, 1995). The CAK purified from budding yeast is encoded by a seperate gene, *CAK1*, with low homology to CDK7 (Kaldis *et al.*, 1996; Espinoza *et al.*, 1996). The CAK1 protein is essential with a G2 arrest in mutant cells. All of the CDK7 and CAK kinases show no variation in activity through the cell cycle.

The CDK kinase subunit that binds Cyclin C in higher eukaryotes has been identified (CDK8) and this complex has been shown to be part of the RNA polymerase II holoenzyme (Tassan *et al.*, 1995). These proteins are homologous to the SRB10 and SRB11 proteins in *S. cerevisiae* which are also part of the RNA Polymerase II holoenzyme (Liao *et al.*, 1995), which interacts with the TFIIH complex during transcriptional initiation. No cell cycle controlling role has so far been shown for the CDK8/Cyclin C complex, although the RNA polymerase II holoenzyme has also been implicated in DNA damage recognition/repair, possibly suggesting a role of these kinase complexes in cell cycle checkpoint control.

Microtubules have been shown to be involved in the cell cycle, and to possibly interact with B type cyclins in *S. pombe*. In fission yeast mutations in both alpha and beta tubulin genes can result in lethally abnormal mitochondrial distribution, showing a link between microtubules and mitochondrial movement (Yaffe *et al.*, 1996). A *cdc10* null mutant of *S. pombe* that causes G1 arrest can be suppressed by an uncharacterised mutation in a transcription factor (*sct1*) which causes low levels of cells to show aberrant cytoskeletal and nuclear DNA morphologies. The DNA sometimes appeared fragmented, the nuclei were occasionally displaced and binuclear cells were seen. Both

microtubular and microfilament perturbations were seen in the mutants (Marks *et al.*, 1992). The *S. pombe sspl* protein kinase gene mutants show a cell cycle arrest in G2 with altered actin deposition patterns. This is accompanied by a failure to correctly regulate the growth polarity of the cells (Matsusaka *et al.*, 1995).

Protein phosphatases have also been shown to be involved in cell morphogenesis in eukaryotes. Mutations of PP2A subunits in *S. cerevisiae* cause cells to become multibudded and multinucleated (Van Zyl *et al.*, 1992). *S. pombe* mutants result in aberrant cytoskeletal organisation and a block in cytokinesis (Kinoshita *et al.*, 1996). Similar mutations in *Drosophila melanogaster* cause mitotic arrest, a lack of microtubule organisation and the uncoupling of the nuclear and centrosome cell cycles in the embryo (Snaith *et al.*, 1996).

1.4.2 *Trypanosoma brucei*, the cytoskeleton and the cell cycle.

A putative RNA polymerase II promoter region has now been isolated from the *T. brucei hsp70* locus (Lee, 1996). This region directs transcription of heterologous genes, but does not result in an upregulation of the level of mRNA under heat stress, once again suggesting that the major control of mRNA levels is post-transcriptional.

The microtubule cytoskeleton of *T. brucei* has been shown by a variety of methods to be highly polarised with the positive ends of the microtubules at the posterior end of the cell (Robinson *et al.*, 1995). Other work has shown that the sub-pellicular array grows by the insertion of new microtubules into the array predominantly in the posterior region of the cell (Sherwin and Gull, 1989). The movement of the kinetoplast during the differentiation between short stumpy and procyclic forms is microtubule mediated, just as the division and movement of the organelle is during the cell cycle (Matthews *et al.*, 1995).

Treatment of procyclic *T. brucei* cultures with the protein phosphatases inhibitor okadaic acid has indicated a role for PP1/PP2A proteins in the coordination of DNA synthesis, mitosis, organellar rearrangement and cytokinesis (Das *et al.*, 1994). The

cells treated with okadaic acid became multinucleate, but while they replicated the kinetoplast DNA they neither divided the organelle nor underwent cytokinesis.

CHAPTER TWO
MATERIALS AND METHODS

2.1 Buffers/Media/Stock solutions

Alkaline lysis buffers

Solution I	50 mM Glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0).
Solution II	0.2 M NaOH, 1 % SDS.
Solution III	6 ml 5 M K acetate, 1.15 ml glacial acetic acid, 2.85 ml dH_2O.

Antibiotics

Ampicillin	Stock (1000x)	50 mg / ml in dH_2O
Tetracyclin	Stock (1000x)	5 mg / ml in 50 % ethanol
Phleomycin	Stock	20 mg / ml in dH_2O
G418	Stock	10 mg / ml in dH_2O

Blot electrode buffer 3.25 g Tris HCl, 14.4 g Glycine, 200 ml Methanol, made up to 1 litre with dH_2O.

Denaturation solution 0.5 M NaOH, 1.5 M NaCl.

50xDenhardts 10 g Ficoll, 10 g polyvinylpyrrolidine, 10 g BSA, made up to 1 litre with dH_2O.

EDTA A 0.5 M solution was made up in dH_2O and adjusted to pH 8.0 with 10 M NaOH.

5 x Final Sample Buffer (5 x FSB) 1.25 ml 0.5 M Tris-HCl, pH 6.8, 1.0 ml Glycerol, 2.0 ml 10 % (w/v) SDS, 0.5 ml 2-Beta mercaptoethanol, 0.25 ml 0.05 % (w/v) Bromophenol blue, 5.0 ml dH_2O

Hybridisation buffer 6 x SSC, 5 x Denhardt's reagent, 0.5 % SDS, 100 μg / ml denatured fragmented salmon sperm DNA

Kinase Assay Buffer (KAB) 50 mM MOPS pH 7.2, 20 mM $MgCl_2$, 2 mM DTT, 10 mM EGTA

Kinase Assay Mix (KAM) (per 200 μl)

5 μl	10 mg / ml Histone H1
1 μl	gamma ^{32}P rATP
8 μl	100 μM rATP
186 μl	KAB

L broth 10 g Tryptone, 5 g yeast extract, 5 g NaCl, made up to 1 litre with dH_2O, adjusted to pH 7.0 with 10M NaOH.

LB agar As LB broth plus 15 g of agar/litre.

5 x Loading Buffer (5 x LB) 0.25 % Bromophenol blue, 0.25 % Xylene cyanol FF, 10 mM EDTA (pH 8.0), 15 % Ficoll.

Lysis Solution (LS) 50 mM MOPS (NaOH to pH 7.2), 100 mM NaCl, 1 mM EDTA, 1 mMEGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 % Triton X-100.

Lysis Solution with Inhibitors (LSI) per 10 ml.

9.75 ml Lysis solution

50 μl 200 mM 1,10 Phenanthroline (in 50 % ethanol)

50 μl 1 mg / ml Pepstatin A (in 100 % methanol)

100 μl 10 mg / ml leupeptin (in dH_2O)

50 μl 10 mg / ml PMSF (in 100 % ethanol)

MB medium 0.5 g KH_2PO_4, 0.36 g K acetate, 0.5 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.1 g $CaCl_2.2H_2O$, 5 g $(NH_4)_2SO_4$, 500 μg H_3BO_4, 40 μg $CuSO_4.5H_2O$, 100 μg KI, 200 μg $FeCl_3.6H_2O$, 400 μg $MnSO_4.H_2O$, 200 μg Na molybynate, 400 μg $ZnSO_4.7H_2O$, 5 g glucose, 10 μg biotin, 1 mg Ca pantothenate, 10 mg nicotinic acid, 10 mg inositol. Make up to 1 litre with dH_2O and filter sterilise. Add leucine to 150 μg / ml for the culture of *leu*- strains.

10 x MMA 10 g KH_2PO_4, 5 g $MgSO_4.7H_2O$, 1 g NaCl, 1 g $CaCl_2.2H_2O$, 50 g $(NH_4)_2SO_4$, 5 mg H_3BO_4, 400 μg $CuSO_4.5H_2O$, 1 mg KI, 2 mg $FeCl_3.6H_2O$, 4 mg $MnSO_4.H_2O$, 2 mg Na molybynate, 4 mg $ZnSO_4.7H_2O$, 100 g glucose, 100 μg biotin, 10 mg Ca pantothenate, 100 mg nicotinic acid, 100 mg inositol. Make up to 1 litre with dH_2O and filter sterilise. For plates, add 1/10 volume to agar autoclaved in 9/10 dH_2O. Final agar concentration 2 %.

Neutralisation solution 1.5 M NaCl, 0.5 M Tris.HCl (pH 7.4).

Phosphate buffered saline (PBS) 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, adjust to pH 7.4, add dH_2O to 1 litre.

5 x SDS-PAGE Running Buffer 15 g Tris base, 72 g Glycine, 5 g SDS, made up to 1 litre with dH_2O.

SM buffer 5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 ml 1 M Tris.HCl (pH 7.5), 5 ml 2 % gelatin solution, made up to 1 litre with dH_2O.

20 x SSC 175.3 g NaCl, 88.2 g Sodium citrate, made up to 1 litre with dH_2O.

50 x TAE 242 g Tris base, 57.1 ml glacial acid, 100 ml 0.5 M EDTA (pH 8.0), made up to 1 litre with dH_2O.

5 x TBE 54 g Tris base, 27.5 g boric acid, 20 ml 0.5 M EDTA (pH 8.0), made up to 1 litre with dH_2O.

TBS-Tween 8 g NaCl, 0.2 g KCl, 3 g Tris base, 0.5 ml Tween-20, adjust to pH 7.4, add $dH_2O$to 1 litre.

TE 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0).

1/2YEL+leu 0.25 % yeast extract, 1.5 % glucose, 30 μg / ml leucine.

2 x YT broth 10 g Tryptone, 10 g yeast extract, 5 g NaCl, made up to 1 litre with dH_2O.

ZPFM 7.71 g NaCl, 0.60 g KCl, 1.14 g Na_2HPO_4 (anhydrous), 0.20 g KH_2PO_4 (anhydrous), 0.20 g Mg acetate.$4H_2O$, 16 mg Ca acetate, made up to 1 litre with dH_2O.

2.2 General Methods

2.2.1 DNA protocols

2.2.1.1 Restriction Enzyme digestion of plasmid and lambda DNA.

Typically 0.5-2 μg of DNA were digested in 20 μl containing the recommended enzyme buffer and 5-10 Units of enzyme. After 1 hour at the appropriate temperature the incubations were placed on ice and 5 μl of 5 x Loading Buffer (5 x LB) were added. For sequential digests the first R.E. was either heat inactivated or removed by Magic DNA Cleanup Columns (Promega) before the second digestion. For transfection of trypanosomes, 25-50 μg of plasmid DNA were digested in 100-200 μl using 30-50 U of each enzyme. The digests were incubated for 3-5 hours before gel electrophoresis and band extraction.

2.2.1.2 Agarose Gel Electrophoresis.

0.7-2 % gels were used for separating plasmid and PCR derived DNA depending on the size of the expected fragments. The gels were normally run in 1 x TBE at 100-150 Volts and contained 0.1 μg / ml of Ethidium Bromide. LMP agarose gels were run in 1 x TAE at 50 V.

Digests of genomic DNA were run on a 300 ml, 0.7 %, 1 x TBE gel without Ethidium Bromide at 50 V overnight. The gel was then stained in 1 x TBE containing 1 μg / ml Ethidium Bromide for 30 minutes and destained in dH_2O for 30 minutes. The DNA was visualised by UV illumination.

2.2.1.3 Extraction of DNA from Agarose.

The bands of interest were cut from agarose gels under UV illumination with a sterile scalpel blade. To extract the DNA, three methods were employed during the Ph.D.:

(1) Low melting point agarose in 1 x TAE was initially used at the start of the Ph.D.

(2) Geneclean (BIO 101 inc.) was later used to extract DNA from high percentage 1 x TBE gels (using the TBE modifier supplied) when the fragments were smaller than 500 bp.

(3) Spin-X columns (Sigma). Used for 1 x TAE gels and fragments larger than 500 bp. The tube containing the agarose slice was frozen at -20 ^{o}C before being centrifuged at maximum speed in a microfuge for 15 minutes.

2.2.1.4 Labelling of DNA fragments by random priming.

25 ng of gel purified DNA were labelled using the Prim-it kit (Stratagene), with 50 μCi of Super ^{32}P-dCTP. The unincorporated label was removed using Nuctrap columns (Stratagene). Before hybridisation the probe was denatured by boiling for 5 minutes, then spun down and kept on ice before adding to the hybridisation tube.

2.2.1.5 Southern Blotting and Hybridisation.

Agarose gels containing digested lambda or genomic DNA were treated with 0.2 M HCl for 15 minutes to ensure transfer of the larger fragments. The gels were then rinsed with dH_2O and placed in denaturing solution for at least 30 minutes. The DNA was then transferred onto Hybaid Nylon membrane by capillary transfer overnight, the blotting buffer being alkali transfer buffer. Gels only containing plasmid DNA smaller than 7 kb were not treated with HCl. After transfer the positions of the wells were marked with pencil, and the membranes washed with 2 x SSC. The DNA was fixed by baking the filters at 80 ^{o}C for 2 hours.

The membranes were pre-hybridised in 20 ml of hybridisation buffer containing 100 μg / ml of denatured herring sperm DNA at the hybridisation temperature. 15 ml of buffer were then removed and the probe added. Hybridisation took place overnight, usually at 65 ^{o}C. In the morning the membranes were washed in 0.1 x SSC, 0.1 % SDS at 65 ^{o}C for 1hour, with 3 changes of wash buffer. The membranes were either sealed

in a plastic bag or wrapped in clingfilm before exposure to X-ray film at -70 ^{O}C in cassettes with enhancer screens.

2.2.1.6 PCR from genomic DNA.

PCR with degenerate oligonucleotides was performed on purified genomic DNA from *T. brucei* and *L. mexicana*. The 'touchdown' method was employed, where the starting annealing temperature is high, and then the temperature is lowered very few cycles before the major amplification cycles (Don *et al.*, 1991). Typically the conditions were:

Step 1	94 ^{O}C for 5 min	(initial denaturation)
Step 2	60 ^{O}C for 30 sec	5 μl of each primer (20 pM stock)
Step 3	72 ^{O}C for 1min	5 μl of 2 mM dNTPs
Step 4	94 ^{O}C for 30 sec	5 μl of 10 x Taq buffer
(repeat steps 2-4 for 3 cycles)		0.5 μl of Taq DNA polymerase (5 U / μl)
Then continue to cycle, and drop the		0.5 μl of template
annealing temperature by 2 ^{O}C every		dH_2O to 50 μl
third cycle until theannealing temperature		
gets to 48 ^{O}C. Then;		

15 cycles with annealing for 30 sec at 48 ^{O}C. Then;

48 ^{O}C annealing for 2 min

72 ^{O}C for 10 min.

10 μl of the reactions were added to 3 μl of 5 x LB and run on agarose gels. Blunt end cloning was performed when the oligonucleotides had no R.E. site engineered in. These oligonucleotides had been kinased and the PCR fragments generated could therefore be ligated into *Sma*I cut and CIPed pBluescript.

2.2.1.7 Preparation of Vector DNA for ligations.

20 μg of DNA were digested in 100 μl total volume in the appropriate restriction buffer. After digestion 5 Units of Calf Intestinal Phosphatase (CIP) were added to the tube and incubated at 37 ^{o}C for 15 minutes. The CIP was then heat inactivated at 65 ^{o}C for 15 minutes. The DNA was EtOH precipitated, dried and resuspended in 10 μl of TE. 1 μl was used in each ligation.

2.2.1.8 Ligations.

When the insert DNA was in LMP agarose, 1 μl of vector DNA was added to 4 μl of 5 x Ligation buffer, 1 μl of T4 DNA ligase and 4 μl of dH_2O. The agarose with insert was melted at 65 ^{o}C and 10 μl added to the ligation mix. This was left at 4 ^{o}C overnight, and 5 μl used for each transfection.

When the insert had been purified by other means, 1 μl of vector DNA was added to enough insert DNA to give a ratio of approximately 1 vector:3 insert free sticky ends. The mixture was ethanol precipitated and resuspended in 7.5 μl of dH_2O. 2 μl of 5 x Ligation buffer and 0.5 μl of T4 DNA ligase were added. The ligation was left at r.t. for at least 3 hours, or overnight at 4 ^{o}C, before using 2 μl for each transfection.

When the fragments were blunt ended the ratio of vector to insert ends was raised to 1:10, or higher, and 1 μl of T4 DNA ligase was used in the 10 μl reaction.

2.2.1.9 Cloning *tbcrk1/2/3* into expression vectors by PCR.

Oligonucleotides were designed to anneal to the 5' and 3' extremities of the respective gene. The oligos included an adaptor sequence of a restriction enzyme site that would allow the cloning of the PCR product into the correct frame of the

expression vector. *Pfu* DNA polymerase was used due to its much reduced mutation rate, instead of *Taq* DNA polymerase . The PCR conditions were:

Step 1	94 ^{o}C for 5 min	(initial denaturation)
Step 2	60 ^{o}C for 30 sec	5 μl of each oligo (20 pM stock)
Step 3	72 ^{o}C for 1 min 30 secs	5 μl of 2 mM dNTPs
Step 4	94 ^{o}C for 30 sec	5 μl of 10 x Pfu buffer
(repeat Steps 2-4 for 30 cycles)		0.5 μl of *Pfu* DNA polymerase (5 U / μl)
Step 5	60 ^{o}C for 30 sec	dH_2O to 50 μl
Step 6	72 ^{o}C for 10 min	

After 5 μl of the reactions were run on a 1 % agarose gel to check the product the rest of the amplified product was purified using Magic PCR cleanup columns (Promega). The DNA was then digested with the appropriate enzymes, and gel purified. After extraction, this DNA was used for ligations.

2.2.1.10 Sequencing of Plasmid DNA.

5-10 μg of plasmid DNA were used to obtain double stranded sequence using a rapid NaOH denaturing protocol (Hsiao 1991). The Sequenase Version 2.0 (United States Biochemical Corporation) kit was used with dITP buffers used for resolving compressions.

Table 2.2.1.1 Plasmids used during this thesis.

Name	Use and/or insert	Source
pBluescript II	*E. coli* cloning vector.	Stratagene
pMNS21L	Regulatable *S. pombe* expression vector.	J. Kinnaird, WUMP.
pMNS/Hscdc2+	pMNS21L with the human *cdc2* ORF in the correct orientation.	J. Kinnaird, WUMP.
pMNS/Hscdc2-	pMNS21L with the human *cdc2* ORF in the reverse orientation.	J. Kinnaird, WUMP.
pSpcdc2	Non-regulated *S. pombe* expression vector with wild type *S. pombe* cdc2 insert.	J. Kinnaird, WUMP.
pBle	pUC18 with the *T. brucei* PARP promoter/ *ble* resistance gene/PARP 3' untranslated region insert.	S. Graham, WUMP.
pNT	pUC8 with PARP promoter/*neo* resistance gene/ *T. brucei* tubulin 3' untranslated region insert.	D. Jeffries, WUMP.

Table 2.2.1.2 Oligonucleotides used during this thesis.

ATG CGS GCS ATY YTS ATY GAC TGG	degenerate 5' cyclin PCR primer
SGG SGG CTA CAT CTC CTC GTA CTT	degenerate 3' cyclin PCR primer
AK CTC CAT SAR GTA CTT SGC	degenerate 3' cyclin PCR primer
C GGA TCC GCN DSN AAR TAY GAR GAR	degenerate 5' cyclin PCR primer
C GAA TTC YTC NAB NAR RTA YTT NGC	degenerate 3' cyclin PCR primer
GAA GTT ATG GGT GTC TCC	*tbcrk3* sequencing.
CCA TCT AAA ATC TGA TTG	*tbcrk3* sequencing.
CAC AGT GCA AAT GGG GTG	*tbmefg* sequencing.
GCT TGG GGG TGA ATG CCG	*tbmefg* sequencing.
GTT CTT GTA GGC TGA ACC	*tbmefg* sequencing.
TAC CTA TTT TAC TGT GCC	*tbmefg* sequencing.
AGG GTT AGC ATT GTC ACG	*tbmefg* sequencing.
GCG ATT CGG CGC ACC ACG	*tbmefg* sequencing.
GA GGA TCC ATG ACA ATG CTT GGG GCG	*tbcrk3*/H PCR 5' primer.
GC AGA TCT AAA CAT GGC ATC ACT AAA	*tbcrk3*/H PCR 3' primer.
AT CCC ATG GAC CGC TAT AAT CGA ATG	*tbcrk3*/H (-5'ext) PCR 5' primer.
G CCC ATG GGG AGT CGT TAC GAG C	*tbcrk1*/H PCR 5' primer.
CGC GGA TCC GAA CTC GAC AGA AAA GTA T	*tbcrk1*/H PCR 3' primer.
G CCC ATG GAG GTG CAG GTG CAG GAA GG	*tbcrk2*/H PCR 5' primer.
CG CAG ATC TAG CTC CGT TGA ACC GCA TC	*tbcrk2*/H PCR 3' primer.
CC ACC ATG GAC CGG TAT AGC CGA ATA	*tbcrk2*/H (-5'ext) PCR 5' primer.
GA CAT ATG ACA ATG CTT GGG	*tbcrk3* 5' PCR primer for the yeast expression vector pMNS21L.
TG GGA TCC GAT CTA AAA CAT GGC ATC	*tbcrk3* 3' PCR primer for the yeast expression vector pMNS21L.
CG GGT ACC GTG TTG GAA TAC AAC CG	*tbmefg* 5' PCR primer for His-tagged Integration Vector fragment.
CC GAG CTC TGA TCC CCC TTT AAC	*tbmefg* 3' PCR primer for His-tagged Integration Vector fragment.

Degeneracy codes used: B - C, G and T; D - A, G and T; K - G and T; N - A, C, G and T; R - A and G; S - C and G; Y - C and T.

The underlined bases are engineered restriction enzyme sites, and may not be identical to the sequence used as template in the PCR reaction.

2.2.2 Protein protocols.

2.2.2.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Protein samples were separated by SDS-PAGE, using the discontinuous gel method (Laemmli, 1970).

Separating gel - 0.375 M Tris-HCl, pH 8.8.

Stacking gel - 0.125 M Tris-HCl, pH 6.8, 4.0 % acrylamide.

Depending on the size range needing separation, 12 to 17.5 % acrylamide separating gels were used. Most gels were run using minigel kits as per the protocol (Biorad). The gels were either stained using Coomassie R250 brilliant blue, or electroblotted for Westerns.

For staining, the gels were placed in dH_2O containing 30 % methanol, 10 % glacial acetic acid and 0.1 % Coomassie R-250 brilliant blue for 30 minutes. Destaining took approximately 2 hours in frequent changes of dH_2O containing 30 % methanol and 10 % glacial acetic acid. The gels were then left in dH_2O overnight before being transferred to 3MM paper and dried down at 80 ^{o}C under vacuum for 45 minutes.

2.2.2.2 Western Blotting.

The Western blots described in this thesis were predominantly performed using PVDF membrane (Bio-Rad) used as per the protocols given. Proteins from unstained gels were transferred to the membrane in Blot Electrode Buffer, either at 0.2 Amperes for 2 hours in the cold room, or at 0.1 A overnight on the bench. The PVDF was allowed to dry to fix the protein. Often the bound protein could be visualised as translucent areas of the membrane using a lightbox, allowing the markers to be labelled with a pencil and the membrane to be cut up with a scalpel blade without staining of the membrane. If necessary the membrane was moistened with 100 % ethanol and

Ponceau-S stained (Sigma) to allow detection of the protein. The blot was then rinsed with Tris Buffered Saline containing 0.05 % Tween-20 (TBS-Tween). The ECL Western blotting protocol (Amersham) was followed, except that the blocking solution contained 10 % Horse Serum as well as 5 % Marvel powder. Primary antibody dilutions varied, and are detailed in the respective figure legends. Preabsorbtion of oligopeptides to the primary antiserum was performed by incubating the antiserum with 1 μg / ml of the oligopeptide in the blocking solution at r.t. for 2 hours before it was applied to the membrane. The secondary antibodies were used at a dilution of 1:2500.

2.2.2.3 Purification of p13^{suc1} and formation of p13^{suc1} beads.

2.5 mg of semi-purified p13^{suc1} protein expressed in *E. coli* was further purified by anion exchange on a HPLC column (See Chapter 5.4.2). Fractions were run on 17.5 % SDS-PAGE and stained with Coomassie R250 brilliant blue to assess the level of purity. Several fractions were pooled and dialysed into phosphate buffer overnight at 4 ^{o}C. These fractions contained a total of 1.5 mg of protein according to O.D.$_{280}$ readings and Bradford assays. This protein was linked to 0.5 ml of Aminolink™ beads (Pierce) as per the protocol. Control beads were prepared in parallel with the p13^{suc1} beads by blocking the reactive sites of 2 ml of the Aminolink beads with 1.0 M Tris-HCl, pH 7.4.

CRK antisera used

GENE	Antiserum name	Rabbit	Type, Raised to:	Source	CRK Proteins detected
LmmCRK1	CITAA	Bess	Peptide 102, C-terminal 16aa	JCM	LmmCRK1,TbCRK1
TbCRK2	EVREE	Mikhael	Pep 103 C-terminal 16aa	JCM	TbCRK2 Only
TbCRK2	PSTAVR	Diego	Pep 98, 16aa of CRK2 PSTAIR box	JCM	LmmCRK1, TbCRK1, TbCRK2, TbCRK3
TbCRK3	-	Athos	Pep XXX, N-terminal 16aa	GS	NONE
TbCRK3	-	Porthos	Pep XXX, N-terminal 16aa	GS	NONE
TbCRK3	T3HG	Gareth	Entire His tagged protein	GS	TbCRK3 ?TbCRK2, LmmCRK3?
TbCRK3	T3HS	Smith	Entire His tagged protein	GS	TbCRK3 ?TbCRK2, LmmCRK3?
-	PSTAIR mAb	-	Monoclonal to 16aa PSTAIR pep.	Nagahama	LmmCRK1 and 3 TbCRK1, 2 and 3

The raising of antisera was performed under the Home Office Project Licence Number 3113.

2.3 Protocols for *E. coli.*

2.3.1 General protocols (*Ec*).

2.3.1.1 Bacterial strains used during this thesis.

Strain	Genotype	Source
XL1-Blue	*RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB* lacI^{q}ZΔM15 Tn*10* (Tet^{r})]	Stratagene
LE392	e14^{-}(McrA^{-}) *hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*	J. Mottram, WUMP.

2.3.1.2 Transformation of *E. coli.*

Competent *E. coli* were prepared using the Calcium Chloride procedure (Sambrook *et al.*, 1989). For storage 100 μl aliquots had sterile glycerol added to 15 % and were then frozen in an ethanol/dry ice bath before storage at -70 ^{o}C in screw top 1.5 ml eppendorf tubes. 10 μl of ligations in LMP agarose, 2 μl of other ligations, or 1 μl of 1 μg / ml super coiled plasmid were added for the transformation. After the heat shock (1 minute at 42 ^{o}C), 1 ml of 2 x YT was added to each tube and the bacteria allowed to recover at 37 ^{o}C for 45 minutes before plating onto selective media. For blue/white selection 20 μl of 0.5 M IPTG and 50 μl of 50 mg / ml X-Gal were spread onto the 90 mm diameter agar plates and allowed to dry before spreading the transformed bacteria. The plates were incubated at 37 ^{o}C for 12-16 hours (typically overnight).

2.3.1.3 Screening libraries.

For screening on circular 87 mm plates, titrations of lambda in SM buffer were added to 100 μl of an overnight culture of LE392 that had been centrifuged and resuspended in 1/10th volume of 10 mM $MgSO_4$. These were incubated for 20 minutes at r.t. to allow preabsorbtion of the phage to the cells. The cells were then added to 3 ml of top LB agar that had been cooled to 45 ^{o}C and then plated onto LB bottom agar, and incubated overnight at 37 ^{o}C.

Plaques were transferred to Hybond-N nitrocellulose filters for 2 minutes. The filter and agar were pierced with an ink filled syringe needle to enable the duplicate filter to be aligned and the autoradiograph orientation to be worked out. The duplicate filter transfer lasted 4 minutes. After transfer the filters were placed in denaturing solution for 2 minutes, neutralising solution for 2 minutes, and washed in 2 x SSC for 1 minute before baking at 80 ^{o}C for 2 hours. The plates were sealed with parafilm and stored at 4 ^{o}C.

Hybridisation was performed as for Southern blots. Positive plaques were removed from the plate by pipetting the agar into a 1 ml pipette tip that had had the end removed with a scalpel blade. This was then placed into 1 ml of SM buffer and briefly vortexed to help release the phage particles. 50 μl of chloroform was then added to suppress bacterial growth and the phage stored at 4 ^{o}C.

2.3.1.4 Stabilate formation.

E. coli strains were grown overnight at 37 ^{o}C in 2 x YT. 0.75 ml of the culture was added to 0.75 ml of 30 % glycerol, mixed by pipetting and stored at -70 ^{o}C.

2.3.2 DNA protocols (*Ec*)

2.3.2.1 Plasmid Preparation.

Single colonies of *E. coli* were picked with a sterile toothpick, and grown overnight in 2 ml of 2 x YT medium at 37 ^{o}C with constant shaking. Alkaline lysis minipreps of 1.5 ml of culture were used for screening ligations (Sambrook *et al.*, 1989). The protocol was followed up to the neutralisation step (addition of potassium acetate). An equal volume of chloroform was then added to the tube, shaken vigourously by hand, centrifuged at full speed in a microfuge for 5 minutes, and the aqueous phase transfered to a clean tube and precipitated with 0.7 volumes of iso-propanol (propan-2-ol). After washing the pellet with 70 % ethanol and drying, the DNA was typically resuspended in 30 μl of TE. Plasmid DNA was extracted from 3 ml of a 5 ml 2 x YT overnight culture using Magic or Qiagen Minipreps for subcloning, sequencing and labelling (Promega and Qiagen respectively).

For Eukaryotic transfections DNA was extracted from 30 ml of a 2 x YT culture grown overnight from a single colony, using Qiagen Midiprep columns (Qiagen).

2.3.2.2 Lambda DNA preparation.

The lambda plaque was placed in 1 ml of SM buffer with 50 μl of chloroform, briefly vortexed, and left at 4 ^{o}C overnight. 0.5 ml of this buffer was added to 20 ml of LB media containing 10 mM $MgCl_2$ 10 μl of an overnight culture of LE392 that had been centrifuged and resuspended in 1/10th volume of 10 mM $MgSO_4$ were added and the tubes incubated at 37 ^{o}C overnight with shaking. At the same time a 40 ml overnight culture of uninfected LE392 was set up from a single colony.

The next morning the lambda culture was cleared by centrifugation at 11 000 g for 5 minutes. The supernatant was added to 140 ml of LB media plus 10 mM $MgCl_2$ and the 40 ml overnight culture of LE392. This was grown at 37 ^{o}C with shaking for 6

hours or until the culture cleared. DNAse I and RNAse A were added to 1 μg / ml and left at r.t. for 30 minutes. The culture was centrifuged at 11 000 g for 10 minutes at 4 ^{o}C and the supernatant decanted into a new flask. Solid NaCl was added to 1 M and PEG 6000 to 10 % (w/v). This was allowed to dissolve slowly at r.t. and the solution then left at 4 ^{o}C overnight.

The tube was then centrifuged at 11 000 g for 10 minutes at 4 ^{o}C and the pellet resuspended in 3.2 ml of SM buffer. An equal volume of chloroform was added, the solutions mixed and spun at 1 600 g for 15 minutes. The aqueous phase was removed to another tube, avoiding disturbing the interface, and the extraction repeated. This was repeated until there was no white material at the interface. Then EDTA was added to 20 mM, Proteinase K to 50 μg / ml and SDS to 0.5 %. This was incubated at 65 ^{o}C for 1 hour. The solution was then extracted twice with phenol/chloroform, once with chloroform, and the DNA precipitated with ethanol/ammonium acetate at -20 ^{o}C. The DNA was washed with 70 % ethanol, dried and resuspended in TE buffer.

2.3.2.3 PCR screening of transfectants.

A numbered bacterial colony was touched with a sterile toothpick. This was then dipped into 20 μl of dH_2O in a 0.5 ml eppendorf tube. The tubes were boiled for 5 minutes, spun briefly in a microfuge and placed on ice. The rest of the PCR ingredients were added to a final volume of 50 μl and overlaid with mineral oil. The PCR conditions were generally:

Step 1	94 ^{o}C for 5 min	(initial denaturation)
Step 2	55 ^{o}C for 30 sec	5 μl of each oligo (20 pM stock)
Step 3	72 ^{o}C for 1 min	5 μl of 2 mM dNTPs
Step 4	94 ^{o}C for 30 sec	5 μl of 10xTaq buffer
(repeat Steps 2-4 for 30 cycles)		0.5 μl of *Taq* DNA polymerase (5 U / μl)
Step 5	55 ^{o}C for 30 sec	dH_2O to 50 μl

Step 6 72 ^{O}C for 10 min

10 μl of each reaction was added to 3 μl of 5 x LB and run on an applicable percentage agarose gel.

2.3.3 Protein protocols (*Ec*)

2.3.3.1 Purification of poly Histidine-tagged fusion proteins.

For screening transformants, the rapid screening of small scale expression cultures protocol was used (Qiagen). Large scale preparation of denatured His-tagged protein used a scaled up version of this method. The 10 ml overnight culture was added to 90 ml of fresh 2 x YT, grown at 37 ^{O}C with shaking. After 1 hour IPTG was added to a final concentration of 0.2 μg / ml. After a further 3 hours the cells were spun down, the supernatant removed, and the cell pellet kept at -20 ^{O}C until the protein purification. The 1.5 ml of Ni^{2+}/agarose used per purification was contained in a column for the large scale purification of the TbCRK3/H protein. The bound protein was eluted by lowering the pH of the urea buffer, instead of using EDTA addition to elute the protein as in the small scale screening protocol.

Native purification of His-tagged protein was as per the Qiagen protocol for cytoplasmic location, with imidazole washes and elution at varying concentrations depending on the experiment.

2.4 *S. pombe* protocols.

2.4.1 Transfection of *S. pombe*.

The lithium acetate procedure was used for yeast transfection. A 150 ml culture of the starting strain was grown at 25 ^{O}C in MB medium with constant shaking to a

concentration of ~1x10^7 cells / ml. The cells were pelleted at 3000 rpm for 5 minutes, washed with 50 ml of dH_2O and re-pelleted. The cells were resuspended at 1x10^9 cells / ml in lithium acetate (pH 4.9), aliquoted into microcentrifuge tubes (100 μl / tube) and incubated at 25^{o}C for 90 minutes. 15 μl of TE containing 1 μg of plasmid DNA was added and the tubes gently vortexed to mix, not allowing the tubes to cool down. 290 μl of prewarmed (25 ^{o}C) 50 % PEG-4000 was added, mixed by gentle vortexing and incubated at 25 ^{o}C for 1 hour. The cells were heatshocked at 43 ^{o}C for 15 minutes, then allowed to cool to r.t. for 10 minutes. The tubes were centrifuged at 5000 rpm for 2 minutes and the supernatant removed. The pellet was resuspended in 1 ml of 1/2YEL+leu by repeated pipetting, transfered to a 50 ml flask containing 9 ml of 1/2YEL+leu and incubated at 25 ^{o}C with shaking for 1 hour to allow recovery. 100 μl of this was spread on MMA plates, with and without thiamine.

2.4.2 Preparation of *S. pombe* protein extracts.

10 ml of yeast culture was grown to a density of approximately 5x10^6 cells / ml in minimal medium ($O.D._{595}$ = ~0.25). The culture was then spun down by gentle centrifugation, washed with 1 ml of ice cold stop buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN_3, pH 8.0), and re-centrifuged and left to drain. The cell pellet was resuspended in 4 μl of RIPA buffer (10 mM sodium phosphate pH 7.0, 1 % Triton X-100, 0.1 % SDS, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1 mM $NaVO_3$, 4 μg / ml leupeptin, 1 mM PMSF). 0.5 ml of acid washed glass beads (0.5 mm diameter, Sigma) were added and the tube vortexed for 1 minute. 20 μl of 1 % SDS was added and the tube boiled for 3 minutes. 200 μl of 2 x FSB was then added before a further 5 minutes of boiling. The extract was then centrifuged at 4 ^{o}C in a microfuge for 15 minutes at maximum speed and the supernatant removed to another tube. 15 μl of the extract was used in each lane for Western blotting.

2.5 *T. brucei* protocols.

2.5.1 General protocols (*Tb*)

2.5.1.1 *T. brucei* strains used.

The procyclic cultures used for DNA extraction, protein extracts and the creation of mutants was from the STIB247 strain, as were the mixed bloodstream forms and the short stumpy forms. The monomorphic long slender line used was IstatC123.

2.5.1.2 The growth of procyclic form *T. brucei* .

The procyclic form *T. brucei* were cultured at 28 ^{O}C in 5 ml cultures of SDM-79 medium (Brun and Schonenberger, 1979) + 10 % fetal calf serum (FCS), passaged approximately every third day to a concentration of 0.5 x 10^6 cells / ml. Antibiotics were added to the flasks where selection was desired.

2.5.1.3 Preparation of *T. brucei* for microscopic examination.

10-25 μl of log phase procyclic form *T. brucei* were spread across each slide. Low density cultures, such as the putative *tbcrk3* null mutants, first had 0.5 ml spun down in a microfuge at 1 000rpm for 5 minutes. 475 μl of the supernatant was removed and the pellet gently resuspended. This was then spread onto the slide. The slides were air dried in a tissue culture hood before the cells were fixed in 100 % methanol for 1 minute. The slides were then washed with PBS for 2 minutes and then rinsed with dH_2O before air drying again. The cells were stained with 1 μg / ml DAPI for 5 minutes before washing with PBS and mounting.

2.5.1.4 Formation of *T. brucei* stabilates.

Stabilates were made by centrifugation of 5 ml of a mid log-phase culture, resuspension in 1 ml of SDM-79 (without FCS), and addition of 0.5 ml of 22.5 % glycerol. The samples were transfered to cryotubes and frozen surrounded by cotton wool in a expanded polystyrene box in a -80 ^{o}C freezer overnight, and then storage in liquid nitrogen.

2.5.2 DNA protocols (*Tb*).

2.5.2.1 Trypanosome Genomic DNA Preparation.

High molecular weight DNA was prepared from pellets of procyclic *T. brucei* kept at -70 ^{o}C, or freshly spun down cultures of procyclic *T. brucei,* using the Nucleon reagents (Scotlab). Typically, 5 x 10^{7} cells were used per extraction. The protocol was followed precisely, without the RNAse step, with the DNA being recovered from the ethanol precipitation step by spooling with a closed glass pipette. A 1/100 dilution was assayed by a spectrometer at 260 nm and 280 nm to assess the concentration and purity of the samples. All manipulations of the genomic DNA were performed with cut down tips.

2.5.2.2 Restriction Enzyme digestion of Genomic DNA.

3-6 μg of *T.brucei* DNA were digested per lane in 30 μl total volume. For each lane, 10 U of enzyme were added at the start of the digest and the mixture gently stirred. After 1.5 hours at 37 ^{o}C 10 more Units of enzyme were added and mixed carefully. After another 1.5 hours 10 μl of 5 x LB were added, and 40 μl loaded onto the agarose gel using cut off pipette tips.

10 μg of Human high molecular weight DNA were digested per lane in a total volume of 100 μl. As above, 10 U of enzyme were added at 0 hours and 1.5 hours into a 3 hour incubation. The DNA was then precipitated with ethanol, and gently resuspended in 30 μl of TE. 10 μl of 5 x LB were added, and 40 μl loaded onto the agarose gel using cut off pipette tips.

2.5.2.3 Transfection of Procyclic *T. brucei.*

Mid-log phase (~6 x 10^6 cells / ml) procyclic form cultures were gently pelleted by centrifugation at room temperature. The pellets were washed once with Zimmerman post-fusion medium (ZPFM), pelleted again, and resuspended in ZPFM at 6 x 10^7 cells / ml. ~50 μg of the respective DNA at ~2 mg / ml in dH_2O was added per 0.5 ml of cells to be transfected. The transfection was by electroporation (Bio-rad Gene Pulser) using 0.4 cm cuvettes at 1500 V and 25 μF. The pulse was applied to the sample twice before transfer of the cells into pre-warmed SDM-79 medium. After incubation overnight for recovery the cells were passaged to a concentration of 1 x 10^6 cells / ml in SDM-79 medium containing the antibiotic needed for selection of the transfected DNA.

2.5.2.4 PCR from *T. brucei* lines.

500 μl of procyclic form culture was placed in a screwtop eppendorf, DNAse I added to 10 μg / ml and incubated at 28 ^{o}C for 1 hour. EDTA was added to 4 mM and the trypanosomes pelleted in a microfuge at 1 000 rpm for 5 minutes. 480 μl were removed, the pellet resuspended, and then incubated in a boiling water bath for 15 minutes. 0.5 μl was used as a template in each reaction. The PCR conditions were generally:

Step 1	94 ^{o}C for 5 min	(initial denaturation)
Step 2	60 ^{o}C for 45 sec	5 μl of each oligo (20 pM stock)

Step 3	72 °C for 1 min 30 sec	5 μl of 2 mM dNTPs
Step 4	94 °C for 45 sec	5 μl of 10 x Taq buffer
(repeat steps 2-4 for 40 cycles)		0.5 μl of *Taq* DNA polymerase (5 U / μl)
Step 5	60 °C for 45 sec	0.5 μl of template
Step 6	72 °C for 10 min	dH_2O to 50 μl

10 μl of the aqueous phase were added to 3 μl of 5 x LB and run on 1-1.4 % agarose gels.

2.5.3 Protein protocols (*Tb*).

2.5.3.1 Trypanosome protein extracts.

Pellets of *T. brucei* procyclic and bloodstream forms were resuspended in approximately 1 ml of protein Lysis Solution + Inhibitors (LSI) per 5 x 10^8 cells. The tubes were kept on ice for 30 minutes. For whole cell extracts, the protein concentration was then determined by the Bradford assay (Biorad), and the extract diluted to 2.5 mg / ml with 5xFinal Sample Buffer (5 x FSB). For S100 extracts, the lysed cells were centrifuged at 35 000 rpm in a Ti50 ultracentrifuge rotor at 4 °C for 30 minutes. The supernatant and pellet (resuspended in the same volume of LSI as used originally for cell lysis) were diluted to 2.5 mg / ml after Bradford assays.

For SDS-PAGE followed by Coomasie staining or Western blotting, the extracts were incubated in a boiling water bath for 5 min and 15 μl of extract were usually loaded per lane on a mini-gel. For larger gels 40 μl of extract were loaded.

2.5.3.2 Suc1/LmmCKS1 selections from S100 protein extracts.

For selections, 100 μl of suc1/CKS1 or control bead slurry was used in each experiment. The beads were washed in Lysis Solution (LS). 50 μl of 50 mg / ml BSA

and 200 μl of LSI were added to the beads, which were incubated at 4 ^{o}C for 2 hours with agitation. 200 μl of *T. brucei* or *S. pombe* S100 extract supernatant was added, and the beads incubated a further 2 hours. The beads were then washed twice with 1 ml of LSI, 3 times with LSI + 1 M NaCl, then once with LSI.

For Western blots, the bound protein was eluted by transferring the beads to a screw topped tube, adding 100 μl of 2 x F.S.B, heating to 50 ^{o}C for 5 minutes, and removing the supernatant.

2.5.3.3 Histone H1 kinase assays.

For Histone H1 kinase assays the Aminolink beads or Ni^{2+}/agarose were washed once with Kinase Assay Buffer (KAB) and transferred to a screw topped tube. The beads and the Kinase Assay Mix (KAM) were prewarmed separately at 30 ^{o}C for 5 minutes, before adding 20 μl of KAM to each screw topped tube and incubating at 30 ^{o}C for 20 minutes. 20 μl of 5 x F.S.B was added before the samples were placed in a boiling water bath for 2 minutes. 15 μl per sample was loaded onto 12.5 % SDS-PAGE. The gels were stained with Coomasie R250 brilliant blue, destained, dried down onto 3MM paper and exposed to Fuji X-ray film.

2.5.3.4 ^{35}S-Methionine labelling of procyclic *T. brucei brucei*.

Procyclic form STIB247 at 6 x 10^{6} cells / ml were pelleted at room temperature, washed with PBS and then resuspended in methionine free MEM-Eagles media at 6 x 10^{6} cells / ml. After 1 hour at 28 ^{o}C, 100 μCi of ^{35}S-Methionine were added and the cells grown for another 6 hours. The cells were then pelleted, and stored at -70 ^{o}C overnight before S100 extracts were made and suc1/CKS1 selections performed. 15 μl of the extracts were run on 12 % SDS-PAGE in duplicate. Both gels were Coomassie R250 brilliant blue stained, to visualise the markers, and one gel was soaked in Intensify™. The gels were then dried onto 3MM paper and exposed to X-ray film.

CHAPTER THREE
ISOLATION OF *T. BRUCEI* GENES HOMOLOGOUS TO THOSE INVOLVED IN CELL CYCLE CONTROL IN OTHER ORGANISMS

3.1 Introduction.

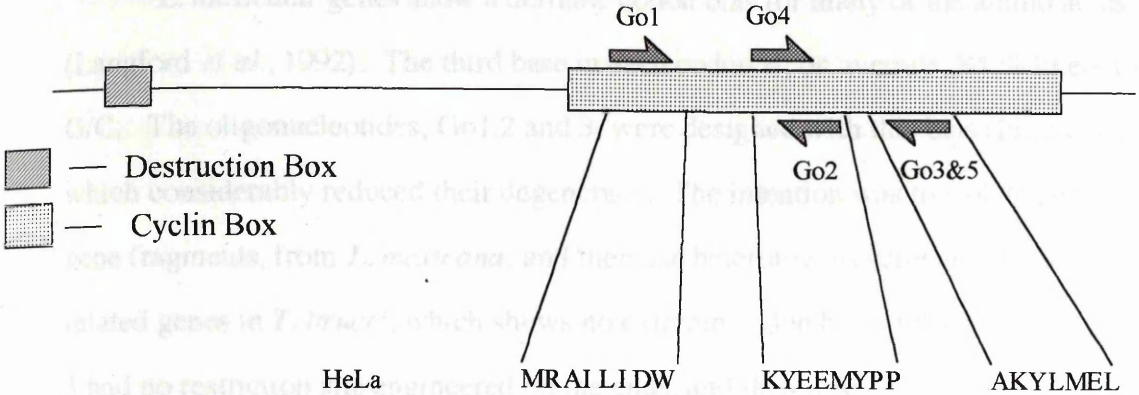
The isolation of genes from the kinetoplastids that are homologous to those from other organisms has been successfully tried using several different techniques. Both PCR with degenerate oligonucleotides (Bangs *et al.*, 1993) and heterologous hybridisation at low stringency (Wong *et al.*, 1993) have isolated genes of interest. It was thought possible that a similar approach could be used to isolate cyclin genes from *T. brucei* and *L. mexicana*.

3.2 Attempted Isolation of Mitotic Cyclin genes from *T. brucei* and *L. mexicana.*

3.2.1 Background and Experiment Design.

In 1990 a number of cyclins had been cloned and the predicted protein sequences were available in the data base. The cyclin proteins could be assigned to a number of different classes on the basis of characteristic sequence motifs (Colas *et al.*, 1993; Gallant and Nigg, 1994). The A and B type cyclins, shown to be involved in G2/Mitosis checkpoints (Pines and Hunter, 1992; Minshull *et al.*, 1990), were better conserved than the CLN cyclins isolated from *S. cerevisiae* (Rowley *et al.*, 1992). Therefore this category of cyclins was thought the most likely to be conserved enough for cloning from the kinetoplastida. Aligning the protein sequences showed that some regions of the A and B type cyclins were very well conserved between species. The domain of highest conservation was called the cyclin box. It was decided to design oligonucleotides that would hybridise to the DNA encoding 3 of the most conserved regions within the cyclin box, with the belief that these regions were the most probable to be conserved in the evolutionary distant protozoa (Figure 3.2.1). These would be used to isolate cyclin genes from *T. brucei* and *L. mexicana* using either PCR or library screening.

Figure 3.2.1 Mitotic cyclin domains and oligonucleotide positions and sequences



HeLa	MRAI LI DW	KYEEMYPP	AKYLMEL
A. punctulata	- - L - - V - -	- - - - - - - -	- - - - - - I
S. solidissima	- - - - - - - -	- - - - - - A -	- - - - - - -
S. pombe	- - G - - T - -	- - - - VNC -	- - - - V - I
X. laevis B1	- - - - - - - -	- - - - - - - -	- - - - - - -
X. laevis B2	- - - - - V - -	- - - - - - T -	- - - - - - -
D. melanogaster	- - - V - - - -	- - - - LF - -	S - - FI - -
CONSENSUS	MRAI LI DW	KYEEMYPP	AKYLMEL

Oligonucleotides Go1, Go2, Go3, Go4 and Go5 — Degeneracy

M R A I L I D W
Go1 ATG CG(C/G) GC(C/G) AT(C/T) (C/T)T(C/G) AT(C/T) GAC TGG — 64 fold

P P Y M E E Y K
Go2 (C/G)GG (C/G)GG CTA CAT CTC CTC GTA CTT — 4 fold

L E M L Y K A
Go3 A(G/T) CTC CAT (C/G)A(A/G) GTA CTT (C/G)GC — 16 fold

A A/S/T K Y E E
Go4 C *GGATCC* GCN (A/G/T)(C/G)N AA(A/G) TA(C/T) GA(A/G) GA(A/G) — 1536 fold
*Bam*HI

E M/L/V L Y K A
Go5 C *GAATTC* (C/T)TC NA(C/G/T) NA(A/G) (A/G)TA (C/T)TT NGC — 768 fold
*Eco*RI

Figure 3.2.1 The sequences of conserved regions of the cyclin box are from B type cyclins from the organisms: *Arbacia punctulata* (sea urchin), *Spisula solidissima* (clam), *Schizosaccharomyces pombe* (fission yeast), *Xenopus laevis* (frog) and *Drosophila melanogaster* (fruit fly).

The nucleotide sequence of each oligonucleotide is written 5' to3'. The bold letters above Go1 and Go4 are the amino acids encoded by the nucleotide sequence. Oligonucleotides Go2, Go3 and Go5 are antisense and the bold letters above the sequence are the amino acids that would be encoded by the opposite strand. The sequences in italics are the added restriction enzyme sites for improved cloning efficiency.

L. mexicana genes show a definate codon bias for many of the amino acids (Langford *et al.*, 1992). The third base in each codon is, on average, 84 % likely to G/C. The oligonucleotides, Go1,2 and 3, were designed with this bias (Figure 3.2.1), which considerably reduced their degeneracy. The intention was to isolate genes, or gene fragments, from *L. mexicana*, and then use heterologous screening to isolate the related genes in *T. brucei*, which shows no extreme codon bias. Oligonucleotides Go1-3 had no restriction site engineered on the ends, and therefore were kinased to help blunt end cloning of the PCR fragments. Of these oligonucleotides, Go1 was sense, to be used as the 5' primer in PCR, while Go2 and 3 were antisense, and were the 3' primers. Later in the project 2 more oligonucleotides were made which were identical to those that had been used to isolate B type cyclin genes from *S. cerevisiae* (Ghiara *et al.*, 1991). Oligonucleotides Go4 and Go5 were designed to the regions also encoded by Go2 and Go3, but Go4 was a 5' primer for PCR, unlike Go2. Go4 and 5 contained *Bam*HI and *Eco*RI sites respectively to improve cloning efficiency (Figure 3.2.1). By comparison with previously cloned cyclins, it was expected that PCR with Go1/2 should result in a product of ~190 bp, while Go1/3 would result in a band at ~380 bp. Oligonucleotides Go4/5 were expected to produce a band at ~200 bp. Due to the lack of *cis* introns in the Kinetoplastida, these sizes could be predicted for PCR from genomic DNA as well as from cDNA.

3.2.2 Cloning Attempts.

One approach to cloning, that had previously been successful in isolating cysteine protease genes from *T. brucei* (Mottram *et al.*, 1989b), was direct screening of cDNA libraries with degenerate oligonucleotides. To ascertain the correct hybridisation conditions, the oligonucleotides Go1-3 were endlabelled with ^{32}P and hybridised individually to an *L. mexicana* genomic DNA Southern blot that had been run in triplicate. All three oligonucleotides gave multiple bands, in multiple restriction enzyme digests, although to the accuracy of the experiment, no two oligonucleotides

gave the same band. (Data not shown) Even so, a *L. mexicana* cDNA library in lambda Zap (supplied by J.C.Mottram) was screened with Go1 and Go2 endlabelled with ^{32}P. These two oligonucleotides were expected to hybridise to genes encoding both Cyclin A and B, unlike Go3, which is Cyclin B specific. ~30 000 plaques were screened in duplicate, with one filter probed with Go1 and the other with Go2. No plaques that hybridised to both probes were found. Of the 6 plaques that appeared to hybridise to Go1, none gave positive signals in the secondary screening. Three lambda clones which hybridised to Go2 were purified, the inserts rescued into Bluescript and single strand partial sequence obtained. No homology to any cyclin sequence in the data base was found. This approach had also been tried in *T. brucei* with an oligonucleotide equivalent to Go2 (M.Carrington, Personal communication). Many cDNA clones were sequenced but in each case the sequence hybridising to the oligonucleotide probe was found to be in the 3' untranslated region and antisense to the Open Reading Frame (ORF). As this approach was not successful in isolating a cyclin gene it was ceased.

PCR with different combinations of the 5 oligonucleotides was also tried, using both *L. mexicana* and *T. brucei* genomic DNA as template. The annealing conditions varied between 37 ^{o}C and 55 ^{o}C. All the oligonucleotide combinations gave a large number of bands and smearing, even at high annealing temperatures. The relative intensities of bands varied between experiments with few bands being consistent. Initially the Go1/Go3 PCR products were seperated on an agarose gel, the DNA was capillary blotted, and the membrane hybridised to endlabelled Go2, an internal oligonucleotide. This attempt to find a specific cyclin fragment amongst the multiple bands never resulted in a signal. In an attempt to reduce spurious annealing, "touchdown" PCR was tried (Don *et al.*, 1991). With this technique the first few rounds of PCR have a higher than optimum annealing temperature, which should result in only target sequences with good matches to the oligonucleotides priming DNA synthesis. The annealing temperature is then reduced in steps over a number of rounds to less stringent conditions, when several more amplifying rounds take place (See Materials and Methods for further details). This should result in a decrease of the products that

rely initially on a poor match annealing. This approach still gave multiple bands, but fewer than before, and with higher consistency. PCR with oligonucleotides Go4 and Go5 gave the most reproduceable results, and so 10 μl of each 50 μl reaction was run out in a 1.8 % lmp agarose gel and bands cut out with sterile scalpel blades (Figure 3.2.2). Bands L1, L2 and T1 were very consistent, though not of the expected size, while T2 was the area of the gel which contained a smear of DNA molecules of the expected size from the *T. brucei* templated reaction. 0.5 μl of lmp agarose from each slice was used as template in another PCR reaction, using the same primers, to increase the proportion of each band in the respective reactions. These reactions were cleaned up by phenol/chloroform extraction and digested with *Eco*RI and *Bam*HI before running out on a 1.8 % lmp agarose gel. The four bands were again cut from the gel and ligated into pBS SK-. Most of the clones had inserts of the expected size, but when sequenced, none of the >15 differing clones showed any similarity to cyclins. Indeed most showed no ORF in any possible frame. This approach was tried many times with minor variations. Other approaches tried included PCR using cDNA as template, which also failed to reduce spurious bands. Nested PCR using cDNA template was also attempted. This involved a first PCR using Go1 and an "adapter" oligonucleotide which hybridised to the sequence downstream of the poly(A) tail. When the cDNA is made, the 3' primer for synthesis of the first DNA strand consists of a poly(T) tract, followed by a region containing a number of restriction enzyme sites, to facilitate cloning of the cDNA into the vector. After the first strand synthesis, an oligonucleotide consisting of just the restriction enzyme sites is used as the 3' primer in a PCR to amplify the cDNA. The annealing of this "adapter" oligonucleotide is more specific, with a higher melting temperature, than that of a poly(T) oligonucleotide. The first stage of the nested PCR should specifically amplify any cyclin messages in the library, giving a smear of DNA due to variation in the length of the poly(A) tail and the position of the initial priming. A second PCR, using the products of the first as template, is then performed with 2 oligonucleotides that are internal to the primers in the first PCR. The

Figure 3.2.2 Touchdown PCR

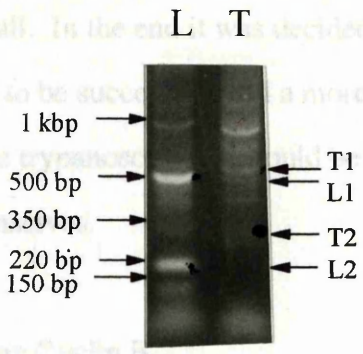


Figure 3.2.2 Touchdown PCR with Go4 and Go5 oligonucleotides. Lanes L and T; reactions with *L. mexicana* or *T. brucei* genomic DNA as template respectively. One fifth of the PCR reactions was run on a 1.8% agarose gel, and stained with Ethidium Bromide. The bands 1 and 2 from each lane were cut from the gel and used as template in another PCR with Go4 and 5.

oligonucleotides used, Go4 and Go5, gave no consistent bands in these reactions, and normally no bands at all. In the end it was decided that the attempt to directly clone cyclins was not going to be successful, and a more productive approach to analysing CDK complexes in the trypanosomatids would be to concentrate on the kinase subunits of the complexes themselves.

3.2.3 Cloning a Human Cyclin B.

Prior to the start of the Ph.D., PCR had been tried by other members of the laboratory, with other oligonucleotides, to isolate cyclin genes from *L. mexicana*. One consistent band of the correct size which hybridised to an labelled internal oligonucleotide, was subcloned into pBS SK- using the *Eco*RI sites added to each primer. The clone, Lcyc12, had the expected insert size of 350 bp and was sequenced on both strands with the T3 and T7 primers (Figure 3.2.3).

As Figure 3.2.4 shows the PCR product is almost identical to the Human Cyclin B1 gene already cloned (Pines and Hunter, 1989). It seems likely that during the early PCR stages of the experiment the *L. mexicana* DNA was contaminated by some HeLa DNA that was being used as a positive control. As expected, the random primed, labelled insert failed to hybridise to *L. mexicana* DNA on a Southern blot (Data not shown).

The sequence differences between Lcyc12 and the previously reported HsCyB1 sequence could be due to a number of reasons. The nucleotide variation could simply be data base error, though the rate would seem considerably higher than previously reported (~1 %). The multiple rounds of PCR are likely to cause a number of base changes. Evidence supporting this is that there is no bias towards changes at the third base in each codon, which results in few silent mutations. This implies that the sequence was not under selection pressure when the changes occured. If the differences are not due to PCR error, then the lack of silent mutations would suggest that the Lcyc12 sequence is unlikely to be allellic to the previously cloned human B1 gene

Figure 3.2.3 DNA and predicted protein sequence of the probable human PCR contaminant.

```
 I  Q  V   Q  M  K  F   R  L  L   Q  E  T   M  Y  M  T
GATACAGGTT CAAATGAAAT TTAGGCTGCT GCAAGAGACT ATGTACATGA

  V  P  I   I  D  R   F  M  Q  D   N  C  A   S  K  K
CTGTTCCCAT TATTGATCGA TTCATGCAGG ATAATTGTGC GTCCAAGAAG

M  L  Q  L   V  G  V   T  A  M   F  I  A  S   K  Y  E
ATGCTGCAGC TAGTTGGTGT CACTGCCATG TTCATTGCCA GCAAATATGA

 E  M  Y   P  P  E  I   G  D  F   A  F  V   T  N  N  T
AGAAATGTAC CCTCCAGAAA TAGGTGACTT CGCTTTTGTG ACTAACAATA

  Y  I  K   H  Q  I   R  P  I  E   M  K  I   L  R  V
CTTACATTAA GCACCAAATT AGACCAATTG AAATGAAGAT TCTAAGAGTT

L  N  F  S   L  G  P   P  L  P   L  H  F  F   H  R  A
CTAAACTTTA GTTTGGGTCC CCCTCTGCCT CTGCACTTCT TCCATAGAGC

 S  K  I   G  E  V  D   V  E  Q   H  T  L
ATCTAAGATT GGAGAGGTTG ACGTTGAACA GCATACTTTG
```

Figure 3.2.3. The above sequence from the insert of pLpCYC12 does not include the regions encoded by the oligonucleotides used in the PCR. The amino acid encoded by each codon is above the first base of the triplet.

Figure 3.2.4a Comparison of the DNA sequence of the PCR contaminant and the HeLa Cyclin B1 gene.

```
pLpCYC12        G ATA CAG GTT CAA ATG AAA TTT AGG CTG CTG CAA
                  || ||| ||| ||| ||| ||| ||  |||  ||  || ||
HeLa Cyclin B1  A GTA CAG GTT CAA ATG AAA TTC AGG TTG TTG CAG

GAG ACT ATG TAC ATG ACT GTT CCC ATT ATT GAT CGA TTC ATG CAG
||| ||  ||| ||| ||| ||| ||  ||  ||| ||| ||| ||  ||| ||| |||
GAG ACC ATG TAC ATG ACT GTC TCC ATT ATT GAT CGG TTC ATG CAG

GAT AAT TGT GCG TCC AAG AAG ATG CTG CAG CTA GTT GGT GTC ACT
 || ||| ||| | |  || ||| ||| ||| ||| ||| ||  ||| ||| ||| |||
AAT AAT TGT GTG CCC AAG AAG ATG CTG CAG CTG GTT GGT GTC ACT

GCC ATG TTC ATT GCC AGC AAA TAT GAA GAA ATG TAC CCT CCA GAA
||| ||| ||  ||| ||  ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
GCC ATG TTT ATT GCA AGC AAA TAT GAA GAA ATG TAC CCT CCA GAA

ATA GGT GAC TTC GCT TTT GTG ACT AAC AAT ACT TAC ATT AAG CAC
||  ||| ||| ||  ||| ||| ||| |||  ||  ||  ||| ||  | | ||| |||
ATT GGT GAC TTT GCT TTT GTG ACT GAC AAC ACT TAT ACT AAG CAC

CAA ATT AGA CCA ATT GAA ATG AAG ATT CTA AGA GTT CTA AAC TTT
||| ||  ||| |   ||  ||| ||| ||| ||| ||| ||| | |  || ||| |||
CAA ATC AGA CAG ATG GAA ATG AAG ATT CTA AGA GCT TTA AAC TTT

AGT TTG GGT CCC CCT CTG CCT CTG CAC TTC TTC CAT AGA GCA TCT
 ||  ||  ||| |   ||| ||  |||  ||  ||| |||  |  |   ||| ||| |||
GGT CTG GGT CGG CCT CTA CCT TTG CAC TTC CTT CGG AGA GCA TCT

AAG ATT GGA GAG GTT GAC GTT GAA CAG CAT ACT TTG
||| ||| ||| ||| ||| ||  ||  ||  ||  ||| ||| |||
AAG ATT GGA GAG GTT GAT GTC GAG CAA CAT ACT TTG
```

Figure 3.2.4a The homology between the two sequences is 88% over the 340bp. Of the differing bases, 29% are at the first position of the codon, 14% at the second, and 57% at the third.

Figure 3.2.4b Comparison of the predicted human Cyclin B1 protein sequence and the translated pLpCYC12 insert.

```
pLpCYC12    IQVQMKFRLL QETMYMTVPI IDRFMQDNCA SKKMLQLVGV
            :|||||||||| |||||||| | |||||||:||:  ||||||||||
Human B1    VQVQMKFRLL QETMYMTVSI IDRFMQNNCV PKKMLQLVGV

TAMFIASKYE EMYPPEIGDF AFVTNNTYIK HQIRPIEMKI LRVLNFSLGP
|||||||||| |||||||||| ||||:||| | ||||  |||| ||:|||.||
TAMFIASKYE EMYPPEIGDF AFVTDNTYTK HQIRQMEMKI LRALNFGLGR

PLPLHFFHRA SKIGEVDVEQ HTL
|||||||.:|| |||||||||| |||
PLPLHFLRRA SKIGEVDVEQ HTL
```

Figure 3.2.4b The predicted protein sequence comparison between the PCR derived insert and the previously cloned Human Cyclin B1 gene shows that the percentage identity is 87% with no gaps in the aligned sequences.

sequence. The obvious possibility, if the changes are not artifacts of cloning, sequencing or data base error, is that this sequence has been amplified from a pseudo-gene. The lack of Southern blot data in the human cyclin gene family leaves this open.

3.2.4 Reasons for the failure to clone a kinetoplastid cyclin.

Although the cell cycle machinery is extremely well conserved throughout the Eukaryotes, with many of the proteins from higher organisms capable of complementing yeast mutants, the Kinetoplastida are a very early branch from the eukaryote lineage (Sogin *et al.*, 1986; Sogin, 1991). Even within the Kinetoplastida the organisms are highly diverged. Therefore the evolutionary distance between *T. brucei* and *S. pombe* is far greater than that between any of the higher eukaryotes (see Figure 1.1.1). This extreme divergence can be seen in the relatively low conservation of the CDC2 related kinases from the Kinetoplastida that will be described later. As more cyclins have been cloned from diverse species it is now clear that the cyclin family is less well conserved than previously thought. It is likely therefore that kinetoplastid cyclins will have significant sequence divergence making cloning by PCR very difficult. A putative mitotic cyclin from *T. brucei* has been recently characterised (Affranchino *et al.*, 1993). The protein described is relatively small, and has an unusual domain structure for a mitotic cyclin. The cyclin box is near the N-terminus, with the destruction box at the C-terminus. The cyclin box has considerably fewer conserved residues than usual with numerous insertions and deletions. One of these insertions, of 12 residues, falls within the region to which Go1 was designed. The normally conserved ASKYEEMYPP motif, parts of which Go2 and Go4 were designed to, is AEHSDSANPK, and the B type cyclin motif of Go3 and Go5 is also totally absent. This is obviously the reason why the methods that I employed failed to isolate this particular *T. brucei* cyclin-like gene.

3.3 Isolation of the *tbcrk3* gene.

3.3.1 The background to cloning the *tbcrk3* gene.

A PCR-based approach to cloning *cdk* related genes had been started in the laboratory prior to this Ph.D. Initially a cDNA from *L. mexicana,* homologous to *cdc2*, was isolated accidentally during a seperate experiment. This gene was designated *lmmcrk1* (Mottram *et al.*, 1993). Oligonucleotides were then designed by Dr. J.C. Mottram to hybridise to DNA encoding conserved regions of CDC2 related protein kinases and used for PCR with *T. brucei* or *L. mexicana* genomic DNA as template (Figure 3.3.1). Two gene fragments were amplified by PCR from *T. brucei* genomic DNA. with the new oligonucleotides. One *T. brucei* fragment showed a high level of homology to *lmmcrk1* and so was named *tbcrk1*. *lmmcrk1* was used to screen a *T. brucei* genomic lambda library under low stringency conditions and a *T. brucei* gene so isolated was named *tbcrk2*. The second gene fragment from the PCR approach was the least conserved, compared to other CDC2 related proteins, within the amplified region and was called *tbcrk3*. The genomic copies of the *tbcrk1/2* and *lmmcrk1* genes had all been cloned and sequenced prior to the start of the Ph.D.

3.3.2 Isolating a Lambda phage clone containing *tbcrk3*.

To isolate the genomic DNA containing the *tbcrk3* gene, a EMBL4 genomic library (supplied by J.C.Mottram) was screened with a 350 bp *tbcrk3* gene fragment which had been gel purified. ~30 000 plaques were screened and one hybridised. The hybridising plaque was purified by 2 further rounds of dilution and hybridisation. One clone, CD47.2, was chosen for a large scale preparation of DNA.

The phage DNA was digested with a number of restriction enzymes including *Eco*RI and *Hin*dIII. A *Hin*dIII site was predicted from the PCR fragment sequence, and was therefore expected to produce 2 hybridising fragments on the Southern blot. The

Figure 3.3.1 Cloning cdc2-related kinases by degenerate oligonucleotide PCR

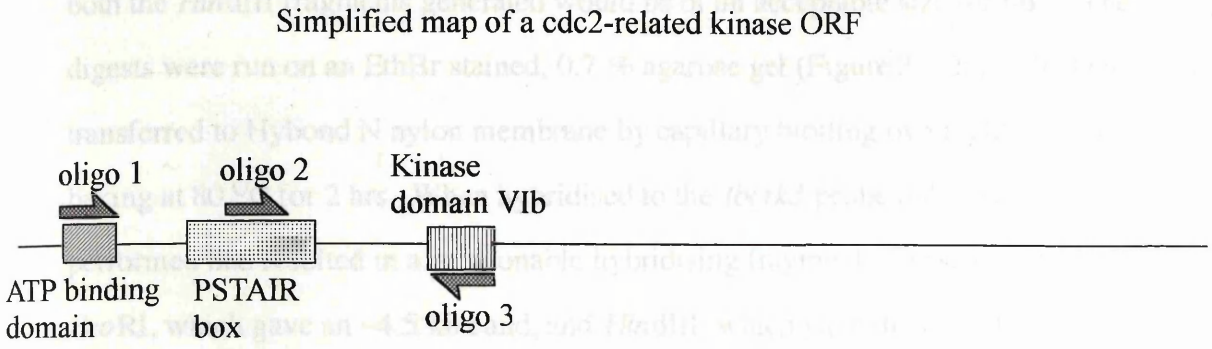


Figure 3.3.1 Degenerate oligonucleotides were designed to anneal to the DNA encoding three well conserved regions of the cdc2 family of serine/threonine kinases by Dr. J. Mottram. *Eco*RI restriction sites were added to oligonucleotides 1 and 3 to facilitate cloning. PCR was performed with the two exterior primers (oligos 1 and 3), and the products screened by Southern blotting using a ^{32}P labelled internal primer (oligo 2). The products were then digested with *Eco*RI and cloned into pBluescript for sequencing. This work was performed prior to the start of my Ph.D.

intention was to subclone a small fragment of the lambda clone into Bluescript plasmid (pBS) to facilitate further mapping and sequencing of the gene, and it was hoped that both the *Hin*dIII fragments generated would be of an acceptable size for pBS. The digests were run on an EthBr stained, 0.7 % agarose gel (Figure 3.3.2a). The DNA was transferred to Hybond N nylon membrane by capillary blotting overnight and fixed by baking at 80 ^{o}C for 2 hrs. When hybridised to the *tbcrk3* probe only two of the digests performed had resulted in a subclonable hybridising fragment. These exceptions were *Eco*RI, which gave an ~4.5 kb band, and *Hin*dIII, which gave the expected 2 fragments, one >12 kb and one at 1.4 kb (Figure 3.3.2b). As the *Eco*RI digest resulted in a double band at 4.5 kb when stained with EtBr, it was difficult to be sure which band was hybridising. Therefore, 20 μg of CD47.2 DNA was cut with *Eco*RI, both bands were removed from lmp agarose and ligated into *Eco*RI cut pBS SK- that had been dephosphorylated using Calf Intestinal Alkaline Phosphorylase (CIP). After transformation of CaCl2-competent XL1-Blue and plating on Tet/Amp plates plus X-Gal/IPTG, a number of white, recombinant colonies were picked for analysis. The plasmid DNA from the overnight cultures was prepared by a modified Birnboim-Doly procedure, restricted with *Eco*RI and Southern blotted. The 1.4 kb band in the *Hin*dIII digest was also subcloned from lmp agarose into pBS SK-, DNA prepared from 8 white colonies, and the restricted DNA blotted, in an equivalent manner. The Southern blots were hybridised to the labelled *tbcrk3* gene fragment, which identified pTgCD70H3 as containing the *Hin*dIII fragment of interest, and the plasmids pTgCD70E(S)2/3/7 and 8 as holding the correct *Eco*RI fragment.

3.3.3 Mapping pCD70E(S)3 and 8.

Digests of the plasmid DNA with *Hin*dIII gave identical patterns with pCD70E(S)2/7 and 8. The pattern of pCD70E(S)3 differed slightly, lacking the 1.2 kb band, with the 2.9 kb pBS band shifting to 4.1 kb. This was due to the insert being reversed in orientation relative to the vector Multiple Cloning Site (MCS). Further

Figure 3.3.2 Subcloning of the *tbcrk3* containing fragments from lambda CD47.2.

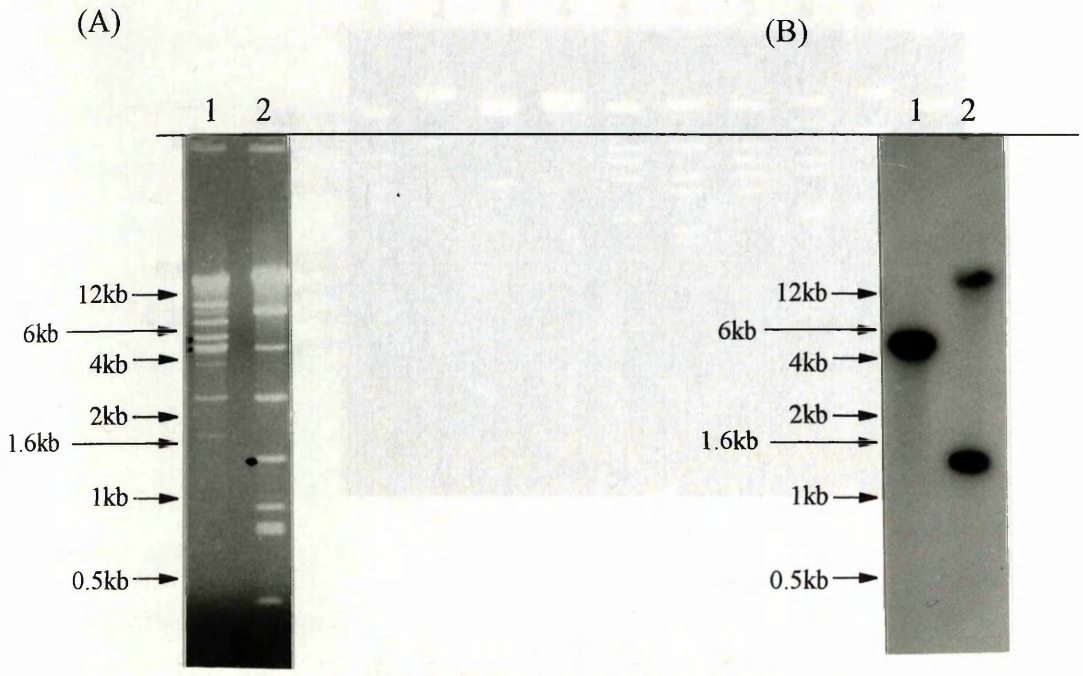


Figure 3.3.2 DNA from lambda CD47.2 was digested with HindIII or EcoRI and run on a 0.7% agarose gel, stained with EtBr (Panel A). The DNA was then transfered (without HCl treatment) to a Hybond N membrane and fixed. The membrane was hybridised to a random primed PCR fragment of tbcrk3 (Panel B).

Figure 3.3.3 Mapping the *tbcrk3* genomic locus.

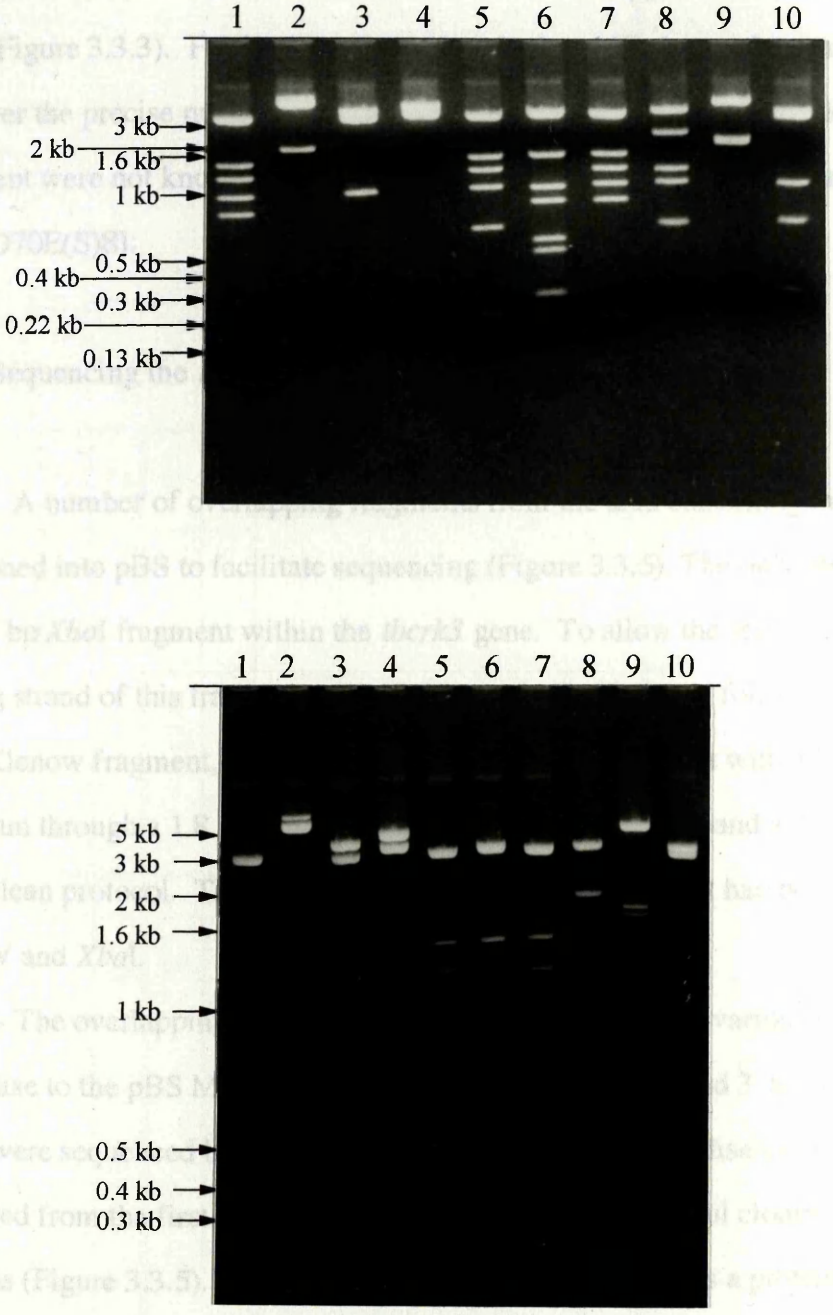


Figure 3.3.3 Purified pCD70E(S)8 plasmid was digested with multiple restriction enzymes to map the insert DNA. Lane 1-*Hin*dIII, lane 2-*Pst*I, lane 3-*Sal*I, lane 4-*Xba*I, lane 5-*Hin*dIII/*Pst*I, lane 6-*Hin*dIII/*Sal*I, lane 7-*Hin*dIII/*Xba*I, lane 8-*Pst*I/*Sal*I, lane 9-*Pst*I/*Xba*I, lane 10-*Sal*I/*Xba*I. The two photographs show the same gel after two different run times.

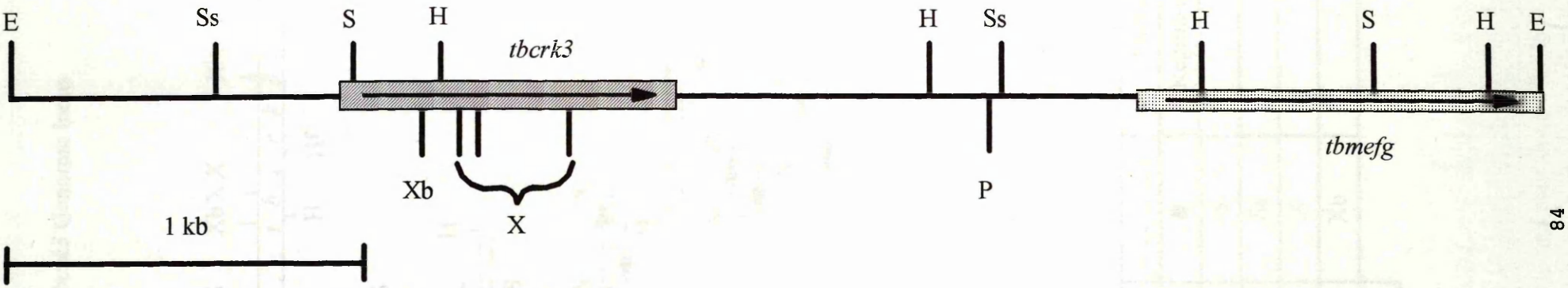
mapping showed no *Bam*HI, *Bgl*II, *Cla*I, *Eco*RV, *Not*I, *Sma*I, *Sst*I or *Sst*II restriction sites within the insert. The 4.4 kb *Eco*RI insert was mapped with *Hin*dIII, *Pst*I, *Sal*I and *Xba*I (Figure 3.3.3). Further mapping using *Ssp*I and *Xho*I was also performed, however the precise number and positions of *Xho*I sites within the 1.4 kb *Hin*dIII fragment were not known until the coding region was sequenced [Figure 3.3.4 is a map of pCD70E(S)8].

3.3.4 Sequencing the *tbcrk3* ORF.

A number of overlapping fragments from the area containing the ORF were subcloned into pBS to facilitate sequencing (Figure 3.3.5). The only difficult region was the 50 bp *Xho*I fragment within the *tbcrk3* gene. To allow the sequencing of the non coding strand of this fragment a *Hin*fI digest of pTgCD70H3, followed by end filling with Klenow fragment, was performed. After a second digest with *Xba*I the products were run through a 1.8 % TAE agarose gel and the required band extracted with the Geneclean protocol. This was then ligated into pBS SK+ that had been digested with *Eco*RV and *Xba*I.

The overlapping subclones were sequenced using the various primers that hybridise to the pBS MCS. The second strand of the far 5' and 3' termini of the *tbcrk3* ORF were sequenced by designing oligonucleotides to hybridise to the sequence obtained from the first strand. This was due to a lack of useful cloning sites in these regions (Figure 3.3.5). The complete ORF of 936 bp encodes a protein of 311 amino acids and a termination codon (Figure 3.3.6). There is a stop codon in frame with the probable start ATG, 18 bp upstream.

Figure 3.3.4 Restriction map of the pCD70E(S)8 insert.

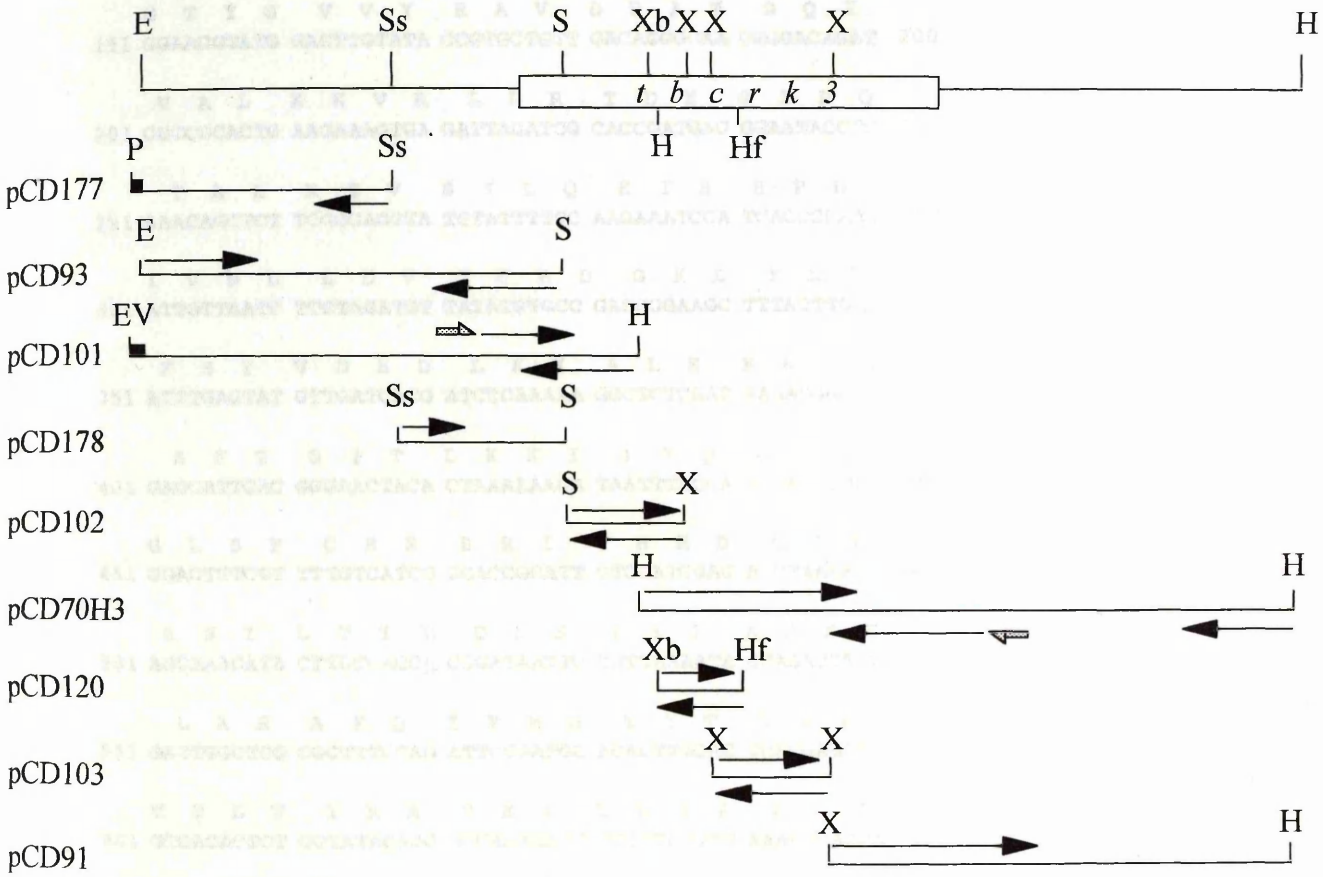


E	*Eco*RI		Ss	*Ssp*I
H	*Hin*dIII		X	*Xho*I
P	*Pst*I		Xb	*Xba*I
S	*Sal*I			

tbcrk3 ORF and direction of transcription.

tbmefg ORF and direction of transcription.

Figure 3.3.5 Sequencing the *tbcrk3* Genomic locus



→	Sequenced DNA	⌐¬	Subcloned Region
◁	Oligonucleotide	■	Region of BS polylinker
E	*Eco*RI	S	*Sal*I
EV	*Eco*RV	Ss	*Ssp*I
H	*Hin*dIII	X	*Xho*I
Hf	*Hin*fI (not the only site)	Xb	*Xba*I
P	*Pst*I		

Figure 3.3.6 Coding strand DNA and the predicted protein sequence of *tbcrk3*

```
                                                    *  V  K  G
1      TTTTGTCTCTGTTTTCCTCTTAAGTTCTTCAGGAAACGTTAGGTTAAAGG   50

     G  D  M  T  M  L  G  A  L  T  G  R  Q  L  S  S  G
51   AGGAGATATG ACAATGCTTG GGGCGTTAAC CGGTCGACAA CTTTCCTCTG  100

     L  K  D  Q  F  D  R  Y  N  R  M  D  I  L  G  E
101  GTCTTAAGGA TCAGTTCGAC CGCTATAATC GAATGGACAT ACTTGGAGAA  150

     G  T  Y  G  V  V  Y  R  A  V  D  R  A  T  G  Q  I
151  GGAACGTATG GAGTTGTATA CCGTGCTGTT GACAGGGCAA CGGGACAGAT  200

     V  A  L  K  K  V  R  L  D  R  T  D  E  G  I  P  Q
201  CGTCGCACTG AAGAAAGTGA GATTAGATCG CACCGATGAG GGAATACCTC  250

     T  A  L  R  E  V  S  I  L  Q  E  I  H  H  P  N
251  AAACAGCTCT TCGGGAGGTA TCTATTTTGC AAGAAATCCA TCACCCCAAC  300

     I  V  N  L  L  D  V  I  C  A  D  G  K  L  Y  L  I
301  ATTGTTAATC TTCTAGATGT TATATGTGCC GATGGGAAGC TTTACTTGAT  350

     F  E  Y  V  D  H  D  L  K  K  A  L  E  K  R  G  G
351  ATTTGAGTAT GTTGATCATG ATCTCAAAAA GGCTCTCGAG AAGAGGGGTG  400

     A  F  T  G  T  T  L  K  K  I  I  Y  Q  L  L  E
401  GAGCATTCAC GGGAACTACA CTAAAAAAGA TAATTTACCA GCTACTCGAG  450

     G  L  S  F  C  H  R  H  R  I  V  H  R  D  L  K  P
451  GGACTTTCGT TTTGTCATCG GCACCGCATT GTCCATCGAG ACCTAAAACC  500

     A  N  I  L  V  T  T  D  N  S  V  K  I  A  D  F  G
501  AGCAAACATA CTTGTCACCA CGGATAATTC CGTTAAAATA GCAGACTTTG  550

     L  A  R  A  F  Q  I  P  M  H  T  Y  T  H  E  V
551  GATTGGCTCG CGCTTTCCAG ATTCCGATGC ACACTTACAC TCACGAGGTT  600

     V  T  L  W  Y  R  A  P  E  I  L  L  G  E  K  H  Y
601  GTCACACTCT GGTATAGAGC GCCAGAGATT CTCCTCGGTG AAAAGCACTA  650

     T  P  A  V  D  M  W  S  I  G  C  I  F  A  E  L  A
651  CACCCCGGCA GTGGATATGT GGAGCATTGG TTGTATTTTT GCTGAACTAG  700

     R  G  K  V  L  F  R  G  D  S  E  I  G  Q  L  F
701  CTCGAGGTAA GGTACTCTTT CGTGGTGACA GTGAGATTGG ACAGTTATTT  750

     E  I  F  Q  V  L  G  T  P  M  D  A  E  G  S  W  L
751  GAGATTTTTC AGGTGCTTGG CACCCCAATG GATGCTGAGG GGTCGTGGTT  800

     G  V  S  S  L  P  D  Y  R  D  V  F  P  K  W  S  G
801  GGGGGTGTCG TCTCTTCCAG ACTATCGTGA CGTCTTTCCA AAGTGGAGTG  850

     K  P  L  T  Q  V  L  P  T  L  D  G  D  A  V  D
851  GAAAACCCCT CACTCAGGTG TTACCAACGC TTGACGGTGA TGCTGTTGAT  900

     L  L  S  Q  M  L  R  Y  N  P  A  E  R  I  S  A  K
901  CTGCTTTCTC AGATGCTGAG GTATAACCCT GCTGAACGTA TTTCAGCCAA  950

     A  A  L  Q  H  P  W  F  S  D  A  M  F  *
951  GGCGGCACTG CAACATCCGT GGTTTAGTGA TGCCATGTTTTAGATCATTATA 1002
```

Figure 3.3.6 The sequence of *tbcrk3* was obtained from overlapping regions of DNA subcloned from pCD70E(S)8. The DNA was sequenced on both strands to ensure accuracy. In frame stop codons and possible initiation codons are underlined. The predicted amino acid is given over the first base in each codon.

3.3.5 Analysis of the protein sequences of TbCRK1-3.

The predicted TbCRK3 protein contains all 15 of the conserved residues indicative of a serine/threonine kinase (Figure 3.3.7) (Hanks and Quinn, 1991). The alignment of the three translated *T. brucei crk* genes with yeast and human CDC2 related proteins (Figure 3.3.7) shows that the TbCRK proteins share the same conserved regions as other members of this family. e.g. the ATP binding region surrounding T-14 and Y-15, the PSTAIR box, and the regions either side of T-161 (HsCDC2 numbering). The TbCRK proteins also show the same regions of low conservation; e.g. S-93 to V-117 (HsCDC2 numbering) and most of the C-terminal 70 amino acids. There are notable differences however, including the N-terminal extensions of TbCRK2 and 3, and the 6 and 5 residue insertions near the C-terminus of TbCRK1 (Figure 3.3.7).

Table 3.3.1 shows the level of amino acid conservation between the kinetoplastid CRK proteins, fission yeast cdc2 and budding yeast CDC28 proteins, and the family of human CDC2 related protein kinases. For TbCRK1 and TbCRK2 the highest levels of identity are 56 % and 52 % respectively. TbCRK1 is approximately equally conserved to HsCDC2, HsCDK3 and HsCDK5 (54 %, 55 % and 56 % respectively). TbCRK2 has a generally lower level of identity with the highest scores being 52 % (HsCDK2) and 50 % (Spcdc2 and ScCDC28). For TbCRK3 the most similar human protein is HsCDK2 (59 %). Even this high level of identity though is notably lower than most recognised cdc2 homologues. Organisms as widely diverged as *S. pombe* and humans have cdc2 proteins that are 65 % identical. *S. cerevisiae* CDC28 though is only 60 % identical to HsCDC2, so the figures for the TbCRK proteins do not rule out the possibility of their being the functional trypanosome homologue.

Figure 3.3.8 shows 4 of the most highly conserved regions of the CDC2/CDK2 family of proteins with the equivalent regions of the kinetoplastid CRK proteins for comparison. The most obvious variation is the lack, in any of the CRK proteins, of a totally conserved PSTAIR box, a region implicated in cyclin binding (Pines and Hunter,

Figure 3.3.7 Comparisons of the TbCRK family with CDC2 related proteins

```
                                                                                *
HsCDC2      .......... .......... .......... .......... ..MEDYTKIE
HsCDK2      .......... .......... .......... .......... ..--NFQ-V-
Spcdc2      .......... .......... .......... .......... ..--N-Q-V-
ScCDC28     .......... .......... .......... .........MS GELAN-KRL-
TbCRK1      .......... .......... .......... .......... .MGSR-ERLQ
TbCRK2      MQVQVQEGQT ACDGSLRPLP SAGPASFVPR SLRPAPLRGT STPDR-SR--
TbCRK3      .......... .......... ...MTMLGAL TGRQLSSGLK DQFDR-NRMD

            * k* k   k              *     k    **  * *  * *  * k**
HsCDC2      KIGEGTYGVV YKG...RHKT TGQVVAMKKI RLESEEEGVP STAIREISLL
HsCDK2      ---------- --A...---L --E---L--- --DT-T---- ----------
Spcdc2      ---------- --A...---L S-RI------ ---D-S---- ----------
ScCDC28     -V-------- --ALDL-PGQ GQR---L--- -----D---- ----------
TbCRK1      -----S---- FRA...-DV- --TI--V-R- ---K------ C-------I-
TbCRK2      -V---S--I- --C...HDNF --RT----R- P-IVNDG--- ---V--V---
TbCRK3      IL-------- -RA...VDRA ---I--L--V --DRTD--I- Q--L--V-I-

            **            *
HsCDC2      KEL....RHP NIVSLQDVLM QD.SRLYLIF EFLSMDLKKY LDSI..PPGQ
HsCDK2      ---....N-- ---K-L--IH TE.NK---V- ---HQ----F M-A...SALT
Spcdc2      --VNDENNRS -C-R-L-I-H AE.-K---V- ---D------ M-R-SETGAT
ScCDC28     ---....KDD ---R-Y-IVH S-AHK---V- ---DL---R- MEG-..-KD-
TbCRK1      ---.....-E ---R-L--CH SE.K--T-V- -CME------ MDH....V-G
TbCRK2      R--....N-- YV-R-L--VL HE.AK-L--- -YMEQ--QGM -KQ....RNT
TbCRK3      Q-I....H-- ---N-L--IC A-.GK----- -YVDH----A -EK....R-G

                                  * *    k  k *k              k
HsCDC2      YMDSSLVKSY LYQILQGIVF CHSRRVLHRD LKPQNLLIDD K.GTIKLADF
HsCDK2      GIPLP-I--- -F-L---LA- ---H------ --------NT E.-A------
Spcdc2      SL-PR--QKF T--LVN-VN- -----II--- ---------K E.-NL-----
ScCDC28     PLGADI--KF MM-LCK--AY ---H-I---- --------NK D.-NL--G--
TbCRK1      DL-AGTIQEF MRSL-S-VR- --E-N----- ---P----SR E.KEL-----
TbCRK2      AFVGGKLRRI MF-L-L-LHE -----FV--- I--S-I---R -ESVV-----
TbCRK3      AFTGTTL-KI I--L-E-LS- --RH-IV--- ---A-I-VTT D.NSV-I---

            k  *  *    * ****        *kk  †    *** k        k
HsCDC2      GLARAFGIPI RVYTHEVVTL WYRSPEVLLG SARYSTPVDI WSIGTIFAEL
HsCDK2      -------V-V -T-------- ---A--I--- -KY---A--- --L-C----M
Spcdc2      ----S--V-L -N----I--- ---A------ -RH---G--- --V-C----M
ScCDC28     -------V-L -A----I--- ---A------ GKQ---G--T ----C----M
TbCRK1      --G------V KKF-Q----- -----D---- -TQ-G----- --V-C----M
TbCRK2      --G---RV-L QT--T--M-- ---A------ DKQ-LPA--V --M-CV----
TbCRK3      ------Q--M HT-------- ---A--I--- EKH-TPA--M ----C-----
```

```
                 † † †*  **                          *          * *   *
HsCDC2      ATKKPLFHGD SEIDQLFRIF RALGTP..NN EVWPEVESLQ DYKNTFPK..
HsCDK2      V-RRA--P-- ---------- -T----..DE V---G-T-MP ---PS---..
Spcdc2      IRRS---P-- ----EI-K-- QV----..-E ----G-TL-- ---S---R..
ScCDC28     CNR--I-S-- -----I-K-- -V----..-E AI--DIVY-P -F-PS--Q..
TbCRK1      -IGA---T-K NDA---L--- QF----..-R Q---SMDTYP NSS-MLSRPE
TbCRK2      -RRRS--A-- TA-N---S-- QL----..TE AT-RG-T--P HHNVN--R..
TbCRK3      -RG-V--R-- ---G---E-- QV----MDAE GS-LG-S--P --RDV---..

                                                       k *
HsCDC2      ....WKPGSL ASHVK..... NLDENGLDLL SKMLIYDPAK RISGKMALNH
HsCDK2      ....-ARQDF SKV-P..... P---DGRS-- -Q--H---N- ---A-A--A-
Spcdc2      . ..--RMD- HKV-P..... -GE-DAIE-- -A--V----H ---A-R--QQ
ScCDC28     ....-RRKD- SQV-P..... S--PR-I--- D-L-A---IN ---ARR-AI-
TbCRK1      FQQTLAATCE EQFQTNPAYA K-GPQ-I--- RWL-R-E-SE -LTAAQ--E-
TbCRK2      ....-TAKP- RTA-P..... A--DD-V--- RR--C-N-RE --TAYE--Q-
TbCRK3      ....-SGKP- TQVLP..... T--GDAV--- -Q--R-N--E ---A-A--Q-


HsCDC2      PYFNDLDNQI KKM
HsCDK2      -F-Q-VTKPV PHLRL
Spcdc2      N-LR-FH
ScCDC28     ---QES
TbCRK1      ---SVEF
TbCRK2      S--DEVREEE VEKLMRFNG
TbCRK3      -W-S-AMF
```

Figure 3.3.7 Pileup comparison of CDC2 related genes from: Hs-*Homo sapiens* (human), Sp-*Schizosaccharomyces pombe* (fission yeast), Sc-*Saccharomyces cerevisiae* (budding yeast) and Tb-*Trypanosoma brucei.*

* - Residues exposed to Solvent.

† - Residues involved in suc1/CKS binding.

Italics - Regions implicated in suc1/CKS binding.

Bold - Residues involved in cyclin binding.

k - Residues conserved throughout the family of serine/threonine kinases.

Table 3.3.1 Conservation between members of the Kinetoplastid CRK family and CDC2 related proteins from Humans and yeast.

	TbCRK1	TbCRK2	TbCRK3	HsCDC2
TbCRK1		47	47	54
TbCRK2	47		53	49
LmmCRK1	72	48	47	56
HsCDC2	54	49	54	
HsCDK2	52	52	59	65
HsCDK3	55	49	56	65
HsCDK4	44	45	48	44
HsCDK5	56	48	51	57
HsCDK6	47	43	47	47
HsPCTAIR-1	50	40	45	52
SpCDC2	53	50	52	65
ScCDC28	52	50	52	60

Table 3.3.1 The figures shown are the percentage identity of the predicted protein sequences for the respective gene. Tb - *T. brucei*, Lmm - *Leishmania mexicana*, Hs - *H. sapiens*, Sp - *Schizosaccharomyces pombe*, Sc - *Saccharomyces cerevisiae*.

Figure 3.3.8 Kinetoplastid CRK protein conservation at CDK control motifs.

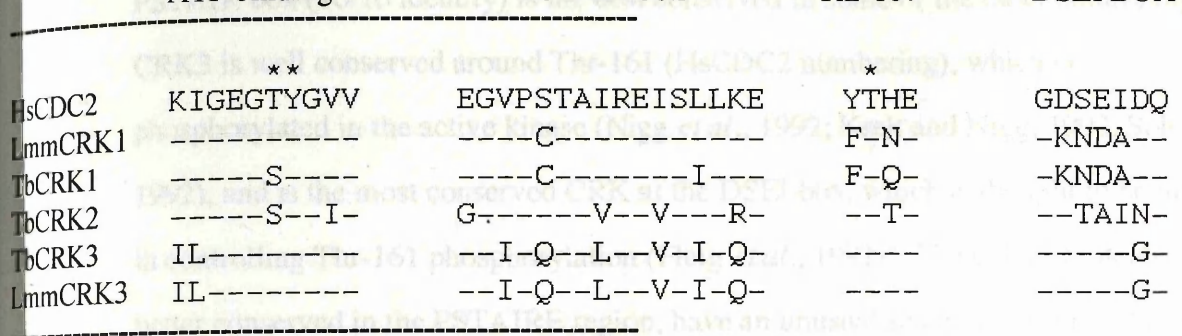


Figure 3.3.8 The predicted protein sequences from the five Kinetoplastid *crk* genes were compared to the predicted sequence of the Human CDC2 kinase subunit in four regions involved in control of kinase activity (see Chapter 3.5.2).

* labels a residue phosphorylated during the cell cycle.

\- shows amino acids identical to the HsCDC2 sequence.

1989; Ducommon *et al.*, 1991b). Unexpectedly, CRK3, which has the least conserved PSTAIR box (10/16 identity) is the best conserved in some of the other control regions. CRK3 is well conserved around Thr-161 (HsCDC2 numbering), which is phosphorylated in the active kinase (Nigg *et al.*, 1992; Krek and Nigg, 1992; Solomon, 1992), and is the most conserved CRK at the DSEI box, which is thought to be involved in controlling Thr-161 phosphorylation (Fleig *et al.*, 1992). The CRK1 proteins, though better conserved in the PSTAIRE region, have an unusual sequence around Thr-161 and are entirely missing the DSEI motif, while TbCRK2 has intermediate conservation in these areas.

The lack of a pattern of conserved, or not conserved, residues continues through the sequences (Figure 3.3.7). Of the residues involved in cyclin binding, TbCRK1 has considerably better conservation with the human and yeast CDC2/CDK2 genes (one conservative change out of seven amino acids) than do either of TbCRK2 (three non-conservative changes) or TbCRK3 (two conservative, two non-conservative). The three *T. brucei* proteins show many alterations of the amino acids implicated in suc1/cks1 binding, however many of these residues are also non-conserved between the human and yeast sequences. A substitution common to the TbCRK family, not seen in other CDK proteins is Aspartic acid for Histidine-23 (HsCDC2 numbering) which is within a region believed to be involved in suc1 binding. TbCRK2 and 3 also have Arg-22 subtituted by Histidine and Valine respectively. In the surface area between R-180 and D-186, implicated in the suc1 interaction, all of the TbCRK proteins have substitutions of S-182, and TbCRK2 and 3 both have the T-183 replaced by a proline residue. TbCRK2 and 3 also have substitutions at R-214 (to serine and glutamic acid respectively) and all three TbCRK proteins have a lysine to glutamic acid alteration at amino acid 274. Many of the other solvent accessible residues are also not conserved by the TbCRK proteins. These changes include:

for TbCRK1, S121E, R123N, Q132P, K238S and P242S;

for TbCRK2, R36P, Q132S, G154R, R158Q, P229R, D236H and K237N;

for TbCRK3, S121R, Q132A, G154Q, R158H and P229L.

All of these residues are perfectly conserved in the yeast and human CDC2/CDC28/CDK2 proteins. Several other surface residues that are conserved in the CDC2/CDK2 proteins are conservatively substituted in the TbCRK proteins.

All three of the TbCRK proteins have substitutions at residues required for checkpoint control in *S. pombe* (Basi and Enoch, 1996). TbCRK1 has the change E8Q, TbCRK2 has N62Y, and TbCRK3 has both E8D and K9I substitutions.

The above data suggest that none of the TbCRK proteins is well enough conserved to be clearly the homologous protein to CDC2 in other organisms. Certainly it is very unlikely that any of the *tbcrk* genes would complement temperature sensitive mutants of the yeast *cdc2/CDC28* genes, a functional assay commonly used. All have mutations in important regulation sites, and it would seem likely that none of the proteins would interact correctly with components of the cell cycle regulation complex from other organisms.

In a further attempt to ascertain the relationship of the 3 trypanosome CRK proteins to the various members of the CDC2 related protein kinase family a dendrogram of the protein sequences was computed. A selection of proteins representative of the various classes were analysed by the PAUP programme (Swofford, 1991), and bootstrap probabilities calculated for the branches of the shortest tree found (Felsenstein, 1985). PHO85 from *S. cerevisiae* was used as the outgroup, as at the time it was thought to be a related cyclin dependent protein kinase with no direct cell cycle function (Kaffman *et al.*, 1994). It has now been shown to be involved in the G1 phase of the *S. cerevisiae* cell cycle, binding a G1 cyclin, and is essential for passage through G1 in the absence of the CDC28 pathway (Espinoza *et al.*, 1994; Measday *et al.*, 1994). The consensus shortest tree found using PHO85 as an outgroup is shown in Figure 3.3.9. Changing the outgroup to another, more divergent, CDC2 related kinase would be unlikely to alter the result that none of the *T. brucei* CRK's are found within the branch containing the CDC2/28 proteins and the HsCDK2/3 and DmCDC2c proteins. Within this branch the 2 yeast proteins diverge first, followed by the split into CDC2 (G2/M control) and CDK2/3 (G1/S control) groups.

Figure 3.3.9 Dendrogram of the TbCRK family and other CDC2 related kinases.

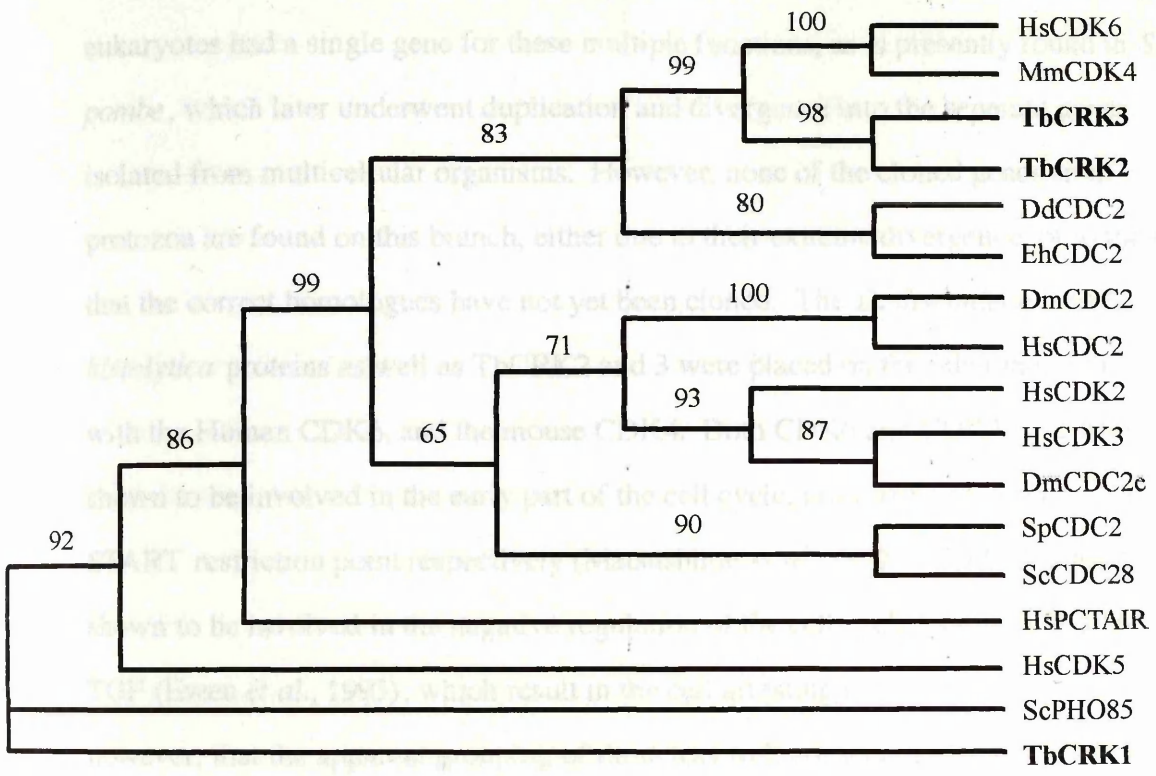


Figure 3.3.9 The dendrogram was calculated by the PAUP (Phylogenetic Analysis Using Parsimony) computer program using the entire open reading frames of each protein sequence, but extensions and insertions were effectively ignored for this comparison. The numbers show the percentage of bootstrap support for each branch.

Dd - *Dictyostelium discoideum*, Dm - *Drosophila melanogaster*, Eh - *Entamoeba histolytica*, Hs - *Homo sapiens*, Mm - *Mus muscularis*, Sc - *Saccharomyces cerevisiae*, Sc - *Schizosaccharomyces pombe*, Tb - *Trypanosoma brucei*,

One interpretation of this data is that the joint ancestor of yeasts and the higher eukaryotes had a single gene for these multiple functions, as is presently found in *S. pombe*, which later underwent duplication and divergence into the seperate genes isolated from multicellular organisms. However, none of the cloned genes from protozoa are found on this branch, either due to their extreme divergence, or to the fact that the correct homologues have not yet been cloned. The *D. discoideum* and *E. histolytica* proteins as well as TbCRK2 and 3 were placed on the other major branch, with the Human CDK6, and the mouse CDK4. Both CDK6 and CDK4 have been shown to be involved in the early part of the cell cycle, prior to START and at the START restriction point respectively (Matsushime *et al.*, 1992). CDK4 has also been shown to be involved in the negative regulation of the cell cycle by anti-mitogens, e.g. TGF (Ewen *et al.*, 1993), which result in the cell arresting in G1. It seems likely however, that the apparent grouping of these less well conserved genes is due to the artifactual clustering of long branches that can occur during phylogeny analysis (Felsenstein, 1978). If the clustering of TbCRK2/3 with these G1/S Phase active kinases is due to a role in G1/S phase control instead of an artifact of the evolutionary divergence then one, or both of TbCRK2/3 could be regulators of the adaptive, G1 cell cycle block in short, stumpy form and/or metacyclic *T. brucei.*

The TbCRK1 protein appears as an outgroup in this analysis, along with proteins which have been shown to have non-cell cycle specific activities. PHO85 is a cyclin dependant kinase involved in controlling the transcription of genes involved in phosphate metabolism (Kaffman *et al.*, 1994), as well as having an apparently redundant role in G1 cell cycle control. The 2 different roles are controlled by association with different cyclins. The PHO85/PHO80 complex can phosphorylate the transcription factor PHO4 resulting in repression of transcription of the secreted acid phosphatase PHO5, while the PHO85/HCS26 or PHO85/OrfD kinase complexes have been found to be essential for passage through G1 when the CDC28/CLN complexes are disrupted (Espinoza *et al.*, 1994; Measday *et al.*, 1994). By analogy it is possible that LmmCRK1/TbCRK1 may play similarly multiple roles, explaining the lack of

relation between LmmCRK1 kinase activity and cell cycle status in *L. mexicana* (Mottram *et al.*, 1993).

CDK5 may also have multiple roles. The protein has been found at low levels in immunoprecipitates with the G1 cyclins, D1 and D3, but no kinase activity was detected (Xiong *et al.*, 1992). The gene is expressed at higher levels in neuronal tissue, and the kinase activity of the CDK5 containing complexes increases with increasing cellullar differentiation. e.g. the highest levels of kinase activity are found in non-dividing cells (Lew *et al.*, 1992; Hisanaga *et al.*, 1992; Helmich *et al.*, 1992). The CDK5 complexes in brain contain an activating subunit with very little homology to cyclins. Unusually, the two subunits, when expressed in *E. coli* and purified, can form an active complex without the regulatory modifications necessary in other CDK systems. Purified CDK5 complexes phosphorylate neurofilament proteins and the tau protein, and *in vivo* co-localisation studies show CDK5 associated with the neurofilaments. The roles of the members of the PCTAIR subfamily is unknown.

The alignments of the TbCRK proteins, and their percentage identity comparisons with other CDK's, are inconclusive in determining which, if any, of the 3 isolated trypanosome genes is the *cdc2* homologue. An accepted test for functional homology is the ability of a CRK to complement yeast temperature sensitive mutants of *cdc2/28* (Hirayama *et al.*, 1991; Paris *et al.*, 1991; Jimenez *et al.*, 1990; Lee and Nurse, 1987). Although lower than the conservation between the yeast and metazoan homolgues of CDC2/28, the TbCRK/yeast level of conservation (50-59 %) is not in itself a barrier to complementation. The *Dictyostelium discoideum cdc2* encoded protein (DdCDC2) displays a similar (61 %) level of identity to yeast CDC2/28 and human CDC2 and is able to weakly complement a *CDC28* temperature sensitive mutant of *S. cerevisiae* (Michaelis and Weeks, 1992a). DdCDC2 also contains conservative changes in the PSTAIR box and the DSEI motif. However, the level of conservation of DdCDC2 is far higher than that shown by TbCRK2 or TbCRK3 in the PSTAIR region, and higher than TbCRK1 and TbCRK2 at the DSEI motif. The TbCRK proteins' low

level of conservation in the motifs shown to be neccessary for CDC2 function means that complemention of yeast cdc2 mutants is unlikely to succeed (see Chapter 5.2).

The use of a dendrogram to assign probable roles to the TbCRK proteins is problematic. Fission yeast has so far been shown to have only one *cdc2* related gene involved in cell cycle control, while budding yeast also has PHO85 as a redundant G1/S CDK. As they branched from the eukaryotic tree long after the Kinetoplastida (Sogin *et al.*, 1986; Sogin, 1991) this means that either the yeasts have lost genes that were present in the joint ancestor or that the vertebrate gene family results from gene duplications that occurred after the divergence from yeast. It seems unlikely that modern yeast require less control over the cell cycle than the primitive ancestor of fission yeast, budding yeast and higher eukaryotes, and so it might be expected that the second explanation is correct. If the second explanation is correct, then any similarity between different TbCRK proteins and classes of human CDK's may be artifactual and not related to shared function. There is the possibility of parallel evolution to result in shared sequence motifs for equivalent functions, i.e. a gene duplication occured on the kinetoplastid branch, followed by specialisation of the extra gene (*tbcrk3*) as a G1/S CDK, and a similar event also occured on the metazoan branch. For this to result in proteins that are apparently related, the substrates for the G1/S CDK, and the regulatory proteins of the G1/S CDK (eg Wee1, CDC25, CIP1, etc) would have needed to share significant homology even after the amount of evolutionary time equivalent to the distance between mammals and the ancestral ancestor of Kinetoplastida and metazoans. Therefore, it is probable that the family of protozoan CRK's are not directly analogous to the isolated mammalian proteins, and the functions of the TbCRK proteins will have to be determined individually. An example of conserved function not being mediated by a conserved gene can be seen in the CDK Activating Kinase (CAK) activity in yeast and metazoans. In these organisms the CDC2/cyclin complex is phosphorylated on T-161 (HsCDC2 numbering) to regulate activity, but the kinases responsible in either *S. cerevisiae* or *S.pombe*/metazoans are not highly homologous at the sequence level.

Although at present the large family of CDKs isolated from mammals has not been duplicated in other organisms, all multicellular organisms examined including plants (Hashimoto *et al.*, 1992; Hirayama *et al.*, 1991) and invertebrates (Lehner and O'Farrell, 1990; Sugaya *et al.*, 1994) have multiple CDK proteins, and in the vertebrate, *Xenopus laevis*, there are reports of CDC2, CDK2 (Eg1), CAK (MO15/CDK7), and a member of the PCTAIR family (Paris *et al.*, 1991; Fesquet *et al.*, 1993; Poon *et al.*, 1993). It seems likely that the large family of CRKs in multicellular organisms is a result of the need to tightly regulate division and differentiation, as unregulated growth would be disastrous, as would uncontrolled differentiation. If these gene duplications did occur as a result of the evolution of multicellular organisms, then the multiple genes isolated from protozoa are due to unrelated events and individual selection pressures. This leads to the expectation that each early branch of the eukaryotes is likely to have its own set of *cdc2* related genes, without clear homologies between them, except in closely related organisms, e.g. no *crk1-3* homologues have so far been isolated from any other protozoa exept the closely related *T. congolense*, which appears to have a *crk1* related gene (reviewed Mottram, 1994). Notably, although CRK1 and CRK3 are conserved between *T. brucei* and *L. mexicana*, the expected leishmanial homologue of *tbcrk2* has not been isolated by PCR, and a 39 kDa protein has not been detected by cross reaction with either PSTAIR reacting antisera or antisera raised to TbCRK2. These reagents would be expected to detect a putative LmmCRK2 protein (See Chapter 4).

There is as yet no pattern of homologous proteins within the protozoa. Each species, or family, seems to have its own group of more or less unique CDC2 related proteins, e.g. *Entamoeba histolytica* (Lohia and Samuelson, 1993), *Plasmodium falciparum* (Ross-McDonald *et al.*, 1994), *T. cruzi* (reviewed Mottram, 1994), *Crithidia fasciculata* (Brown *et al.*, 1992) and *Theileria annulata* (Kinnaird *et al.*, 1996). All of these genes share ~50 % identity with each other and with the CDC2 family from the multicellular organisms. Presumably this is due to the large evolutionary distance between each member of the protozoa resulting in the

conservation of only the essential residues of the ancestral CRK protein. This is further evidence that the duplications and specialisation of CRK's within the various protozoan lineages happened after branching from the major eukaryote line, and therefore it will not be possible to assign roles to CRK's by analogy with other organisms. All of these protozoan genes have been cloned by PCR, and as yet none have been shown to complement yeast temperature sensitive mutants of *cdc2/28*, neither have they been shown by other means (e.g. mutagenesis, synchronisation) to be invoved in control of the cell cycle.

It is possible that the *CDC2* homologue in *T. brucei* has not yet been cloned. Further PCR reactions have been tried by J. C. Mottram with CDC2 related kinase specific oligonucleotides, and one other gene has been cloned (Personal communication). This complements other evidence for the presence of further kinetoplastid CRK proteins. Western blots of protein extracts from kinetoplastids using PSTAIR reactive antisera often show more bands than the number of isolated CRK genes from that organism (see Chapter 4.4). A histone H1 kinase activity from *Leishmania*, associated with the division status of the life cycle stage, can be bound to the *S. pombe* p13suc1 protein (Mottram *et al.*, 1993), which has been shown to bind tightly to CDC2 related proteins from a variety of organisms. It is possible that this protein is LmmCRK3 (J. Mottram, personal communication) expressed at a very low level. This would suggest that the CRK3 proteins are likely to be at least involved in the execution of cell cycle specific modifications of proteins, even if they are not the CDC2 homologue responsible for cell cycle regulation.

3.4 Partial Cloning of the *T. brucei Mitochondrial Elongation Factor G* gene (*tbmefg*).

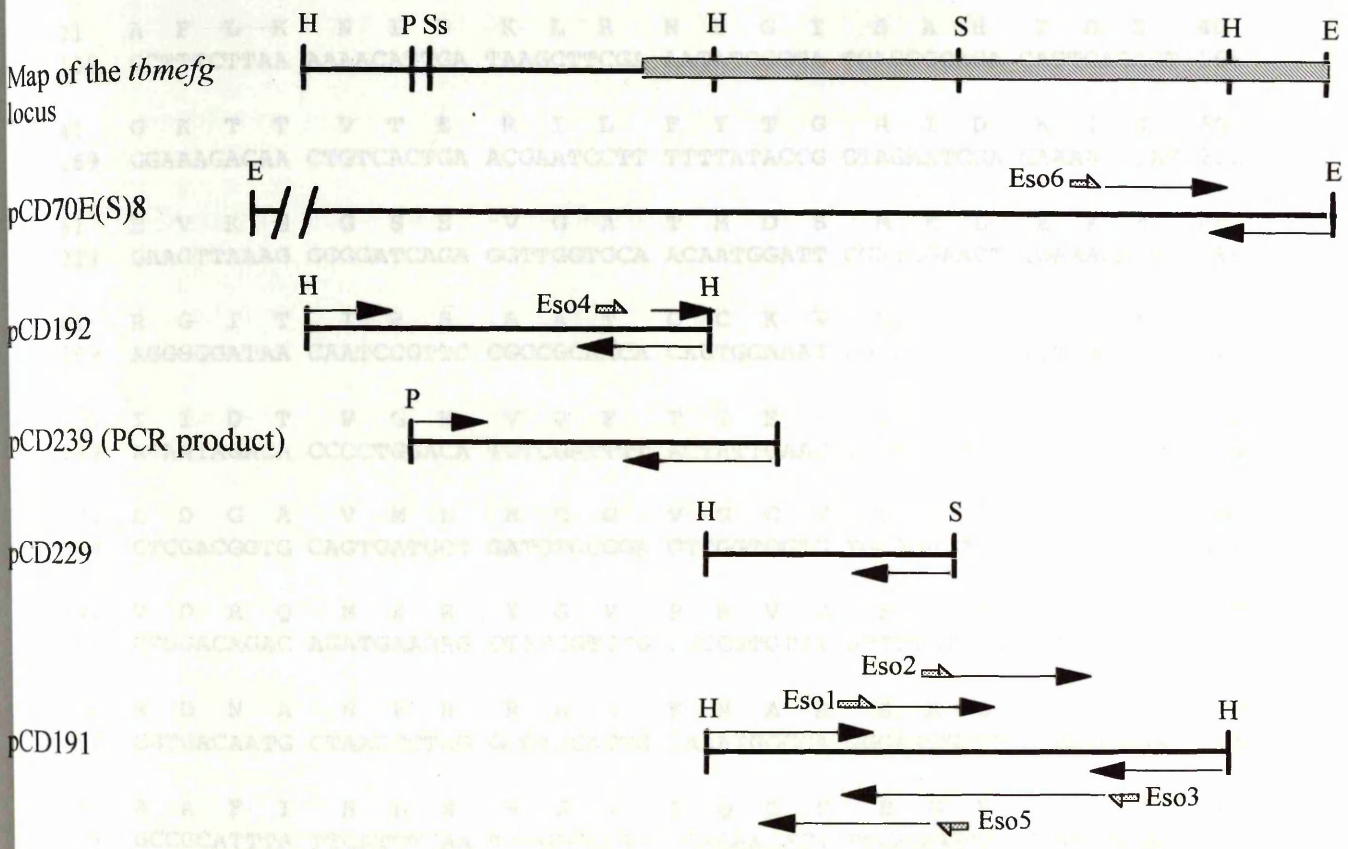
3.4.1 Cloning and Sequencing *tbmefg*.

When mapping and subcloning the pCD70E(S)8 plasmid the 0.9 kb and 0.7 kb *Hin*dIII fragments downstream of the *tbcrk3* ORF (Figure 3.3.4) were also ligated into pBS SK+, and the ends of the inserts sequenced. The predicted translation product of one end of the 0.9 kb fragment (pCD191) showed a high level of homology to the Elongation Factor G (EF-G) proteins of *S. cerevisiae* and eubacteria. EF-G is a GTP binding protein that controls the translocation of the eubacterial ribosome during translation of the mRNA. It has also been found in *S. cerevisiae,* where it is nuclear encoded, and then targetted to the mitochondria (Vanbutas *et al.*, 1991). The equivalent protein in eukaryotic cytoplasmic translation is designated EF-2, and shows limited homology to EF-G. The rest of the ORF that was within pCD70E(S)8 was sequenced by designing oligonucleotides that hybridised to the ends of previously sequenced areas (Figure 3.4.1). The rest of the gene, predicted to be approximately another 900 bp encoding 300 aa may be present on CD47.2 but has not been subcloned to date.

3.4.2 Comparisons with other EF-G proteins.

The partial sequence of the *tbmefg* gene encoded 450 amino acids at the N-terminus of the protein (Figure 3.4.2). This was compared to that of the predicted protein sequences for the reported mitochondrial EF-G proteins, from *S. cerevisiae* (Vanbutas *et al.*, 1991) and rat (Barker *et al.*, 1993), and eubacterial EF-G proteins using the pileup programme (Figure 3.4.3). The level of conservation of these proteins is high, except at the extreme amino terminus. There is a 58 amino acid extension at the N-terminus in the yeast protein. The extension is 17 aa in TbmEF-G, and is not present in eubacterial proteins. The extension is expected to be a mitochondrial

Figure 3.4.1 Sequencing *tbmefg*



	Sequenced DNA		Subcloned Region
	Oligonucleotide		*tbmefg* ORF
E	*Eco*RI	S	*Sal*I
H	*Hin*dIII	Ss	*Ssp*I
P	*Pst*I		

Figure 3.4.2 The DNA and predicted protein sequence of *tbmefg*

```
1                                                    TTTGTCTATATACCTATTTTACTGTGCCTTTTCAATCCGTAGTCTGAG 48

1   M  R  R  F  C  D  V  V  F  Y  P  N  R  F  Y  R  L  A  S  S  20
49  ATGAGGAGGT TTTGTGATGT CGTCTTTTAT CCGAACCGAT TTTACCGATT GGCGTCGTCG 108

21  A  F  L  K  N  I  D  K  L  R  N  I  G  I  S  A  H  I  D  S  40
109 GCTTTCTTAA AAAACATTGA TAAGCTTCGA AATATCGGTA TCAGCGCACA CATTGACAGT 168

41  G  K  T  T  V  T  E  R  I  L  F  Y  T  G  R  I  D  K  I  H  60
169 GGAAAGACAA CTGTCACTGA ACGAATCCTT TTTTATACCG GTAGAATCGA CAAAATCCAC 228

61  E  V  K  G  G  S  E  V  G  A  T  M  D  S  M  E  L  E  K  E  80
229 GAAGTTAAAG GGGGATCAGA GGTTGGTGCA ACAATGGATT CGATGGAACT TGAAAAAGAA 288

81  R  G  I  T  I  R  S  A  A  T  Q  C  K  W  G  D  H  L  I  N  100
289 AGGGGGATAA CAATCCGTTC CGCCGCAACA CAGTGCAAAT GGGGTGATCA TTTGATTAAT 348

101 I  I  D  T  P  G  H  V  D  F  T  I  E  V  E  R  A  L  R  V  120
349 ATAATAGACA CCCCTGGACA TGTCGATTTT ACTATTGAAG TGGAACGTGC CCTTCGTGTC 408

121 L  D  G  A  V  M  L  M  C  G  V  G  G  V  Q  S  Q  T  L  T  140
409 CTCGACGGTG CAGTGATGCT GATGTGCGGA GTCGGTGGTG TGCAGAGTCA GACCCTTACG 468

141 V  D  R  Q  M  K  R  Y  G  V  P  R  V  C  F  I  N  K  L  D  160
469 GTGGACAGAC AGATGAAGAG GTATGGTGTG CCCCGTGTTT GTTTTATCAA TAAACTAGAT 528

161 R  D  N  A  N  P  R  R  A  L  E  M  A  R  E  R  L  G  V  N  180
529 CGTGACAATG CTAACCCTAG GCGTGCATTG GAAATGGCGA GGGAACGCTT GGGGGTGAAT 588

181 A  A  F  I  H  L  N  M  G  V  A  Q  D  F  E  G  V  V  D  V  200
589 GCCGCATTTA TTCATCTCAA TATGGGAGTT GCACAAGACT TTGAAGGTGT TGTCGACGTC 648

201 I  E  S  R  A  V  Y  F  D  G  K  N  G  E  K  I  R  F  E  D  220
649 ATAGAGTCCC GTGCTGTATA CTTTGACGGT AAAAATGGGG AAAAGATCCG ATTTGAGGAT 708
```

```
221  I  P  S  Y     I  A  D     D  V  V     A  T  R  K     E  L  I     S  R  L   240
709  ATACCAAGCT ACATTGCCGA TGACGTGGTG GCAACACGAA AGGAGCTTAT ATCAAGATTG 768

241  A  D  C  D     A  E  M     E  D  V     F  L  N  D     V  E  P     T  A  E   260
769  GCTGACTGTG ATGCCGAAAT GGAAGATGTT TTCTTAAACG ATGTGGAGCC TACAGCAGAG 828

261  Q  I  H  S     A  I  R     R  T  T     I  A  N  K     F  V  P     V  L  V   280
829  CAGATTCACT CAGCGATTCG GCGCACCACG ATTGCAAACA AGTTTGTGCC TGTGTTAGTG 888

281  G  S  A  Y     K  N  K     G  I  Q     L  L  L  D     A  V  C     R  Y  L   300
889  GGTTCAGCCT ACAAGAACAA AGGCATACAA CTTCTTCTTG ATGCCGTCTG TCGTTACCTA 948

301  P  S  P  M     E  K  P     N  S  G     Y  S  V  T     K  V  K     D  D  E   320
949  CCTTCCCCAA TGGAGAAGCC AAATTCGGGT TACTCGGTGA CCAAGGTAAA GGACGATGAA 1008

321  G  N  V  A     N  V  K     G  E  I     V  P  L  A     T  D  D     E  K  P   340
1009 GGTAACGTTG CCAACGTTAA GGGCGAGATC GTTCCTTTAG CGACTGATGA CGAAAAACCT 1068

341  L  V  A  A     I  F  K     L  E  E     T  K  K  T     G  L  L     N  Y  I   360
1069 CTCGTGGCAG CTATATTTAA GCTTGAGGAA ACGAAGAAAA CCGGCCTTCT TAATTACATT 1128

361  R  V  Y  Q     G  K  M     R  R  E     H  L  L  N     V  R  S     G  K  T   380
1129 CGTGTTTACC AGGGTAAAAT GAGAAGGGAA CATTTATTGA ATGTTCGTAG CGGAAAAACG 1188

381  F  L  P  Q     K  L  V     R  M  H     A  N                                 392
1189 TTTCTTCCTC AGAAGCTGGT CCGTATGCAC GCGAATTC                                   1226
```

Figure 3.4.2 The region of *tbmefg* that had been subcloned was sequenced on both strands to ensure accuracy, with ITP used to resolve compressions. The amino acid encoded for each codon is above the first base of each triplet.

targetting signal in the eukaryotic proteins, and as in this case, these signalling peptides are usually very poorly conserved (Hendrick *et al.*, 1989).

The percentage identities given in Table 3.4.1 were calculated by comparing the predicted polypeptides over the region from the start of the eubacterial EF-G to the end of the available TbmEF-G sequence, therefore ignoring the N terminal extensions. TbmEF-G is more similar to the *S. cerevisiae* mEF-G than to either of the eubacterial proteins. In fact the 2 eukaryotic proteins share 52 % identity over this region, while only having 43-46 % identity to the bacterial EF-Gs, which have 59 % identity to each other.

A dendogram of the TbmEF-G protein with other reported sequences was computed (Figure 3.4.4). The sequences used included several eubacterial proteins, mitochondrial proteins from both rat and *S. cerevisiae*, and the partial sequence from the gene isolated from *Leishmania mexicana* which is expected to be the homologue of *tbmefg* (J. Mottram, unpublished results). It also includes a possible chloroplast EF-G from Soybean (Torres *et al.*, 1993) and, as an outgroup, the protein (EF-2) which performs the equivalent function in the archaebacterium *Methanococcus vannielii*. It is immediately obvious that, as expected, the mitochondrial EF-G proteins form a separate group from the bacterial proteins, and that the Kinetoplastida proteins are more similar to each other than they are to either of the other eukaryote mEF-Gs.

3.4.3 Discussion of the Trypanosome mEF-G.

The *T. brucei* EF-G gene isolated is highly conserved, with 46 % identity to the equivalent *E. coli* protein over the 433 amino acids presently predicted (Table 3.4.1). As EF-G is expected to be essential, and it interacts with a large number of proteins in the ribosome and other parts of the translational machinery, it is under a high degree of functional constraint, which is presumably the reason for the high degree of conservation present. It has been found that the *L. mexicana crk3* gene also has a downstream ORF that is also homologous to other *EF-G* genes and the N-terminus of

Figure 3.4.3 Comparison of TbmEF-G with the *S. cerevisiae* homologue and bacterial EF-G proteins

```
          1                                                  50
TbEF-G.   ... ..... ......... ......... ......... .MRRFCDVVF
ScEF-G    MSVQKMMWVP RKMVGGRIPF FTCSKVFSGF SRRSFHESPL ARSTYEEEKV

         51                                                 100
TbEF-G    YPNRFYRLAS SAFLKNIDKL RNIGISAHID SGKTTVTERI LFYTGRIDKI
ScEF-G    LVDEIKQKLT PDDIGICN-- ---------- -----F---V -Y--K--KA-
EcEF-G    .......... .-RTTP-ARY ---------- A----T---- -----VNH--
TaEF-G    ........MA VKVEYDLKR- -----A---- A----T---- -Y-----H--

        101                                                 150
TbEF-G    HEVKGGSEVG ATMDSMELEK ERGITIRSAA TQCKWG.... D...HLINII
ScEF-G    ---R-RDN-- -K----D--R -K----Q--- -Y-S-DKEGK N...YHF-L-
EcEF-G    G--HD-...A ----W--QEQ ------T--- -TAF-SGMAK QYEP-R----
TaEF-G    G--HE-...A ----F--Q-R ------TA-V -T-F-KD... ....-R----

        151                                                 200
TbEF-G    DTPGHVDFTI EVERALRVLD GAVMLMCGVG GVQSQTLTVD RQMKRYGVPR
ScEF-G    -----I---- ---------- ---LVV-A-S ------V--- ---R--N---
EcEF-G    ---------- ----SM---- ----VY-A-- ---P-SE--W --ANK-K---
TaEF-G    ---------- ----SM---- --IVVFDSSQ --EP-SE--W --A-K-K---

        201                                                 250
TbEF-G    VCFINKLDRD NANPRRALEM ARERLGVNAA FIHLNMGVAQ DFEGVVDVIE
ScEF-G    -T----M--M GSD-F--IEQ LNSK-KIP-- AVQIPV-SES SLS----L-N
EcEF-G    IA-V--M--M G--FLKVVNQ IKT---A-PV PLQ-AI-AEE H-T----LVK
TaEF-G    IA-A--M-KT G-DLWLVIRT MQ----ARPV VMQ-PI-RED T-S-II--LR

        251                                                 300
TbEF-G    SRAV.YFDGK NGEKIRFEDI PSYIADDVVA TRKELISRLA DCDAEMEDVF
ScEF-G    RV-I.-NK-D ---I-EKGPV -ENLKPLMEE K-QL--ET-- -V-D--AEM-
EcEF-G    MK-INWN-AD Q-VTFEY--- -ADMVELANE WHQN--ESA- EASE-LMEKY
TaEF-G    MK-YTYGN.D L-TD--EIP- -EEYL-QARE YHEK-VEVA- -F-ENIMLKY

        301                                                 350
TbEF-G    LNDVEPTAEQ IHSAIRRTTI ANKFVPVLVG SAYKNKGIQL LLDAVCRYLP
ScEF-G    -EEK---TQ- -KD----S-- -RS-T---M- --LA-T---P V---IVD---
EcEF-G    -GGE-L-EAE -KG-L-QRVL N-EIIL-TC- --F----V-A M----ID---
TaEF-G    -EGE---E-E LVA---KG-- DL-IT--FL- --L----V-- -----VD---

        351                                                 400
TbEF-G    SPMEKPNSGY SVTKVKDDEG NVANVKGEIV PLATDDEKPL VAAIFKLEET
ScEF-G    N-S-VL-TAL D-SN...... .....NEAK- N-VPAVQQ-F -GLA-----G
EcEF-G    --VDV-AI.. ........N- ILDDG-DTPA ERHAS-D--F S-LA--IATD
TaEF-G    --LDI-PI.. ........K- TTPEG-..V- EIHP-PNG-- A-LA--IMAD

        401                                                450+
TbEF-G    KKTGLLNYIR VYQGKMRREH LL.NVRSGKT FLPQKLVRMH AN    +++
ScEF-G    .-Y-Q-T-V- ----RL-KGN YIT--KT--K VKVAR----- SS    +++
EcEF-G    PFV-N-TFF- --S-VVNSGD TVL-SVKAAR ERFGRI-Q-- --    +++
TaEF-G    PYV-R-TF-- --S-TLTSGS YVY-TTK-RK ERVAR-L--- --    +++
```

Figure 3.4.3 Comparison of predicted EF-G protein sequences from; Tb - *T. brucei*, Sc - *Saccharomyces cerevisiae*, Ec - *E. coli*, Ta - *T. aquaticus*.

- identical amino acid to the TbEF-G predicted residue.

· Gap in the sequence for alignment purposes.

Table 3.4.1 Conservation between *T. brucei* mEF-G, yeast mEF-G and eubacterial EF-G.

	ScmEF-G	EcEF-G	TaEF-G
TbmEF-G	52 (69)	46 (64)	46 (64)
ScmEF-G		43 (66)	45 (64)
EcEF-G			59 (76)

Table 3.4.1 The predicted protein sequences of the *efg* genes were compared using the GAP program. Sc - *S. cerevisiae* , Ec - *E. coli* , Ta - *T. aquaticus* , Tb - *T. brucei.* The area compared was just the region of TbmEF-G that has been sequenced, and also did not include the N-terminal extensions of the eukaryotic proteins. The first figure in each box is the percentage identity, the figure in brackets is percentage similarity.

Figure 3.4.4 Dendrogram of the putative Kinetoplastida mitochondrial Elongation Factor-G protein with EF-G proteins from other organisms.

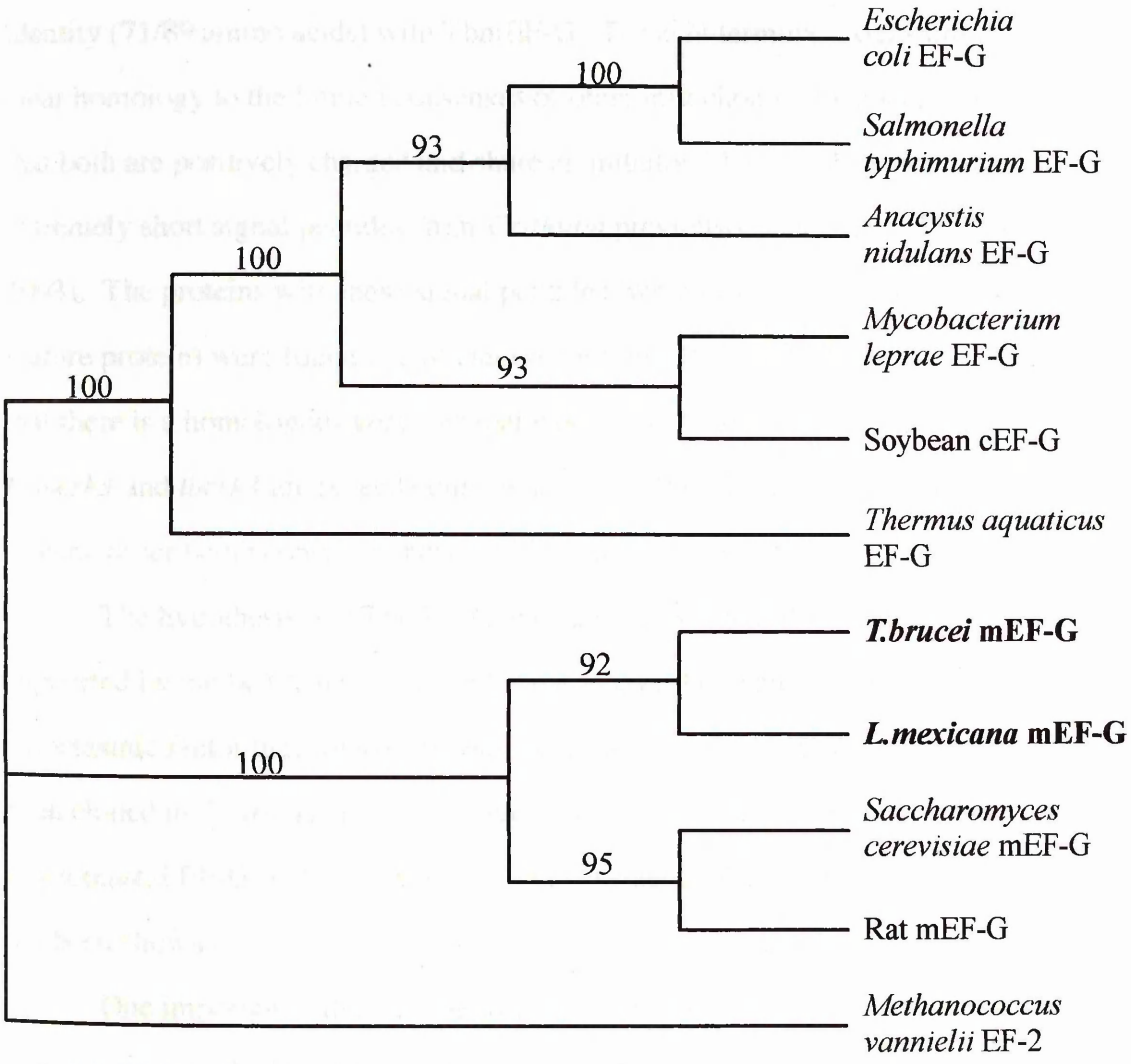


Figure 3.4.4 The dendrogram shows the relationship between the putative Kinetoplastida mitochondrial Elongation Factor-G proteins and other mitochondrial EF-G proteins (mEF-G), a chloroplast EF-G (cEF-G), bacterial EF-Gs and the archaebacterial homologue (EF-2). The numbers represent the bootstrap support for each branch point as a percentage.

the *L. mexicana mefg* has been sequenced (J. Mottram, unpublished results). The predicted protein has an N-terminal extension of the same length as TbmEF-G, but this is poorly conserved. Within these 20 residues only 5 are identical, including the initiating methionine. The rest of the sequenced gene gives a protein that shares 80 % identity (71/89 amino acids) with TbmEF-G. These N-terminal extensions show no clear homology to the limited consensus of other mitochondrial signal peptides, except that both are positively charged and share an initiator-Met-Arg-Arg motif with the extremely short signal peptides from *Crithidia* previously characterised (Xu and Ray, 1993). The proteins with these signal peptides (which were apparently cleaved from the mature protein) were found in association with the DNA of the kinetoplast. The fact that there is a homologous gene downstream of *tbcrk3* and *lmmcrk3* is evidence that *lmmcrk3* and *tbcrk3* are indeed equivalent genes. It is also an, at present rare, example of gene order being conserved between diverged species within the Kinetoplastida.

The hypothesis that TbmEF-G and LmmEF-G are mitochondrial proteins is supported by the fact that all other eukaryotes have the related protein, EF-2, as the cytoplasmic factor that controls ribosomal translocation. However, this protein has not been cloned in *T. brucei* or *L. mexicana*, and mitochondrial targetting of the kinetoplastid EF-G proteins, or proteolytic cleavage of the N-terminal extension, has not been shown.

One important difference between the EF-G and the EF-2 proteins is that EF-G does not contain the histidine residue responsible for EF-2's susceptibility to Diptheria toxin. This toxin causes the ribosylation of the conserved histidine in EF-2 resulting in the halting of translation. The rat *efg* gene has been isolated (Barker *et al.*, 1993), and inhibition experiments with Diptheria toxin imply that this EF-G protein is part of the mitochondrial translation machinery only, as no cytosolic translation could be detected when cells were treated with the toxin. I am unaware of any attempts to block kinetoplastid translation by Diptheria toxin. If the experiment is possible (the toxin requires an activation step while crossing the plasma membrane into the cell, which

may not occur in the trypanosome), and cytosolic translation is blocked, then it is likely that the *efg* genes isolated are only involved in mitochondrial translation.

3.5 Discussion.

The successful cloning of the *tbcrk* genes, using PCR hybridising to conserved domains of CDC2 related kinases shows that the isolation of trypanosome genes homologous to those in other organisms is possible when the functional constraints, and therefore selection, acting on a protein are high. By analogy with CDK's from other organisms, it is expected that the CRK proteins must interact with several other proteins in order to function correctly, and loss of activity would be lethal unless there were functional redundancy, or unless the product was only needed during specific life cycle stages. If there was redundancy, the fact that *S. cerevisiae* has retained an extremely high level of redundancy within the CDK/cyclin complexes, implies that if not lethal, the decrease in viability caused by CDK loss of function may result in selection against the mutated organism in the enviroment, even though cultured forms may show no effect on growth or viability. For instance, bloodstream form *T. brucei* that contain no kinetoplast (dyskinetoplastic) can be isolated, and grown, in culture (Agbe and Yielding, 1994), but only low levels (1-2 %) of such organisms have been found *in vivo*. Dyskinetoplastic strains of *T. evansi*, a parasite which only exists in the bloodstream form, have been isolated (Ou et al., 1991), although most strains still retain the kinetoplast. Equally, even if the protein was only essential in a specific life cycle stage, the loss of the encoding gene would result in an organism unable to complete the life cycle, and so unable to be transmitted by the normal routes of infection, as occurs is the case with *T. equiperdum*.

Although *CDC2* related genes were isolated, it should be noted that the *tbcrk* genes are notably more diverged from the *cdk* genes of other eukaryotes than other *CDC2* related genes previously found, probably due to the extreme evolutionary

distance, and possibly also the high level of selection undergone by parasites. For instance, the region most commonly used to define a CDC2 related kinase, the PSTAIR box, is not well conserved in either TbCRK2 or TbCRK3, and therefore oligonucleotides designed to anneal to the PSTAIR box encoding region might not have isolated *tbcrk2* or *tbcrk3*.

This variation in which regions are homologous, and the failure to clone a kinetoplastid cyclin, shows how important it is to choose the correct area for oligonucleotide design, especially when proteins are not expected to have perfectly conserved domains. A cyclin-like gene from *T. brucei* was successful cloned by PCR (Affranchino *et al.*, 1993) using only one cyclin specific oligonucleotide. The second oligonucleotide was homologous to the Spliced Leader sequence, and the template used for the reactions was cDNA. This putative cyclin has an unusual structure with a possible destruction box situated towards the C terminus of the protein, and very little predicted protein sequence N-terminal to the cyclin box. The level of homology within the cyclin box between TbCYC1 and either A or B type cyclins from other organisms, is considerably lower than usual, with a large number of both gaps and insertions necessary for alignment. The number of cyclins now isolated is far higher than in 1990, and the regions that are well conserved between widely diverged species are not necessarily those chosen at the start of this project. With hindsight, the divergence of the CRK PSTAIR regions implies that the kinetoplastid cyclins are likely to be notably different from cyclins isolated from other organisms, as this region appears to be involved in CDK/Cyclin complex formation (Pines and Hunter, 1989). The unusual nature of the *T. brucei* cyclin is probably going to be matched by other components of the kinetoplastid cell cycle machinery, e.g. the activating kinase CAK, Wee1, and CDC25 homologues, but it is expected that these proteins will be conserved enough to be recognised as the *Leishmania mexicana* homologue of the *S. pombe* CDC2 complex component, $p13^{suc1}$, has been successfully isolated (Mottram and Grant, 1996).

Aligning the CRK sequences with that of HsCDK2, for which a crystal structure has been achieved (De Bondt *et al.*, 1993), shows that many of the solvent accessible

residues, which are expected to be involved in most of the binding reactions with other proteins, are not conserved (see Figure 3.3.7). This includes those residues implicated in suc1/HsCKS1 binding (Bourne *et al.*, 1996; Hayles *et al.*, 1986; Ducommon *et al.*, 1991a), those in cyclin binding regions (Ducommon *et al.*, 1991a; Ducommon *et al.*, 1991b), as well as those without any known function. This lack of conservation of the CRK surface residues means that assessment of their function by interactions with components from other systems, e.g. $p13^{suc1}$, is likely to be difficult, with low affinity binding, and therefore problems with signal/noise ratios.

There are several feature that distinguish members of the CRK family. TbCRK2 has a 40 amino acid N-terminal extention with no homology to any putative signalling peptide found in *T. brucei*, either from proteins expected to be targetted to the mitochondria (Peterson *et al.*, 1993) or to those transported into glycosomes (Swinkels *et al.*, 1986; Sommer and Wang, 1994). The extentions of TbCRK3 and LmmCRK3 also have no homology to these putative signalling peptides from *T. brucei*, the N terminal extensions of kinetoplastid associated proteins from *Crithidia* (Xu and Ray, 1993), or signalling peptides from other eukaryotes. Interestingly the CRK3 extensions also show little shared sequence with each other, with only 6/20 identical residues, including the starting methionine (Figure 3.5.1). The protein sequence in the rest of the TbCRK3 and LmmCRK3 proteins is very well conserved (80 % identity over 291 amino acids), but at the residue that is normally the initiator methionine for the CDC2/CDK2/3 family, the conservation stops. The role of this extension could be structural rather than involved in targetting. Of the 6 conserved residues, 2 are glycine, and could be involved in ß-turns, and 2 are an aspartic acid and an arginine, that could form a salt bridge.

The TbCRK extensions bear no similarity to the N-terminal extensions of any of the putative mitochondrial or chloroplast EF-G proteins from other organisms, or TbmEF-G. There is therefore no evidence for either of the TbCRK extensions playing a role in synchronising the division cycles of the nucleus and kinetoplast by causing the complexes to shuttle between the cytosol and mitochondria.

Figure 3.5.1 Comparison of TbCRK3 and LmmCRK3

```
                                        *M*
TbCRK3     1  MTMLGALTGR QLSSGLKDQF DRYNRMDILG EGTYGVVYRA VDRATGQIVA
              |: .| .|.|     .| :| . ||||| |.|| |||||||||| ||:.||| ||
LmmCRK3    1  MSSFGRVTAR SGDAGTRDSL DRYNRLDVLG EGTYGVVYRA VDKITGQYVA

           51  LKKVRLDRTD EGIPQTALRE VSILQEIHHP NIVNLLDVIC ADGKLYLIFE
               |||||||||: |||||||||| |||||| || |||||||||| .|||||.||
           51  LKKVRLDRTE EGIPQTALRE VSILQEFDHP NIVNLLDVIC SDGKLYLVFE

          101  YVDHDLKKAL EKRGGAFTGT TLKKIIYQLL EGLSFCHRHR IVHRDLKPAN
               ||: |||||| ||  |.::|   ||::||||| :|| |||||| |.||||||||
          101  YVEADLKKAI EKQEGGYSGM DLKRLIYQLL DGLYFCHRHR IIHRDLKPAN

          151  ILVTTDNSVK IADFGLARAF QIPMHTYTHE VVTLWYRAPE ILLGEKHYTP
               ||.|: | .| :|||||||||| |.|||||||| |||||||||| ||||||||.|
          151  ILLTSGNVLK LADFGLARAF QVPMHTYTHE VVTLWYRAPE ILLGEKHYAP

          201  AVDMWSIGCI FAELARGKVL FRGDSEIGQL FEIFQVLGTP MDAEGSWLGV
               ||||||.||| |||||| ||| |||||||||| |||||||||| |.||||.||
          201  AVDMWSVGCI FAELARRKVL FRGDSEIGQL FEIFQVLGTP TDTEGSWPGV

          251  SSLPDYRDVF PKWSGKPLTQ VLPTLDGDAV DLLSQMLRYN PAERISAKAA
               | |||||||| |||:.| | | ||| |  ||. |||| ||:|: | |||||| |
          251  SRLPDYRDVF PKWTAKRLGQ VLPELHPDAI DLLSKMLKYD PRERISAKEA

          301  LQHPWFSDAM F
               ||||||||   :
          301  LQHPWFSDLR W
```

Amino acid 21, under the *M* symbol, is the consensus start methionine in the CDC2/CDK2/3 family of proteins. The two proteins share 80% identity over the region from residue 21 to the C-terminus.

The partial sequence of *tbmefg* clearly shows a high level of homology to genes from both eukaryotes and bacteria (Table 3.4.1). The phylogeny analysis using the partial sequences of predicted EF-G proteins does not show any unexpected results. Assuming the theory that the eukaryotic nuclear encoded *efg* genes were ancestrally part of the genomes of symbiotes which evolved into either cloroplast or mitochondria, then the chloroplast symbiotic event was, as may be predicted, different from that of the mitochondria. The data shown does not differentiate between a single symbiosis leading to Kinetoplast and higher eukaryote mitochondria, or separate symbiotic events in the different lines, as has been postulated to explain the presence of editing in the Kinetoplasts.

The high level of conservation seen in both protein and DNA comparisons between bacterial and eukaryotic *efg* genes shows again that the isolation of homologous genes which encode proteins that are both essential for *in vivo* viability and which interact with multiple other protein or RNA factors, can be achieved even when the organisms are extremely diverged.

CHAPTER FOUR
ORGANISATION AND EXPRESSION OF THE *TBCRK* GENE FAMILY

4.1 Introduction.

The three *tbcrk* genes isolated were one of the first family of CDC2-related genes cloned from a single-celled organism. We were interested to determine whether they played a role in the complex trypanosome life cycle. As explained in Chapter 1.1.2, *T. brucei* pass through stages which do not divide and which are essential to transmission between species. After transmission the non dividing forms undergo a transformation to a rapidly proliferating cell type to set up the infection.

After analysing the genomic organisation of the genes we then attempted to raise antisera to the three proteins using both conjugated peptides and fusion proteins expressed in *E. coli*. These antisera were used to characterise the expression of TbCRK1-3 in different life cycle stages.

4.2 Gene organisation.

4.2.1 Introduction.

As previously described (see Chapter 1.1.3), many of the genes isolated from trypanosomes have been found to be multicopy. These can be at one locus in a tandem array or at several locations within the genome. It was important to determine whether the *tbcrk* genes were single or multi-copy so that the attempt to create null mutants of the *tbcrk* genes via homologous recombination could be devised.

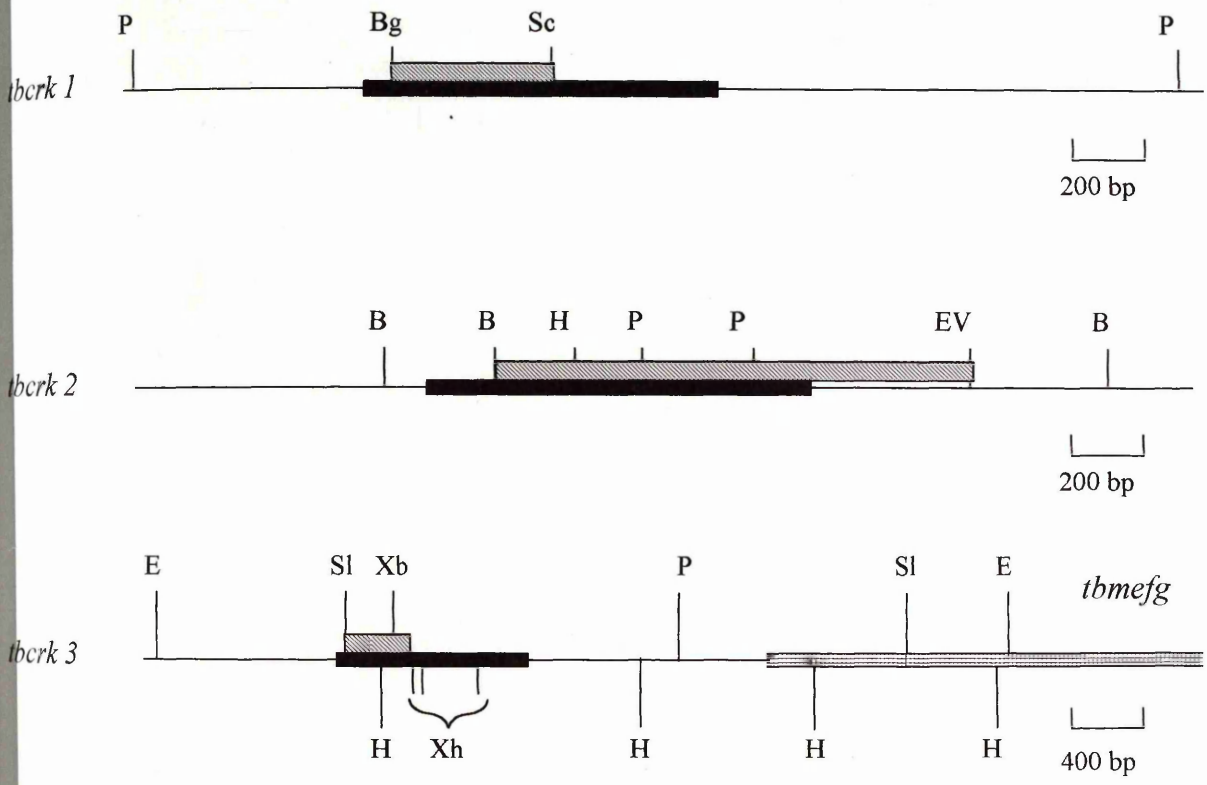
4.2.2 Organisation of the *tbcrk* gene family.

From the sequencing of the *tbcrk3* locus cloned into pBS it was clear that *tbcrk3* at least was not in a tandem array, unless pCD70E(S)8 contained the most 3' gene of such an array. Partial mapping of *tbcrk1* and *tbcrk2* had already been done, and consulting these maps (see Figure 4.2.1) DNA fragments from each open reading frame were prepared. These fragments were labelled with ^{32}-P by random priming and hybridised onto Southern blots of *T. brucei* DNA digested with different restriction enzymes (see Figure 4.2.2). Panels A, B and C are *T. brucei* DNA digested and run on a 0.8 % agarose gel in triplicate, blotted to Hybond-N and hybridised to labelled *tbcrk1*, *2* and *3* respectively. Single bands were detected for all 3 genes in all four digests, with the band size ranging from 1.8 kb to >23 kb. All the bands in each digest are of a similar intensity taking into account the reduction in transfer efficiency for large DNA molecules. There is no evidence of other lower intensity bands which would be caused by regions flanking a tandem array, the implication being that all 3 genes are single copy. The lack of any bands the same size hybridising to different probes in any of the digests suggests that the genes are not closely linked in the genome. To further address the chromosomal location of each of the genes, Pulse Field Gel Electrophoresis (PFGE) was performed with *T. brucei* chromosomes.

4.2.3 Pulse Field Gel Electrophoresis of *T. brucei* chromosomal DNA.

It is now possible to separate many of the *T. brucei* chromosomes in agarose gels using PFGE (see Chapter 1.1.3). Depending on the parameters used, the small, intermediate and many of the megabase chromosomes can be separated, although the largest are often unresolved within the compression zone and in many stocks different chromosomes migrate at the same rate. PFGE with chromosomes from STIB247 and STIB386 was run in triplicate, Ethidium Bromide stained [see Figure 4.2.3(i)] and transferred to Nylon membrane (performed by N. Buchanan and A. Tait).

Figure 4.2.1 Restriction maps of the *tbcrk1-3* genes.



B	*Bam*HI		Bg	*Bgl*II		E	*Eco*RI
EV	*Eco*RV		H	*Hin*dIII		P	*Pst*I
Sc	*Sac*I		Sl	*Sal*I		Xb	*Xba*I
Xh	*Xho*I						

P^{32} labelled fragments

tbcrk ORFs

tbmefg ORF

Figure 4.2.2 Southern blot of STIB247 genomic DNA hybridised to *tbcrk1*, *tbcrk2* and *tbcrk3* gene specific probes.

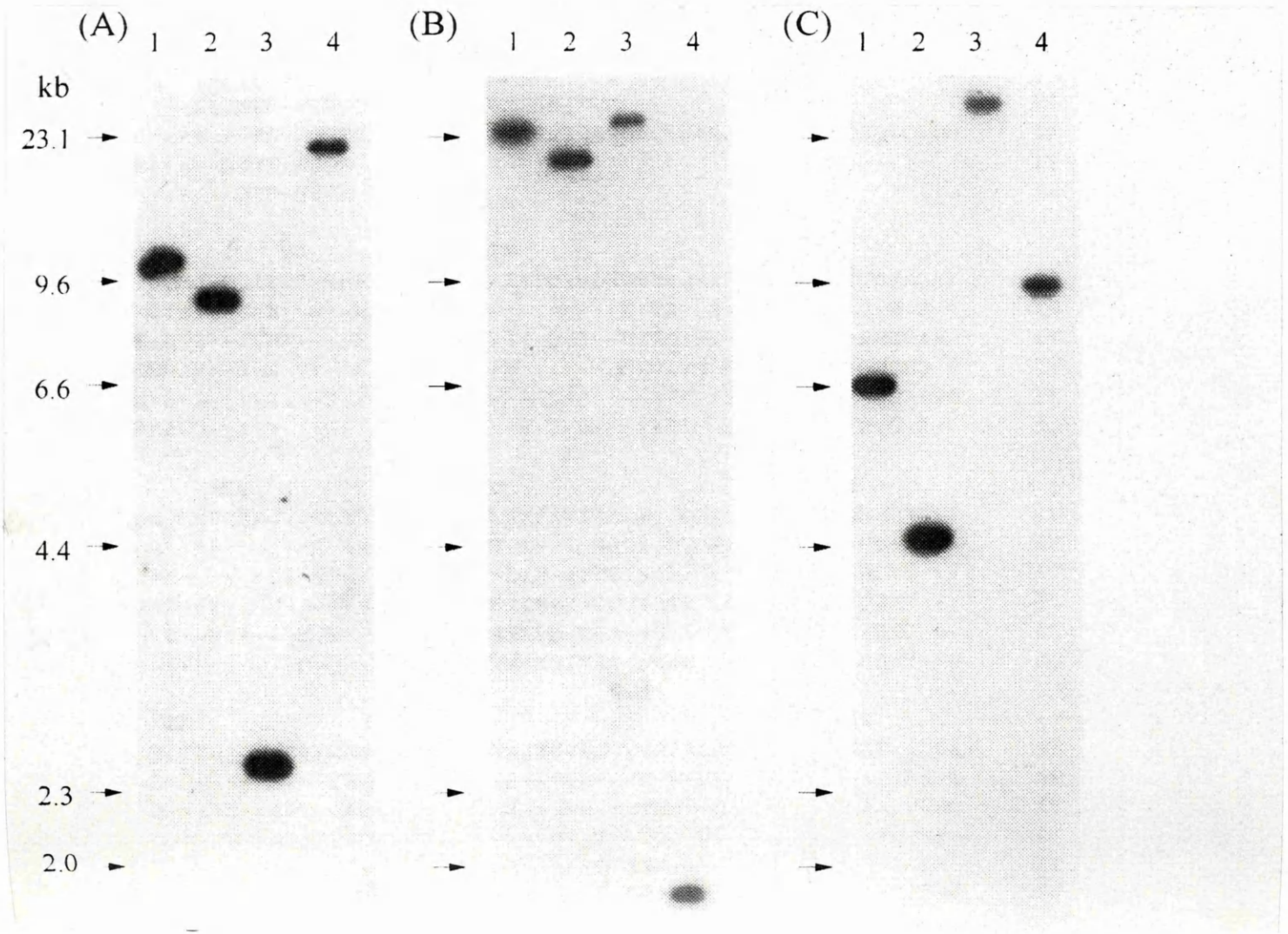


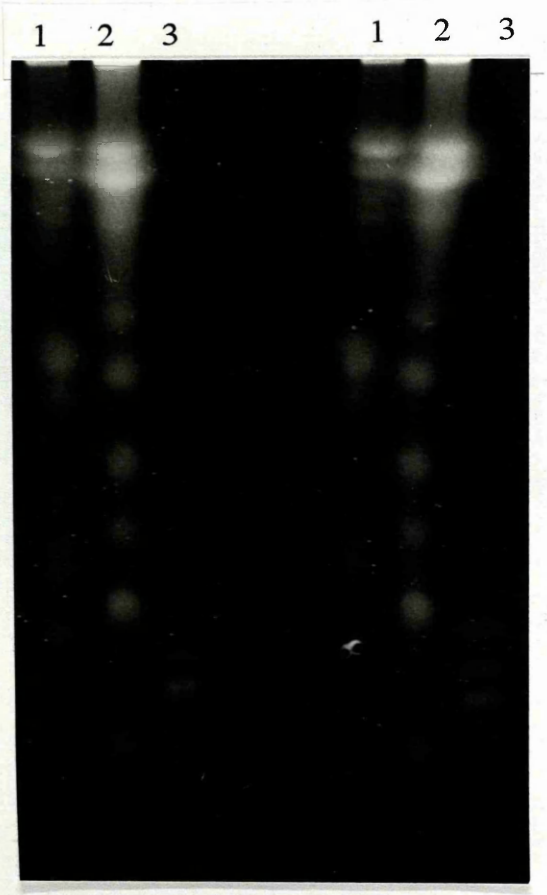
Figure 4.2.2 Southern blot of *T. brucei* genomic DNA. 7.5 μg of STIB247 genomic DNA was digested with each restriction enzyme, and 2.5 μg of this DNA run per lane through a 0.8 % TBE/agarose gel. The gel was blotted to Hybond-N and hybridised to *tbcrk* gene specific probes. Lane 1-*Xho*I, lane 2-*Eco*RI, lane 3-*Bgl*II and lane 4-*Bam*HI. Panel (A)-probed with *tbcrk1* fragment, (B)-*tbcrk2*, (C)-*tbcrk3*. See Figure 4.2.1 for labelled fragments. The hybridisation was performed overnight at 65 ^{o}C with 25 ng of P^{32} random primed probe and washed for 2 hours at 65 ^{o}C in 0.1 x SSC / 0.1 % SDS (see Materials and Methods).

The karyotype clearly varies between the two stocks with all three types of chromosome showing different patterns.

The 3 strips were then hybridised to the gene specific probes previously used [Figure 4.2.3(ii)]. The three *tbcrk* genes were located to the megabase chromosomes in both of the stocks. 2 bands in the STIB247 extract were detected hybridising to the *tbcrk2* probe (Panel B, lane 1), indicating that *tbcrk2* is located on a different chromosome to the other two genes. It is common for homologous chromosomes in *T. brucei* to be different sizes and this is likely to be the case for *tbcrk2*. In this experiment the *tbcrk2* probe also hybridised to some of the intermediate sized chromosomes. This had not been seen in previous PFGE blots, and may be due to this hybridisation being carried out in a higher salt buffer than usual, which may result in a higher level of non-specific hybridisation. It should also be noted that the *tbcrk2* probe is the only one of the three which includes some non-coding region, from the 3' untranslated region, which may be the reason for it containing DNA elements that hybridise to other parts of the genome.

Figure 4.2.3 Pulse field gel electrophoresis of *T. brucei* chromosomal DNA and hybridisation to *tbcrk1*, *tbcrk2* and *tbcrk3* gene specific probes.

(i)



(ii)

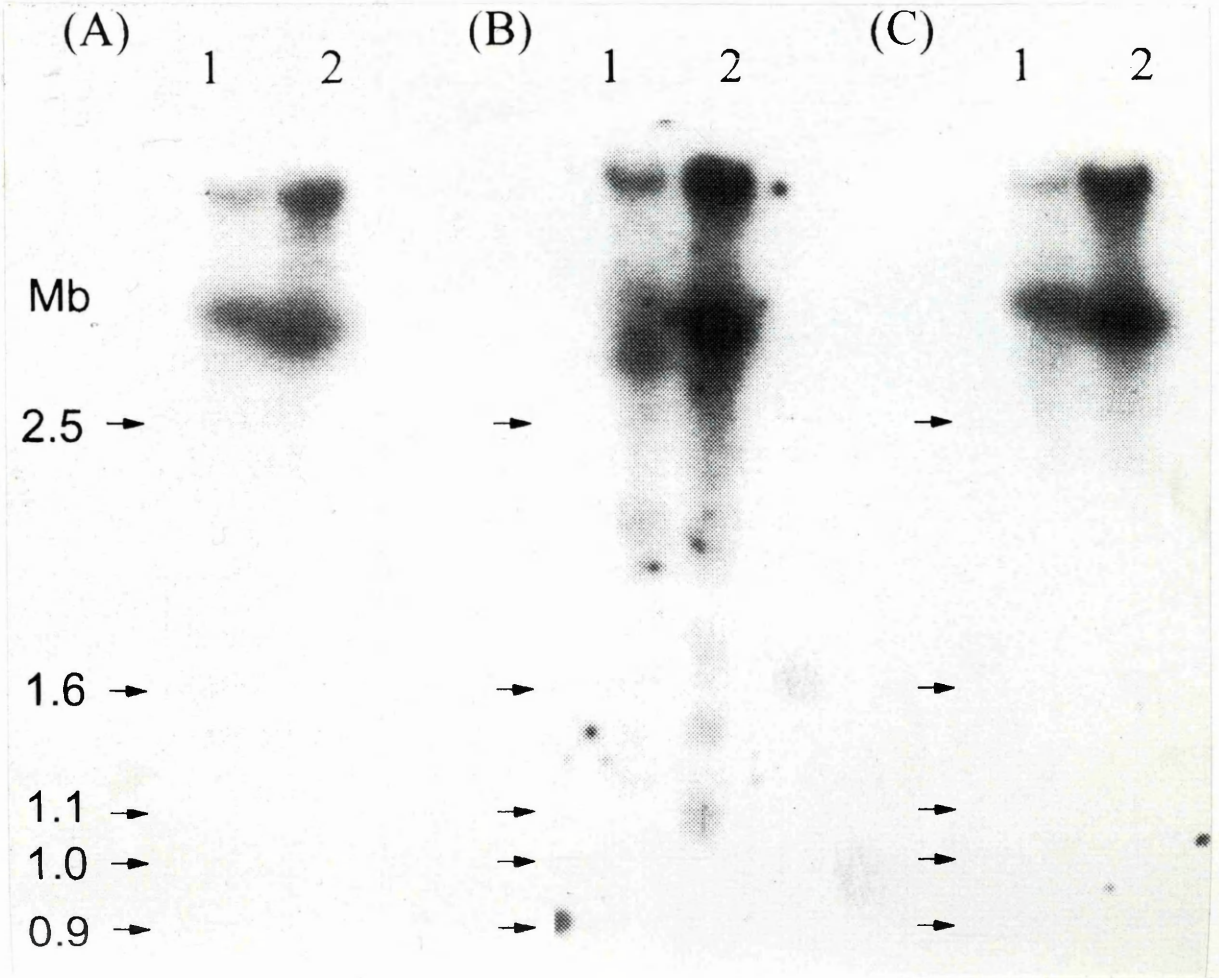


Figure 4.2.3. Panel (i) Ethidium bromide stained PFG. Lanes 1-STIB247, lanes 2-STIB386, lanes 3-*S. pombe* chromosome markers. The gel was run as described (Schweizer *et al.*, 1994), blotted to Hybond-N and hybridised as before (see Figure 4.2.2). Panel (ii) Autoradiograph of blotted PFG. Panel (A)-probed with *tbcrk1* fragment, (B)-*tbcrk2*, (C)-*tbcrk3*.

4.3 Fusion protein expression for antibody production and analysis of antisera.

4.3.1 Introduction.

To study the expression of the *tbcrk* genes it was necessary to raise antibodies, preferably specific, to each encoded protein. These proteins would also allow the assessment of how much cross-reactivity was present between the TbCRK proteins when using the available antisera. Antisera available in the laboratory (full list in Materials and Methods, Table 2.2.2.1) included Monoclonal antibodies (mAb) raised to a 16-mer oligopeptide with the sequence of the human CDC2 PSTAIR box, which were a kind gift of Y. Nagahama (Yamashita *et al.*, 1991), and had been previously shown to react to at least 2 proteins in *T. brucei* (Mottram *et al.*, 1993). Also in the laboratory were antisera which had been raised to the C-terminal 16 residues of LmmCRK1 (named CITAA), the C-terminal 16 residues of TbCRK2 (EVREE), and to the 16 amino acids of the substituted PSTAIR box of TbCRK2 (PSTAVR). These 3 antisera had previously been affinity purified using the respective oligopeptide bound to Aminolink coupling gel. It was intended that this project would raise antisera to the TbCRK3 protein.

Several attempts were made to raise specific antisera to TbCRK3. The first method tried was to generate a fusion of the *tbcrk3* PCR fragment from pCDTrpL1 with Glutathione-S Transferase. The entire *Eco*RI fragment was cloned into the correct reading frame of a pGEX vector and the junction sequenced to show that the *tbcrk3* gene fragment was in the correct orientation and in frame. The protein formed was insoluble, and so could not be purified on an affinity column. Small amounts could be made soluble by treatment with 1 % Triton X-100 and DTT but the yield was very low and unreliable. An alternative strategy was therefore adopted. An oligopeptide (Pep191) homologous to the N-terminus of TbCRK3 was synthesised commercially (see Materials and Methods). This region was chosen as the N terminal extension was, as far as we were aware, unique to this protein. It was designed to begin just after the

second methionine in the protein, as we can not be sure if translation *in vivo* starts at the first ATG.

Predicted TbCRK3 sequence MTMLGALTGRQLSSGLKDQ...
Oligopeptide Pep191 sequence CLGALTGRQLSSG

The first residue in the oligopeptide is a non coded Cysteine necessary for the manufacturing process. Several milligrams of the peptide had been linked to Keyhole Limpet Haemocyanin by the manufacturers ready for injection. Two rabbits were inoculated. Sera were taken from the rabbits after one month. They were then given booster injections and sera again taken after two weeks. These antisera were tested for cross reaction with western blots of *T. brucei* whole cell protein extracts. No proteins of approximately the correct size were detected. It was later shown that the sera from the two rabbits also did not react to a full length TbCRK3 fusion protein expressed in *E. coli* (see Figure 4.4.7). It may be that this region is very poorly immunogenic

A third method was tried to raise antisera to TbCRK3. A construct was generated containing the entire *tbcrk3* ORF tagged with 6 histidine residues at the C-terminus (see Materials and Methods) and expressed in *E. coli.* This $(His)_6$ tag allows the purification of proteins on a Ni^{2+}/agarose matrix, even in denaturing agents such as 6 M Urea, which allows the purification of proteins that are usually insoluble. The protein extract is bound to the column and then washed with buffers of either decreasing pH, or increasing concentrations of imidazole (a competitor of the histidine). Most contaminating proteins should be removed by pH 6.0 buffer or 100-200 mM imidazole. The tagged proteins typically need a pH of 5.4, for monomer elution, or 4.0 for multimer elution. The equivalent figures for imidazole are 250 and 500 mM. The binding and washing of the column has to be performed with solutions without EDTA as chelation of the nickel ions results in the complete loss of histidine binding. As the expression and purification of full length TbCRK protein would allow the assessment of antisera reaction to the protein without interference from other sources, it was decided

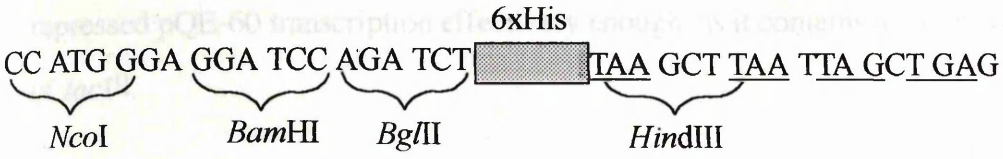
to express TbCRK1 and TbCRK2 as His-tagged proteins in *E. coli* as well for an equivalent analysis.

4.3.2 Cloning of the histidine tagged TbCRK proteins.

The expression vector pQE-60 (see Figure 4.3.1) contains restriction enzyme sites to enable the cloning of ORFs in front of 6 histidine codons followed by a termination codon. The initiator ATG is formed as part of an *Nco*I site at the 5' end of the MCS. Oligonucleotides were designed to amplify the coding region of each of the *tbcrk* genes (see Figure 4.3.1). The sequences of the oligonucleotides were altered so that the sequence coding for the starting methionine included an *Nco*I site, while at the 3' end, the stop codon was removed and replaced with either a *Bam*HI or a *Bgl*II site in the correct frame for the histidine codons. As these enzymes leave compatible overhangs the vector for all the ligations could be treated with *Nco*I and *Bam*HI in duplicate. The *tbcrk2* 3' oligonucleotide needed to use a *Bgl*II site as the gene includes a *Bam*HI site (see Figure 4.2.1). In each case the PCR, from plasmid [pCD70E(S)8 for *tbcrk3*] or lambda (for *tbcrk1* and *tbcrk2*) template, gave a single band of approximately 1 kb as expected. *Pfu* DNA Polymerase was used for the PCR as this enzyme has a 3'-5' proof-reading activity, unlike *Taq* DNA Polymerase, and so the mutation rate is twelve fold reduced (see Materials and Methods). The PCR products were purified, the DNA was digested with *Nco*I and the 3' enzyme (either *Bam*HI or *Bgl*II), and ligated into *Nco*I/*Bam*HI cut pQE-60. Transformed colonies of Amp resistant XL-1 Blue were screened by touching with a sterile toothpick, to transfer some of the colony to a 0.5 ml eppendorf tube with 20 μl of water which was then boiled for 5 min. This was then used as template for PCR with *Taq* DNA polymerase and the original oligonucleotides. Colonies thought to contain the correct insert were grown up overnight, plasmid DNA was prepared and then digested with several restriction enzymes to show that the correct gene had been inserted properly into the expression vector. It is recommended that pQE-60 recombinants are grown in a bacterial strain containing a multicopy

Figure 4.3.1 The Qiagen his-tagged expression vector and *tbcrk* oligonucleotides.

The pQE-60 Polylinker sequence



G C**CC ATG G**GG AGT CGT TAC GAG C	*tbcrk1/H* PCR 5' primer.
CGC **GGA TCC** GAA CTC GAC AGA AAA GTA T	*tbcrk1/H* PCR 3' primer.
G C**CC ATG G**AG GTG CAG GTG CAG GAA GG	*tbcrk2/H* PCR 5' primer.
CG C**AG ATC T**AG CTC CGT TGA ACC GCA TC	*tbcrk2/H* PCR 3' primer.
CC A**CC ATG G**AC CGG TAT AGC CGA ATA	*tbcrk2/H (-5'ext)* PCR 5' primer.
GA **GGA TCC** ATG ACA ATG CTT GGG GCG	*tbcrk3/H* PCR 5' primer.
GC **AGA TCT** AAA CAT GGC ATC ACT AAA	*tbcrk3/H* PCR 3' primer.
AT C**CC ATG G**AC CGC TAT AAT CGA ATG	*tbcrk3/H (-5'ext)* PCR 5' primer.

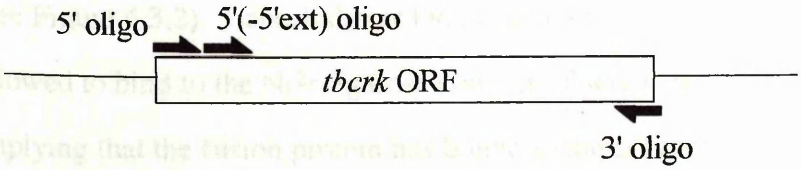


Figure 4.3.1. The oligonucleotides used to perform PCR so that the *tbcrk* genes could be cloned into pQE-60 are shown. The bases in bold are the engineered restriction sites for the in frame insertion into the pQE-60 polylinker. The underlined bases in the polylinker are the termination codons for all three reading frames.

plasmid encoding the *lac*I^{q} gene, which ensures almost complete repression of the His-tagged gene's transcription, and so minimises any toxic effects. I found this was unnecessary in the case of the *tbcrk* genes, and so to make the analysis of plasmid DNA restriction digests easier, the subcloning and expression were done in XL-1 Blue, which repressed pQE-60 transcription effectively enough, as it contains a chromosomal copy of *lac*I^{q}.

4.3.3 Expression and purification of TbCRK3/His.

Initial small scale expression screening of colonies containing the *tbcrk3/h* plasmid (pQCD236) showed that the protein was expressed and could be purified in denatured form using 6 M urea. However attempts at purification of the native form of the protein were unsuccessful. No soluble protein could be purified, but by SDS-PAGE and coomassie staining it was found that the insoluble pellet contained large amounts of a ~34 kDa protein.

The 6M urea extraction was scaled up for a 50 ml culture of cells that had been induced with IPTG for 4 hr. The extract was added to the Ni^{2+}/agarose column, washed with increasingly acidic buffer, and finally eluted with pH 4.0 buffer. 20 μl of 0.5 ml samples were boiled in SDS-Final Sample Buffer (FSB) and run on 12 % SDS-PAGE (see Figure 4.3.2). Lane 1 shows the protein remaining after the cell extract has been allowed to bind to the Ni^{2+}/agarose column. There is no intense band at 34 kDa, implying that the fusion protein has bound to the column. The next two lanes show early and late fractions from the first wash, which was performed with 5 ml of the column binding buffer B (pH8.0). The proteins washed off in this buffer (lane 2) are the proteins that do not bind to the Ni^{2+}/agarose column. Washing with buffer C (pH 6.0) eluted a large number of proteins (lanes 4-8), with a different profile to the flow through (lane 1). These are proteins that bind weakly to the Ni^{2+}/agarose matrix.

Figure 4.3.2 Coomassie stained SDS-PAGE showing purification of denatured TbCRK3/H.

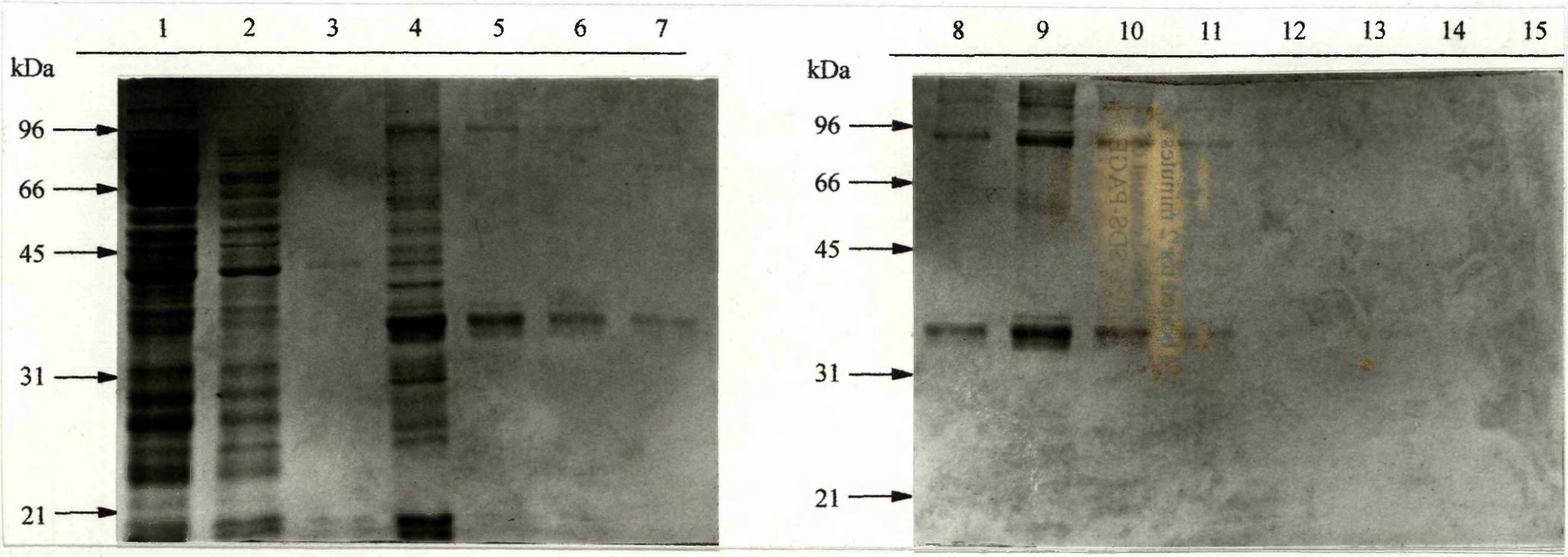


Figure 4.3.2 A 50 ml culture of induced XL-1 Blue/pQCD236 was pelleted, resuspended in 5 ml of 6 M urea, Buffer B, then spun at 10 000 g for 15 minutes at 4 ^{o}C. The supernatant was loaded onto a 0.5 ml Ni^{2+}/agarose column. This was washed with 5 ml of Buffer B. 0.5 ml fractions were collected after ~1 ml (early fraction) and ~4.5 ml (late fraction) of wash buffer had passed through the column. The Buffer C wash consisted of 5 ml collected in 9 fractions of ~0.5 ml. The bound protein was eluted with 4 ml of pH4.0 Buffer D, collected as ~0.5 ml fractions. 20 μl of the fraction to be run on the gel was boiled for 2 minutes with 5 μl of 5 x Final Sample Buffer (FSB) before loading onto 12 % SDS-PAGE.

Lanes 1-15. (1)-Extract after passage through Ni^{2+}/agarose column. (2)-Early fraction of the Buffer B wash. (3)-Late fraction of the Buffer B wash. (4-7)-Fractions 2/4/6/8 of the Buffer C wash. (8-15)-Fractions 1-8 of Buffer "D" pH 4.0 elution. For Buffers A-D see Materials and Methods.

The major band in the buffer C wash is actually the TbCRK3/H protein at 34 kDa. The elution of large amounts of TbCRK3/H in the wash buffer (Lanes 4-7) may be partially due to this buffer being a lower pH than planned when rechecked with indicator paper. However, substantial loses of protein during the washing of the column were seen with other protein purifications using other buffers. The protein coming off the column early may have been trimmed at the C-terminus, which would result in release from the matrix under less stringent conditions than usual. The purified protein seen on the coomassie stained gel does indeed consist of several closely spaced bands, but there appears to be no preferred elution of the lower bands. It is likely that most of the loss is due to the weak nature of the histidine/Ni^{2+} interaction resulting in the gradual loss of protein from the column. Treatment of the column with buffer D (pH 4.0) elutes more of the bound TbCRK3/H protein, this time in a predominantly pure form. The higher molecular weight bands that co-purify with the 34 kDa band are apparently a form of TbCRK3/H multimer, formed due to the high level of expression, that has not broken down in the FSB when boiled (see Figure 4.4.7, lane 1). The larger bands were not always seen when purified TbCRK3/H samples were run on SDS-PAGE and coomasie stained.

The protocol used above was scaled up further, and protein prepared from 500 ml of cells. The purified protein from the equivalent of lanes 8-12 was pooled, and then dialysed into lower dilutions of urea every 10-14 hr. The 6/4/2/1/0 M solutions of urea also contained 0.05 M Tris-HCl (pH 8.0). In an effort to keep the TbCRK3/H soluble, the dialysis solutions included 0.005 % Tween-20, 2 mM reduced glutathione and 0.02 mM oxidised glutathione as suggested by the QIAGEN manual. When the concentration of urea was between 4 M and 2 M the protein precipitated out of solution. The TbCRK3/H protein was prepared for injection into rabbits by centrifugation of the urea free material, at 15 000 rpm for 45 min in a microfuge. The supernatant was carefully removed and the pellet dried under a vacuum before weighing. The pellet was resuspended in dH_2O by vortexing and repeated pipetting. Some of the urea free pellet and supernatant were run on SDS-PAGE and coomassie stained. All of the TbCRK3/H

was in the pellet. Some of the TbCRK3/H was kept for analysis of antisera cross reaction (see Chapter 4.4.4).

4.3.4 Expression and purification of TbCRK1/His.

Small scale urea purifications of pQCD278.1h showed that *tbcrk1/h* was being expressed at high levels when IPTG was added to the culture medium. Unlike TbCRK3/H though, considerable amounts of a 34 kDa protein could also be seen in the supernatant of cells lysed without urea present. A native protein purification was performed on a 50 ml culture, induced for 4 hr. Instead of using buffers of varying pH for the purification, the wash and elution in this case was performed with a step gradient of 100/200/300/500 mM imidazole. 3 ml of each imidazole buffer were passed through the 0.5 ml column and 1 ml samples of the eluate taken (except for the 500 mM imidazole buffer which was collected as four 0.75 ml fractions. 20 μl of each sample were heated to 37 ^{O}C in FSB for 5min before running on 12 % SDS-PAGE (see Figure 4.3.3). The protein samples containing imidazole were not boiled as the imidazole can promote proteolytic cleavage. It appears that although the volume of Ni^{2+}/agarose was only 0.5 ml, the column is acting as if there is a 'dead space' of approximately 2 ml. This may be due to the mixing of previous buffers with the each new imidazole buffer, so only slowly raising the competitor concentration with each step of the gradient. With this interpretation Figure 4.3.3, lanes 3-5 of show that the majority of soluble proteins bound to the Ni^{2+}/agarose column can be washed off using 100 mM imidazole. The 200 mM imidazole buffer elutes most of the remaining protein (lanes 6-8), but again , the histidine tagged protein (at 34 kDa) is eluted in considerable amounts by the wash buffer. While TbCRK1/H is preferentially bound to the column, and is the major component of the protein eluted by the 300 mM imidazole buffer (lanes 9 and 10), there is a 45 kDa protein contaminating these extracts.

Figure 4.3.3 Coomassie stained SDS-PAGE showing purification of native TbCRK1/H.

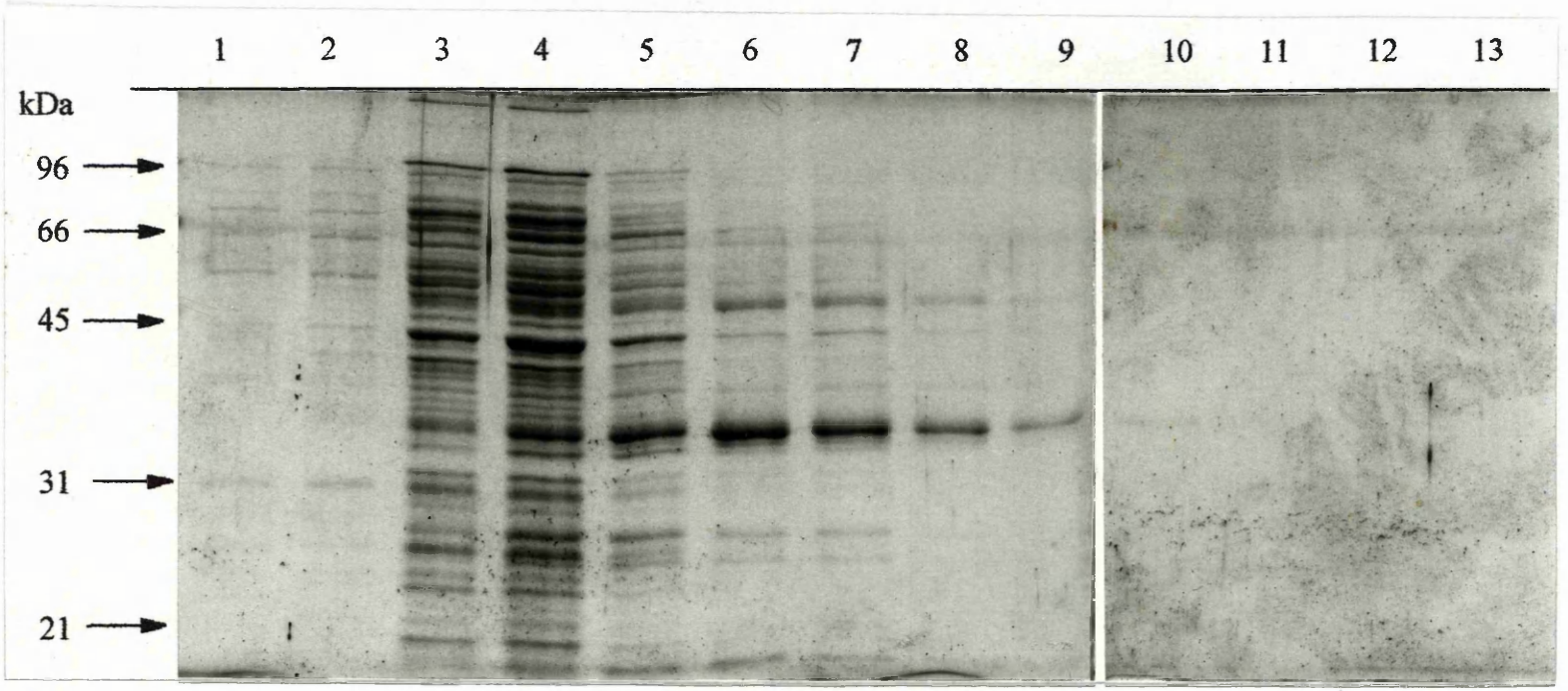


Figure 4.3.3 A 50 ml culture of induced XL-1 Blue/pQCD278.1h was pelleted, resuspended in 5 ml of Sonication Buffer, and lysed by incubation with lysozyme followed by sonication. After centrifugation at 10 000 g for 15 minutes the supernatant was added to a 0.5 ml Ni^{2+}/agarose column. The column was washed with 10 ml of Sonication Buffer, 10 ml of Wash Buffer and then the protein was eluted with a step gradient of 3 ml of Wash Buffer containing 100/200/300/500 mM imidazole. The imidazole containing buffer was collected as 1 ml fractions, except for the 500 mM elution which consisted of 4 x 0.75 ml fractions. 20 μl of each fraction to be run on the gel was heated to 37 ^{o}C for 5 minutes with 5 μl of 5 x FSB before loading onto 12 % SDS-PAGE.

Lanes 1-13. (1-3)-100 mM imidazole wash. (4-6)-200 mM imidazole wash. (7-9)-300 mM imidazole elution. (10-13)-500 mM imidazole elution.

This protein bound relatively well to the Ni^{2+}/agarose in other native purifications, with or without TbCRK1/H. No protein could be seen in the fractions eluted with 500 mM imidazole. Presumably none of the TbCRK1/H was multimeric, and so all had been eluted by the buffers with a lower concentration of imidazole.

TbCRK1/H protein samples equivalent to Figure 4.3.3, lane 9 were used for the antiserum cross-reaction tests (see Chapter 4.4.2), and samples from the equivalent of lane 10 were used in the attempts to activate TbCRK1/H (see Chapter 5.3).

4.3.5 Expression of TbCRK2/His.

Small scale urea purifications of pQCD278.2 cultures failed to show any protein corresponding to the expected size of TbCRK2/H (approximately 40 kDa). When a 50 ml culture was treated in parallel with the TbCRK1/H native purification in Figure 4.3.3 no purification of TbCRK2/H was seen (see Figure 4.3.4). Urea based purifications were also unsuccessful. Comparisons between induced and non-induced pQCD278.2/XL-1 Blue, whether of pellet or supernatant, revealed very little difference, with a major band at 40 kDa in the uninduced bacteria masking any low level TbCRK2/H expression. I was unable to convincingly purify TbCRK2/H, but whole cell extracts boiled in FSB could be used for the assessment of antibody cross-reaction (see Chapter 4.4.3), if the antiserum had not been raised to protein made in *E. coli* (i.e. antipeptide antisera could be used).

4.3.6 Cloning and expression of TbCRK2 & TbCRK3 without the N-terminal extensions.

One possible reason for TbCRK2/H and TbCRK3/H not being expressed and being insoluble respectively, when TbCRK1/H was both highly expressed and soluble, could have been the presence of the N-terminal extensions. It was decided to make new

Figure 4.3.4 Attempted purification of native TbCRK2/H.

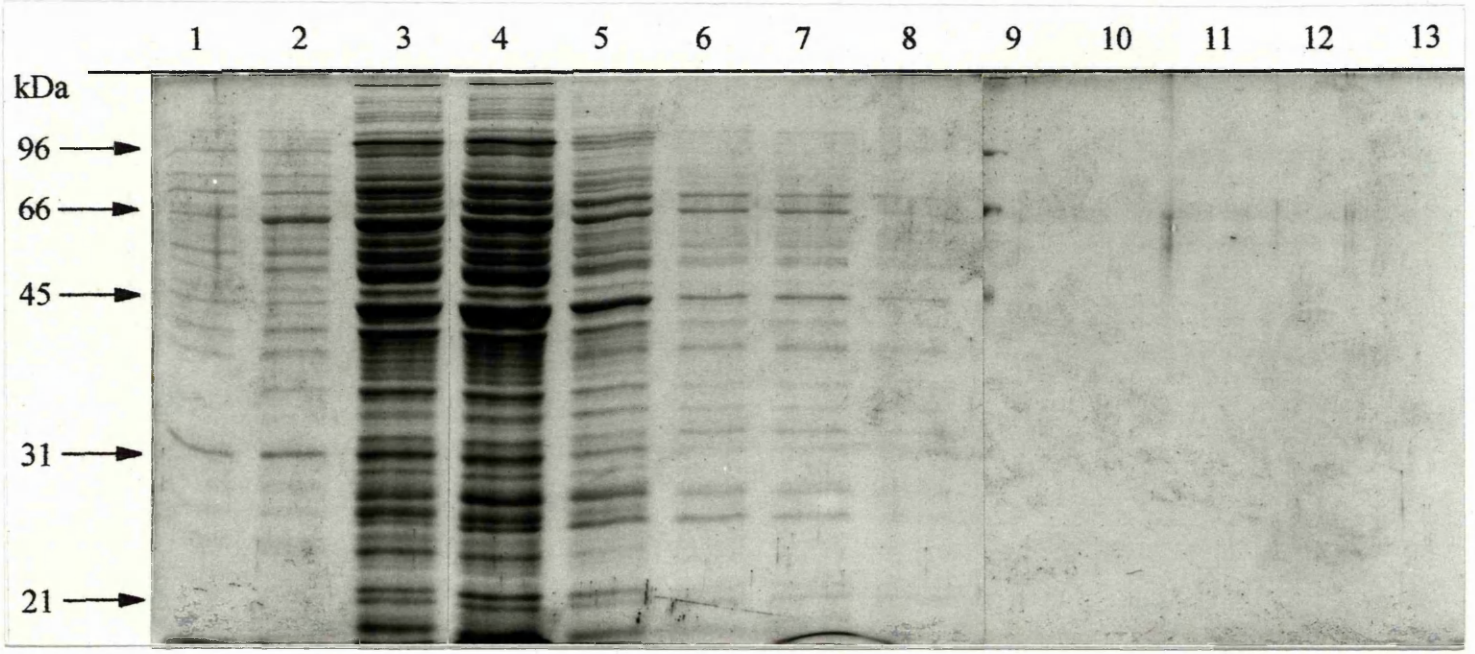


Figure 4.3.4 The 50 ml culture of XL-1 Blue/pQCD278.2 was treated, and analysed in an identical manner to the TbCRK1/H native purification (see Figure 4.3.3).

Lanes 1-13. (1-3)-100 mM imidazole wash. (4-6)-200 mM imidazole wash. (7-9)-300 mM imidazole elution. (10-13)-500 mM imidazole elution.

constructs by PCR to remove these sequences. Oligonucleotides were designed to anneal just 5' to the DNA encoding the ATP binding region (see Figure 4.3.1). As before, the oligonucleotides included a *Nco*I site for subcloning. The same 3' oligonucleotides as before were used, and transformants again screened by PCR and plasmid restriction mapping. Positive clones for both TbCRK2/H/-5'ext [pQCD307/2(2)] and TbCRK3/H/-5'ext [pQCD307/3(9)] were identified.

100 ml cultures of both pQCD307/2(2) and pQCD307/3(9) were induced for 4hr, and a native protein purification performed without success, apparently for the same reasons as with the full length proteins. There was no clear expression of TbCRK2/H/-5'ext and no 40 kDa band could be preferentially bound to the Ni^{2+}/agarose column (see Figure 4.3.5). TbCRK3/H/-5'ext was insoluble [compare Figure 4.3.6, lane 1 (insoluble fraction) with lane 2 (the soluble extract)]. By reducing the induction time to 1.5-2 hr, and lowering the growing temperature during this time to 30 ^{o}C it seemed possible to get a low level of expression of soluble TbCRK3/H/-5'ext. For unknown reasons this protein was washed off the Ni^{2+}/agarose column at low concentrations of imidazole (50-100 mM), and so could not be purified (Data not shown). For this reason, the goal of attaining soluble TbCRK3 was abandoned.

Figure 4.3.5 Attempted purification of native TbCRK2/H/-5'ext.

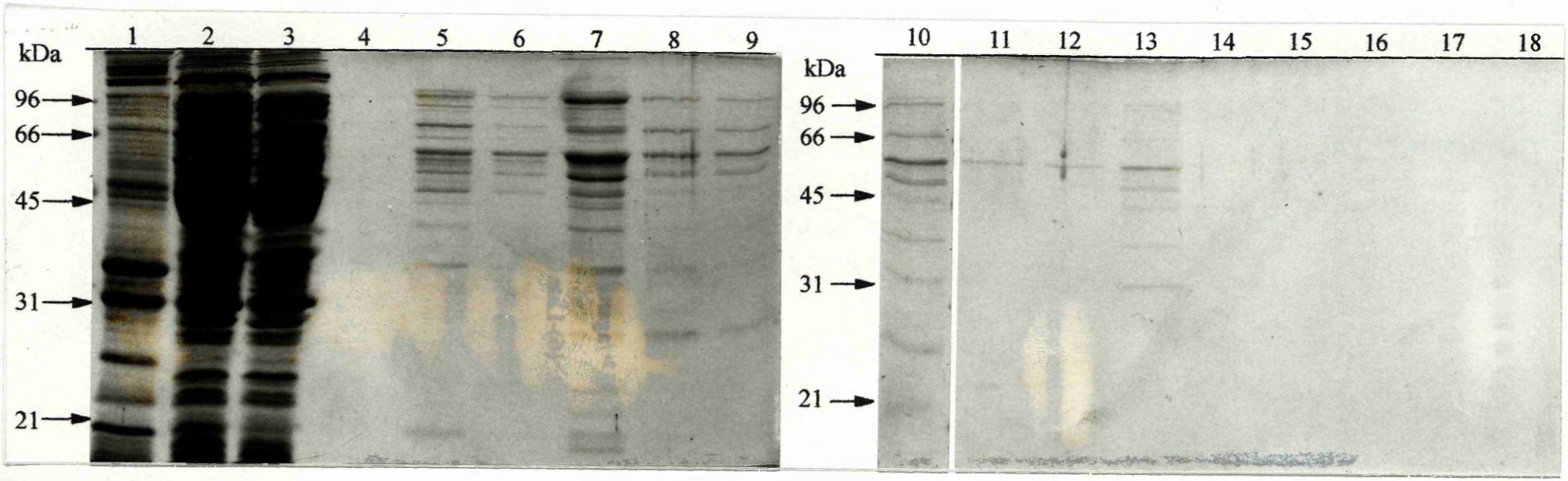


Figure 4.3.5 A 100 ml culture of induced XL-1 Blue/pQCD307/2(2) was pelleted, resuspended in 10 ml of Sonication Buffer, and lysed by incubation with lysozyme followed by sonication. After centrifugation at 10 000 g for 15 minutes the supernatant was added to a 1 ml Ni^{2+}/agarose column. The column was washed with 15 ml of Sonication Buffer and a sample collected of the final 0.5 ml. 1 ml of Wash Buffer containing 25 mM imidazole was added to the column, mixed for 5 minutes at 4 ^{o}C, then collected as the Wash Equilibrate fraction (WE). The column was then washed with a further 14 ml of the 25 mM imidazole solution, with 0.5 ml samples collected after 7 ml (Mid-wash fraction) and 13 ml (Late wash fraction) had flowed through the column. The protein was eluted with a step gradient of 6 ml of 50/100/200/500 mM imidazole in Wash buffer. Each concentration was first added as a 1 ml fraction, which was incubated with agitation to allow the column to equilibrate, as with the 25 mM Wash Buffer, then allowed to drain (called fraction E). The next 5 ml were then added to the column and collected in 1 ml fractions (I-V). The pellet was resuspended in 10 ml of sonication buffer, and 20 μl boiled with 5 μl of 5 x FSB before loading on 12 % SDS-PAGE. 20 μl of the other fractions to be run on the gel were heated to 37 ^{o}C for 5 minutes with 5 μl of 5 x FSB.

Lanes 1-18. (1)-Resuspended pellet. (2)-Supernatant. (3)-Supernatant after passage through Ni^{2+}/agarose column. (4)-Sonication Buffer wash, final 0.5 ml. (5)-Mid-wash fraction. (6)-Late wash fraction. (7-9)-Fractions E/II/IV of the 50 mM imidazole wash. (10-12)-Fractions E/II/IV of the 100 mM imidazole wash. (13-15)-Fractions E/II/IV of the 200 mM imidazole wash. (16-18)-Fractions E/II/IV of the 500 mM imidazole elution.

Figure 4.3.6 Attempted purification of native TbCRK3/H/-5'ext.

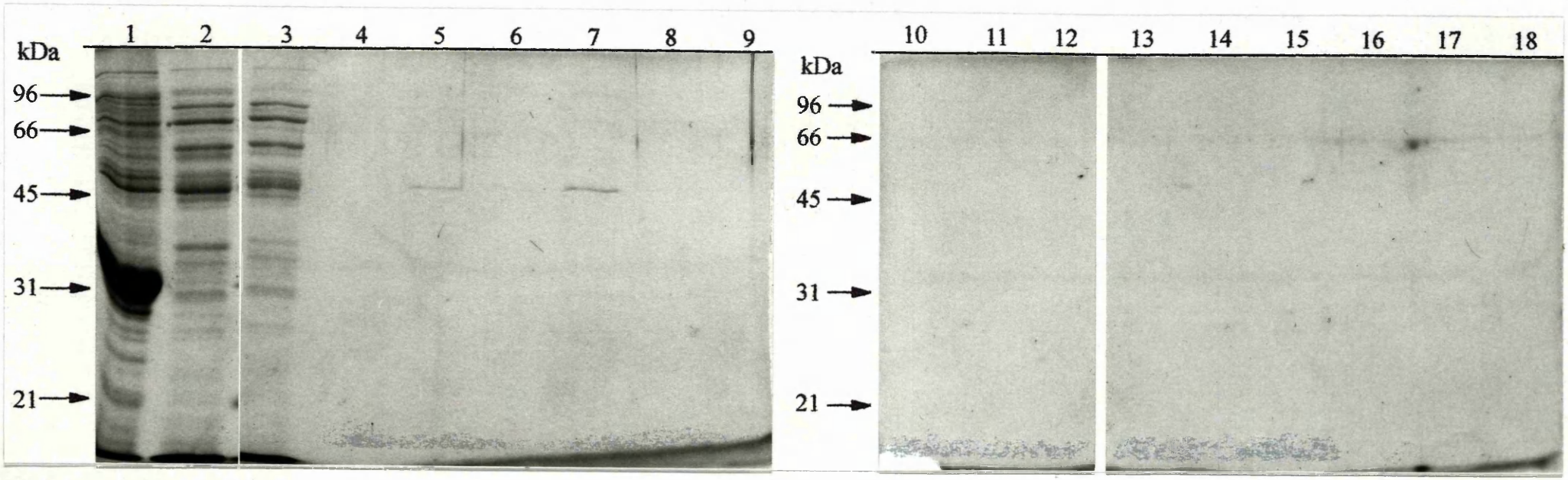


Figure 4.3.6 The 100 ml culture of XL-1 Blue/pQCD307/3(9) was treated in the same manner as for the purification of native TbCRK2/H/-5'ext (see Figure 4.3.5), except only 5 μl of each fraction was loaded in each lane.

Lanes 1-18. (1)-Resuspended pellet. (2)-Supernatant. (3)-Supernatant after passage through Ni^{2+}/agarose column. (4)-Sonication Buffer wash, final 0.5 ml. (5)-Mid-wash fraction. (6)-Late wash fraction. (7-9)-Fractions E/II/IV of the 50 mM imidazole wash. (10-12)-Fractions E/II/IV of the 100 mM imidazole wash. (13-15)-Fractions E/II/IV of the 200 mM imidazole wash. (16-18)-Fractions E/II/IV of the 500 mM imidazole elution.

4.4 The detection of *E. coli* expressed TbCRK proteins by Western blotting.

4.4.1 Antisera believed to cross-react with the TbCRK proteins.

Antisera raised to PSTAIR or PSTAIR related sequences, (the PSTAIR mAb and PSTAVR) were available to be tested for their cross-reactivity to the TbCRK/H fusion proteins. There was also antiserum that was expected to be specific for TbCRK1 (CITAA) and an antiserum raised to the TbCRK2 C-terminus (EVREE) (see Chapter 4.3.1). There were also antisera from two rabbits injected with the denatured, purified TbCRK3/H (see Chapter 4.3.3). The rabbit antisera were designated TC3HG and TC3HS.

4.4.2 Antibody detection of TbCRK1/H.

To test if TbCRK1/H cross reacts with the PSTAIR mAb, partially purified TbCRK1/H protein from the Ni^{2+}/agarose column selection (see Figure 4.3.3, lane 9) was run on 12 % SDS-PAGE in duplicate, and then electroblotted to PVDF membrane. TbCRK2/H (unpurified whole cell extract) and purified TbCRK3/H (see Chapter 4.3.3) were also run in duplicate on this gel. Figure 4.4.1 shows the result of incubating one set of the 3 proteins with the PSTAIR mAb alone, and the duplicate set with antiserum which had been pre-absorbed with the PSTAIR oligopeptide (see Materials and Methods). Figure 4.4.1, lanes 1 and 4, show that the PSTAIR mAb detects TbCRK1/H, and that the recognition is blocked by addition of an oligopeptide consisting of the PSTAIR sequence.

To test which of the other antisera recognised TbCRK1, TbCRK1/H protein from the Ni^{2+}/agarose column selection was run on a 12 % Polyacrylamide preparation gel and then electroblotted to PVDF membrane. Strips of the membrane were incubated with various antisera (see Figure 4.4.2). Figure 4.4.2 shows that TbCRK1/H is recognised by PSTAVR (lane 5) and CITAA (lane 6), but not EVREE

Figure 4.4.1 PSTAIR mAb detection of TbCRK/H proteins.

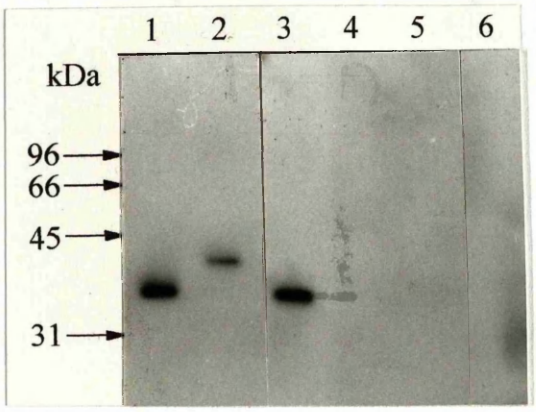


Figure 4.4.1 Extracts of proteins were run on 12 % SDS-PAGE and electroblotted to PVDF membrane. Lanes 1 & 4, 10 ng of TbCRK1/H; lanes 2 & 5, whole cell *E. coli* extract expressing TbCRK2/H; lanes 3 & 6, 10 ng of TbCRK3/H. Lanes 1-3 were probed with PSTAIR mAb (1:250 dilution); lanes 4-6 were probed with PSTAIR mAb that had been preincubated with 1 μg / ml PSTAIR oligopeptide.

Figure 4.4.2 TbCRK1/H detection by CRK and CDK antisera.

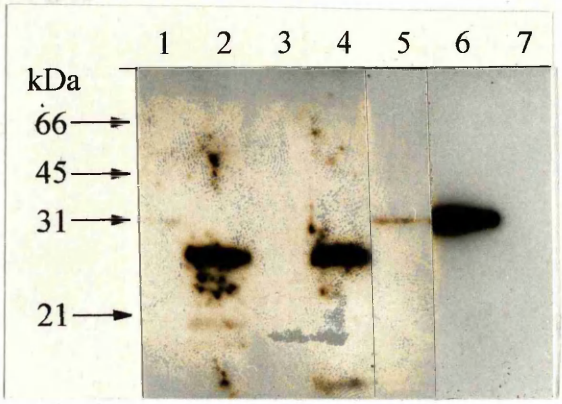


Figure 4.4.2 10 ng of semi-purified TbCRK1/H was loaded per lane onto 12 % SDS-PAGE and electroblotted to PVDF membrane. Lanes probed with: (1)-TC3HG Pre-Immune serum (1:800), (2)-TC3HG Immune serum (1:800), (3)-TC3HS Pre-Immune serum (1:800), (4)-TC3HS Immune serum (1:800), (5)-PSTAVR antiserum (1:50), (6)-CITAA antiserum (1:50), (7)-EVREE antiserum (1:50).

(lane 7) or either of the TC3H antisera (lanes 2 and 4). The reacting bands in lanes 2 and 4 are impurities that are detected by anti-*E. coli* antibodies in the polyclonal TC3HG and TC3HS sera.

4.4.3 Antibody detection of TbCRK2/H.

Although TbCRK2/H was not purified from *E. coli* it was thought that whole cell extracts from IPTG induced bacteria could contain enough TbCRK2 fusion protein to enable assessment of antisera reactivity by Western blotting. If the antisera had been raised to a protein expressed in *E. coli*, however, then the level of cross reaction with bacterial proteins precluded assessing TbCRK2/H reactivity. This is presumably due to contamination of the inoculated protein and was seen in antisera raised to both histidine tagged and GST fusion proteins purified from *E. coli*. For this reason it was not possible to show whether or not TbCRK2/H was recognised by either TC3HG or TC3HS. Of the antipeptide antisera; PSTAIR mAb (Figure 4.4.1, lane 2), PSTAVR (Figure 4.4.3, lane 2), and EVREE (Figure 4.4.4, lane 2) bind TbCRK2/H specifically, while CITAA did not (Figure 4.4.5, lane 2). There was found to be a 34 kDa protein in the *E. coli* extract that was detected by the affinity purified PSTAVR antisera (Figure 4.4.3, lane 2), but this recognition was not blocked by the addition of PSTAVR oligopeptide (see Figure 4.4.3, lane 5). Although this protein from the bacterial whole cell extract ran at approximately the same position as TbCRK1 and TbCRK3, the loss of reacting bands in the lanes containing the purified histidine tagged proteins after the blocking oligopeptide addition showed that the recognised *E. coli* protein must be lost during the Ni^{2+}/agarose purification. The 40 kDa band detected by the EVREE antiserum in the *E. coli* extract (Figure 4.4.4, lane 2) was notably larger than the 39 kDa band seen in the *T. brucei* long, slender extract (lane 1). The 6 histidine residues cause the bacterially expressed protein to be over 0.8 kDa larger.

Figure 4.4.3 PSTAVR antiserum detection of TbCRK/H proteins.

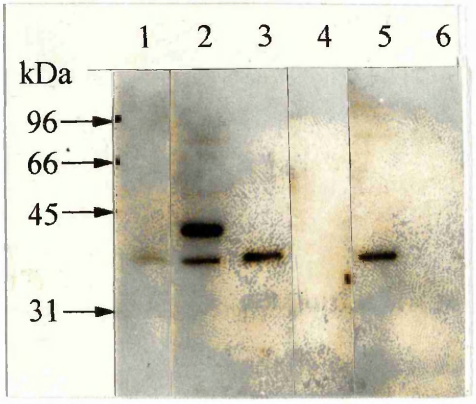


Figure 4.4.3 Extracts of proteins were run on 12 % SDS-PAGE and electroblotted to PVDF membrane. Lanes 1 & 4, 3 ng of TbCRK1/H; lanes 2 & 5, whole cell *E. coli* extract expressing TbCRK2/H; lanes 3 & 6, 10 ng of TbCRK3/H. Lanes 1-3 were probed with PSTAVR antiserum (1:50); lanes 4-6 were probed with PSTAVR antiserum that had been preincubated with 1 μg / ml PSTAVR oligopeptide.

Figure 4.4.4 EVREE detection of TbCRK2/H.

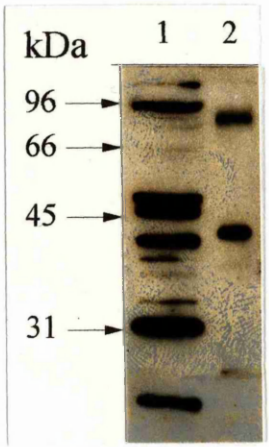


Figure 4.4.4 Extracts of proteins were run on 12 % SDS-PAGE and electroblotted to PVDF membrane. Lane 1, whole cell long, slender *T. brucei* protein extract; lane 2, whole cell *E. coli* extract expressing TbCRK2/H. Both probed with EVREE antiserum (1:50).

Figure 4.4.5 CITAA antiserum does not recognise TbCRK2/H.

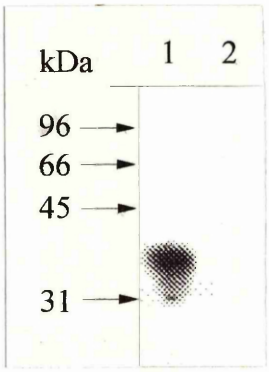


Figure 4.4.5 Extracts of proteins were run on 12 % SDS-PAGE and electroblotted to PVDF membrane. Lane 1, 10 ng of LmmCRK1/H (Purified by J.C.Mottram); lane 2, whole cell *E. coli* extract expressing TbCRK2/H. Both probed with CITAA antiserum (1:50).

4.4.4 Antibody detection of TbCRK3/H.

TbCRK3/H purified using the method described in Chapter 4.3.3 was used for Western blotting. The protein was run on 12 % SDS-PAGE and electroblotted to PVDF membrane which was then cut into strips to be incubated with various antisera (Figure 4.4.6). These strips were processed in the same solutions as the TbCRK1/H western blot (Figure 4.4.2). No bands are visible when TbCRK3/H is incubated with either of the pre-immune TC3H antisera (Figure 4.4.6, lanes 1 and 3). When incubated with the TC3HG and TC3HS immune sera intense bands are seen at the expected size of approximately 34 kDa (lanes 2 and 4). The 34 kDa band is also present on the membrane exposed to the PSTAVR antiserum (lane 5), while no bands are seen in lanes incubated with CITAA (Lane 6) or EVREE (lane 7) antisera. Therefore TbCRK3/H is recognised by the TC3HG and the TC3HS antisera as well as the PSTAVR affinity purified antiserum. The TbCRK3 fusion protein is not recognised by either the CITAA antiserum, raised to the LmmCRK1 C-terminal oligopeptide sequence, or the EVREE antiserum, raised to the TbCRK2 C-terminal oligopeptide sequence.

When membranes carrying TbCRK3/H were incubated with PSTAIR mAb a 34 kDa band could be visualised (Figure 4.4.1, lane 3). This recognition could be abolished by pre-incubation of the antiserum with PSTAIR oligopeptide (Figure 4.4.1, lane 6), showing that the PSTAIR mAb cross reacts with the TbCRK3/H fusion protein. In the same manner recognition of TbCRK3/H by the PSTAVR antiserum (Figure 4.4.3, lane 5 and Figure 4.4.3, lane 3) could be blocked by pre-incubation of the antibody solution with the PSTAVR oligopeptide (Figure 4.4.3, lane 6).

Figure 4.4.7 shows that the antisera raised to the oligopeptide homologous to the N terminal extension of TbCRK3 failed to recognise the histidine tagged TbCRK3 fusion protein (lanes 2 and 3). Lane 1 shows that protein recognisable by the PSTAIR mAb is on the membrane, and that the higher molecular weight bands co-purifying with the 34 kDa band in Figure 4.3.2 are also recognised by the PSTAIR mAb, and are therefore likely to be multimeric forms of the TbCRK3/H protein.

Figure 4.4.6 TbCRK3/H detection by CRK and CDK antisera.

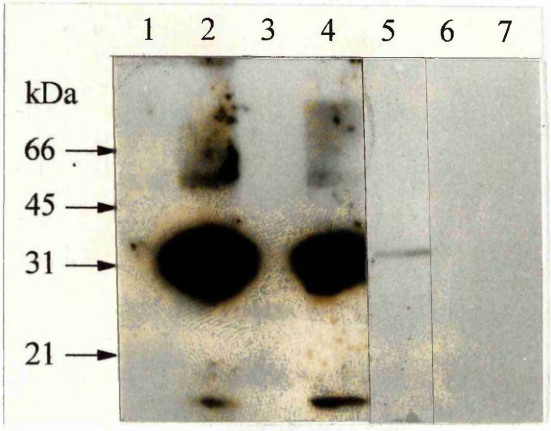


Figure 4.4.6 10 ng of semi-purified TbCRK3/H was loaded per lane onto 12 % SDS-PAGE. The gel was electroblotted to PVDF membrane. Lanes probed with: (1)-TC3HG Pre-Immune serum (1/800), (2)-TC3HG Immune serum (1:800), (3)-TC3HS Pre-Immune serum (1:800), (4)-TC3HS Immune serum (1:800), (5)-PSTAVR antiserum (1:50), (6)-CITAA antiserum (1:50), (7)-EVREE antiserum (1:50).

Figure 4.4.7 TbCRK3/H is not detected by antisera raised to the N-terminal oligopeptide.

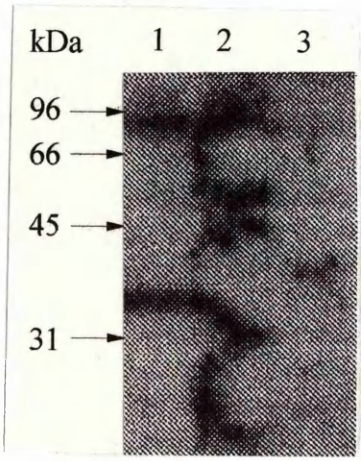


Figure 4.4.7 Purified TbCRK3/H, equivalent to that shown in Figure 4.3.2 lane 2, was run on 12 % SDS-PAGE and electroblotted to PVDF membrane. Lanes incubated with: (1)-PSTAIR mAb (1:250), (2)-N-terminal oligopeptide antiserum A (1:250); (3)-N-terminal oligopeptide antiserum B (1:250).

4.4.5 Discussion of antisera specificity.

The cross reactivity of the available antisera is compared in Table 4.4.1. It is immediately obvious that all three TbCRK proteins are detected by both antisera directed to the PSTAIR region. Figures 4.4.1 and 4.4.3 show that TbCRK1 and TbCRK3 are very similar in size and may run together on SDS-PAGE, which is a possibly explanation for why only two bands were apparent in previous Western blots of *T. brucei* (of 33 and 39 kDa). The cross reactivity of the three TbCRK proteins with the PSTAIR mAb is surprising considering all of them contain substitutions in the PSTAIR box. TbCRK3/H has only 10/16 residues conserved with the 16mer oligopeptide to which the monoclonal antiserum was raised. While the conservative changes, I to L and L to I, would not be expected to radically alter the antibody/epitope recognition the non-conservative changes, S to Q and K to Q might. It is possible for a single residue change to destroy antibody recognition (Barnett *et al.*, 1990).

The anti-C-terminal antisera are specific for the CRK family member they were raised to. EVREE only detects TbCRK2/H (see Figures 4.4.2, 4.4.4 and 4.4.6), while CITAA detects both LmmCRK1/H (Figure 4.4.5, lane 1) and TbCRK1/H (Figure 4.4.2, lane 6) but not TbCRK2/H (Figure 4.4.5, lane 2) or TbCRK3/H (Figure 4.4.6, lane 6). The 5 changes between the 16 C-terminal residues of LmmCRK1 and TbCRK1 did not appear to greatly affect the antibody recognition. The addition of the histidine tag to the C-terminus also did not appear to affect antibody binding in any of these cases.

As the TC3HG and TC3HS antisera had been raised to the entire ORF of TbCRK3/H it was important to test if the antisera cross reacted with the other TbCRK proteins especially as some regions of the CRK proteins are well conserved. The monoclonal PSTAIR and the polyclonal PSTAVR antisera had already shown the ability of antibodies to recognise sequences related to those that the antibodies had been raised to. The histidine tag is reported to be a poor immunogen, and this was confirmed when TC3HG and TC3HS were tested against TbCRK1/H as no binding to this protein

Table 4.4.1 Recognition of the TbCRK proteins by various antisera.

	PSTAIR mAb	PSTAVR	CITAA	EVREE	TC3HG&S
TbCRK1/H	XXX	XXX	XXX	---	---
TbCRK2/H	XXX	XXX	---	XXX	ND
TbCRK3/H	XXX	XXX	---	---	XXX

Table 4.4.1 Antiserum/protein recognition.

+++ Protein recognised by antiserum.
--- No specific reaction seen.
ND Not Done, due to contaminant *E. coli* protein.

was detected. The intense band around 27 kDa in these western blots (Figure 4.4.2, lanes 2 and 4) shows the strength of reaction to contaminating proteins from *E. coli.* Because of the recognition of this, and other, bacterial proteins, it was not possible to test if TC3HG or TC3HS cross reacted with the TbCRK2/H fusion protein. In an effort to reduce the cross reaction with *E. coli* proteins a 0.05 ml aliquot of the TC3HG antiserum was diluted 1:20 with 10 mM Tris pH 7.5. This was then incubated with amino-link beads to which a protein extract from *E. coli* had been bound (previously prepared in WUMP). The flow through from this column [TC3HG(-Ec)]was used for some Western blots without a marked reduction in extraneous recognition, presumably due to overloading of the column.

4.5 Analysis of TbCRK expression in different life cycle stages.

4.5.1 Available life cycle stages and protein extracts.

Of the major life cycle stages of *T. brucei*, it is possible to harvest and perform expression analysis with Western blotting techniques on three; the long slender (ls) and the short stumpy (ss) bloodstream forms, and *in vitro* grown procyclic (pc) forms. The long slender and procyclic forms are rapidly dividing stages, while the short stumpy form is blocked in G1 (see Chapter 1.1.2). It is not possible to purify short stumpy forms to homogeneity from a mixed population, however by using laboratory strains with high rates of short stumpy formation and infecting irradiated rats it is possible to get populations of >75 % short stumpy trypanosomes. 100 % long slender forms can be obtained from monomorphic strains of *T. brucei*. These strains have been selected for rapid growth in the rat due to the repeated bloodstream passage via inoculation, and a number of alterations have occurred. The rate of VSG switching has apparently decreased significantly (Barry and Turner, 1991) and, by definition, they have lost the ability to differentiate to the short stumpy form, a transition which must have cell cycle controlling protein involvement. Due to this, experiments on the CRK proteins using these strains should be interpreted with caution. For some of the Western blots mixed bloodstream form trypanosomes (BS) from a pleomorphic strain of *T. brucei* were used instead of purified populations of long slender and short stumpy forms.

Originally S100 extracts were used for Western blotting as the CDK proteins from other organisms are generally soluble. Using these extracts however, it was not possible to detect TbCRK3 and it was found necessary to use either whole cell extracts or both S100 and pellet fractions.

Unless otherwise noted, the procyclic extracts were made from freshly pelleted STIB247 cultures during log phase growth. The mixed bloodstream forms and the short stumpy forms are also from the STIB247 line. The monomorphic long slender line usually used was IstatC123. Bradford assays were used to estimate the protein

concentration and the extracts were first run on SDS-PAGE and stained with coomassie blue to check that there was approximately equal loading before the extracts were used for Western blots. Typically, 30 μg of total protein was run in each lane of a small gel, 80 μg were run per lane on the larger equipment.

4.5.2 TbCRK expression in three life cycle stages of *T. b. brucei.*

EVREE antiserum, previously shown to recognise TbCRK2/H, was incubated with S100 extracts of mixed bloodstream form, short stumpy form and procyclic form *T. b. brucei* that had been run on SDS-page and electroblotted to PVDF membrane (see Figure 4.5.1). This Western blot was performed by Dr. Mottram. A 40 kDa protein is recognised in all three life cycle stage extracts. The antisera also recognises an unknown 50 kDa protein in all of the stages. Relative to the unknown protein it appears that the short stumpy forms have an increased amount of TbCRK2 but we do not know if the expression of the 50 kDa protein is constant between the three stages tested (although total protein loading should have been approximately equal in each lane).

Figure 4.5.2 shows whole cell extracts of long slender form (lane 1), short stumpy (lane 2) and procyclic (lane 3) *T. brucei* incubated with the TbCRK1 specific CITAA antiserum [Plus one lane of long slender extract incubated with the PSTAIR mAb (lane 4)]. In all three life cycle stage extracts the predominant band is at 33 kDa. There are also varying lower molecular weight proteins recognised which may be due to proteolytic cleavage, which occasionally occurred even in the presence of protease inhibitors. The 33 kDa protein, presumably TbCRK1, runs at the same size as the lower band of a 34 kDa doublet seen when the long, slender form extract was incubated with the PSTAIR mAb

Figure 4.5.1 TbCRK2 expression in *T. brucei.*

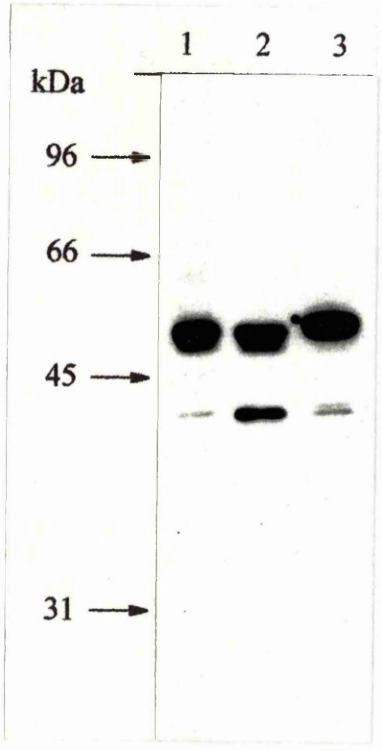


Figure 4.5.1 (Supplied by J.C.Mottram) S100 supernatant protein extracts were run on 10 % SDS-PAGE, electroblotted to PVDF membrane and incubated with EVREE antiserum (1:50). Lane 1-mixed bloodstream form, lane 2-predominantly short stumpy, lane 3-cultured procyclic.

Figure 4.5.2 PSTAIR mAb reacting proteins in long slender form *T. brucei* and TbCRK1 expression in different life cycle stages.

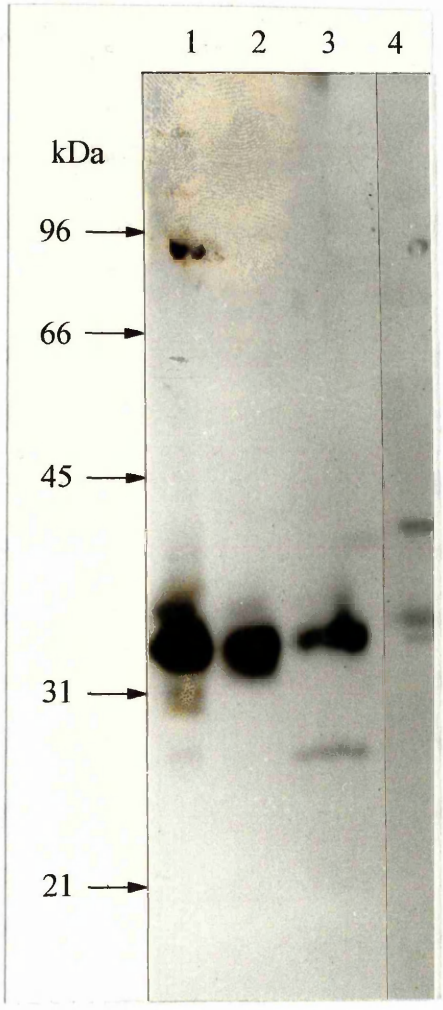


Figure 4.5.2 Whole cell protein extracts from long slender (lanes 1 & 4), short stumpy (lane 2), and procyclic (lane 3) *T. brucei* were run on 12 % SDS-PAGE, electroblotted to PVDF membrane and probed with; lanes 1/2/3- CITAA antiserum (1:50); lane 4- PSTAIR mAb (1:500).

antiserum (lane 4). As well as the 34 kDa doublet the long slender extract also shows a PSTAIR reactive band at 40 kDa. It is likely that the proteins recognised by the mAb are TbCRK1 (33 kDa), TbCRK3 (34 kDa) and TbCRK2 (40 kDa).

Figure 4.5.3 shows the short stumpy form S100 extracts probed with the PSTAIR mAb. Only the 34 kDa and 40 kDa bands were visualised, but this is likely to be due to the underloading/underexposure of the blot. Therefore it is expected that the non-dividing short stumpy form trypanosomes contain TbCRK2 (the 40 kDa band) and at least one of TbCRK1 or TbCRK3.

A Western blot of S100 procyclic extract incubated with the PSTAIR mAb results in four distinguishable bands at 33, 40, 48 and 60 kDa (see Figure 4.5.4). It is unlikely that the 34 kDa doublet of TbCRK1 and TbCRK3 would be resolved adequately on the small Bio-Rad gel kit used in this experiment. It is also possible that the doublet is not seen because TbCRK3 is insoluble under these conditions (see Figure 4.5.7, lanes 3 and 4). The 40 kDa band was expected to be TbCRK2, but the identities of the 48 and 60 kDa proteins are unknown. A PCR generated fragment of a fourth TbCRK gene (TbCRK4) has been isolated and may be found to encode one of these proteins (J. Mottram, personal communication)

Figure 4.5.5 is a Western blot of mixed bloodstream form whole cell extract incubated with the PSTAIR mAb (lane 1) and TC3HG Immune antiserum (lane 2). The mAb detects the 34 kDa doublet, the 40 kDa protein and an 80 kDa protein as well. The TC3HG antiserum recognises many proteins in the mixed bloodstream extract, one of which runs at the same size as the higher of the band sin the 34 kDa doublet. Another band co-migrates with the 40 kDa band from the PSTAIR mAb probed Western blot. This strongly suggests that TbCRK3 is expressed in bloodstream form *T. brucei*, and that the larger of the 34 kDa PSTAIR reactive bands is TbCRK3. It also implies that TC3HG may cross react with TbCRK2.

Figure 4.5.3 PSTAIR mAb reacting proteins in short stumpy form *T. brucei.*

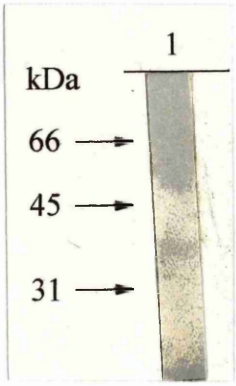


Figure 4.5.3 Whole cell protein extract from short stumpy *T. brucei* was run on 12 % SDS-PAGE, electroblotted to PVDF membrane and probed with PSTAIR mAb (1:1000).

Figure 4.5.4 PSTAIR reacting proteins in procyclic *T. brucei* S100 supernatants.

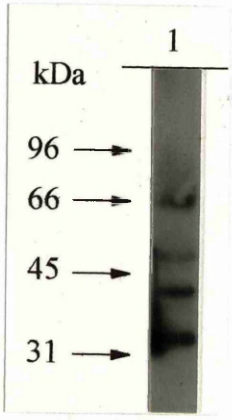


Figure 4.5.4 S100 protein extract from procyclic form *T. brucei* was run on 12 % SDS-PAGE, electroblotted to PVDF membrane and incubated with PSTAIR mAb (1:25) that had been affinity purified by J.C.Mottram.

Figure 4.5.5 TbCRK expression in bloodstream form *T. brucei.*

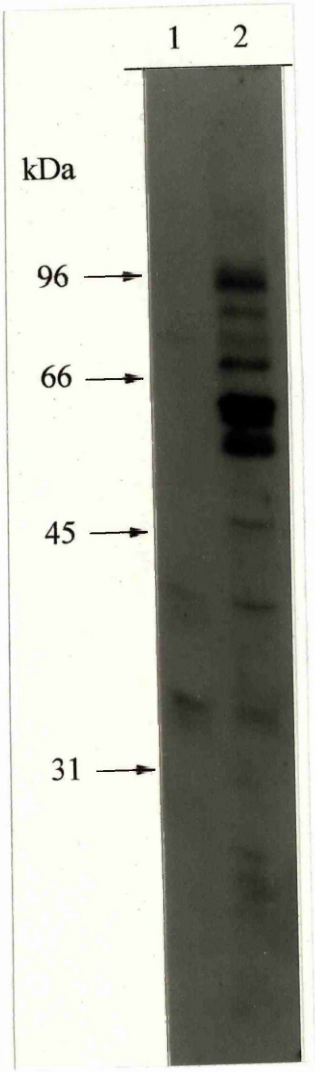


Figure 4.5.5 Whole cell protein extract from mixed bloodstream form *T. brucei* was run on 12 % SDS-PAGE, electroblotted to PVDF membrane and probed with; lane 1-Affinity purified PSTAIR mAb (1:50); lane 2, TC3HG(-Ec) (1:50).

Figure 4.5.6 TbCRK3 expression in long slender form *T. brucei.*

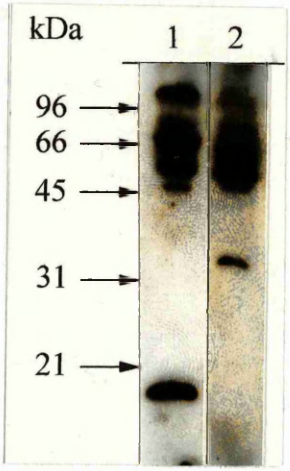


Figure 4.5.6 Whole cell protein extract from long slender *T. brucei*, run on 12 % SDS-PAGE and electroblotted to PVDF membrane, was probed with; lane 1-TC3HG Pre-Immune antiserum (1:800); lane 2-TC3HG Immune antiserum (1:800).

Long slender whole cell extracts were run on SDS-PAGE, electroblotted to PVDF membrane and incubated with Pre-Immune or Immune TC3HG antisera (Figure 4.5.6). No bands between 21 and 45 kDa are seen to react to the Pre-Immune (lane 1).A protein of 34 kDa is detected by the TC3HG Immune antiserum, presumably TbCRK3 (see Figure 4.5.6, lane 2), but in this extract no 40 kDa protein was recognised.

In Figure 4.5.7 short stumpy S100 and pellet extracts have been run separately on SDS-PAGE, and the Western blot incubated with Pre-Immune (lanes 1 and 2) or Immune (lanes 3 and 4) TC3HG antisera. Again, the Pre-Immune TC3HG did not detect any proteins between 21 and 45 kDa in either the supernatant (lane 1) or the pellet fraction (lane 2). The Immune antiserum cross reacts with a 34 kDa protein in the pellet fraction (lane 4) and a 26 kDa protein in the S100 supernatant (lane 3). There is also a possible very faint band in the supernatant at 40 kDa when incubated with the Immune TC3HG. The association of TbCRK3 with the pellet implies that it is bound to the cytoskeleton or DNA within the cell. The 26 kDa protein has not been detected with the PSTAIR mAb, but if the change in size was due to the removal of 70-80 residues from the N-terminus (consistent with the mobility change) then the PSTAIR box would be missing from the protein. Proteolysis of a CDC2-related kinase in this manner has not been seen previously, and as with the lower molecular weight CITAA reactive bands (see Figure 4.5.2) it is likely to be an artefact of the sample preparation.

The TC3HG antiserum gave high background when incubated with the procyclic whole cell extracts but it was just possible to detect a 34 kDa band as predicted for TbCRK3, reacting to the Immune serum (Figure 4.5.8, lane 2), while the Pre-Immune antiserum (lane 1) detected no bands in this region. Therefore TbCRK3 is being expressed in procyclic form *T. brucei.* No cross reaction of the TC3HG Immune antiserum with TbCRK2 was seen due to the level of non-specific signal.

The evidence strongly suggests that all three *tbcrk* genes so far cloned are expressed in the three life cycle stages tested.

Figure 4.5.7 Insolubility of TbCRK3 in short stumpy form *T. brucei.*

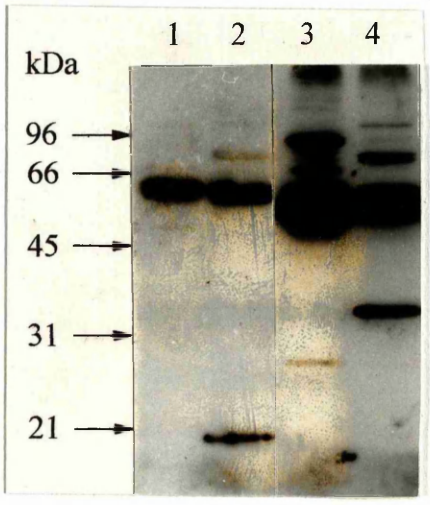


Figure 4.5.7 S100 protein extracts of short stumpy form *T. brucei* were run on SDS-PAGE and electroblotted to PVDF membrane. Lanes 1 & 3-supernatant; Lanes 2 & 4-pellet fraction. Lanes 1 & 2-TC3HG Pre-Immune antiserum (1:400). Lanes 3 & 4-TC3HG Immune antiserum (1:400).

Figure 4.5.8 TbCRK3 expression in procyclic *T. brucei.*

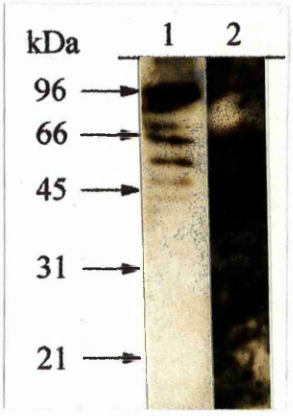


Figure 4.5.8 Whole cell protein extract from procyclic *T. brucei* was run on 12 % SDS-PAGE and electroblotted to PVDF membrane. Lane1-TC3HG Pre-Immune antiserum (1:600); lane 2-TC3HG Immune antiserum (1:600).

4.6 Discussion.

The data obtained from Southern blots of the *tbcrk* genes shows that they are single copy per haploid genome. At the present time, few *T. brucei* genes isolated are single copy. It would seem unlikely that multiple copies are essential for high level expression as the high levels of VSG protein are expressed from a single telomere, therefore the VSG gene effectively has a copy number of 1/2 per haploid genome. The VSG, however, is transcribed by an alpha-amatin resistant RNA polymerase which affects transcription levels. More pertinent is the fact that deletion of two thirds of the tubulin genes in *Leishmania enriettii* by homologous recombination only results in a lowering of mRNA levels to 62-76 % of the normal (de Lafaille and Wirth, 1992). The evidence so far amassed suggests that mRNA levels for RNA polymerase II transcribed genes is regulated by post transcriptional methods. Because of the apparent general lack of promoter activation in the kinetoplastids it may be that the method used to allow rapid increases and decreases in mRNA levels is constant transcriptional initiation followed by variable stability. A single copy gene under these conditions would only slowly build up its RNA levels when the message was stabilised.

The TbCRK2/H protein expressed in *E. coli* had a very low level of expression, the reason for which is unknown. The codons used in TbCRK2/H synthesis do not vary in frequency fundamentally from the codon bias seen in *E. coli* ribosomal proteins, so limiting tRNA factors should not have caused reduced expression (Zhang *et al.*, 1991). In addition Western blots show no sign of protein degradation, with the 40 kDa band being the only antisera specific band recognised (see Figures 4.4.1 and 4.4.3).

The TbCRK3/H protein expressed in *E. coli* was extremely insoluble. In this case the result does agree with the finding that TbCRK3/H is associated with the pellet fraction of S100 extracts (see Figure 4.5.7). In contrast to TbCRK3/H, LmmCRK3/H was soluble and could be purified in the native form (J. Mottram, personnel communication). Apart from the N-terminal extension, which has 6/20 amino acids

identical (including the initial Methionine), the two proteins are 80 % identical, yet one is soluble in *E. coli* while the other forms inclusion bodies.

The N-terminal extensions of both TbCRK2 and TbCRK3 were shown to not be responsible for these problems. Engineered constructs lacking the extensions behaved in the same manner as the full length clones (see Chapter 4.3.6).

The detection of p33, p34 and p40 TbCRK1-3 proteins by the PSTAIR mAb and the affinity purified polyclonal PSTAVR antibodies shows that considerable variation in the protein sequence can be tolerated in the antibody/epitope recognition. Epitopes are typically 3-8 aa in length (Rajadhyaksha and Thanavala, 1995; Thomson *et al.*, 1995). The nature of an antibody/epitope recognition makes it likely that the region of the PSTAIR box that is recognised by the monoclonal antibody is one in which the *T. brucei* proteins have only conservative substitutions. These would make only small changes to the epitope's steric, hydrophobic and/or electrical characteristics. The probable epitope is contained in the sequence, TAIREISLL, which is bounded by the TbCRK3 substitutions, S55Q and K65Q (TbCRK3 numbering). Within this region the TbCRK proteins have the conservative changes, I to V, I to L and L to I. The detection of TbCRK1/H with the CITAA antiserum is another example of antibody tolerance of conservative changes. Within the 16 amino acids of LmmCRK1 sequence to which the antiserum was raised, TbCRK1 has 5 substitutions, with the longest string of perfectly conserved residues being only 5 long.

```
LmmCRK1 C-terminal oligopeptide  CITAADALNHPYFSLQF
      TbCRK1 C-terminus                L---Q--E-----VE-
```

The initial Cysteine of the CITAA peptide was necessary for the manufacturing process. The conserved residues are represented by the - marks. All of the substitutions are relatively conservative (I to L, D to Q, N to E, L to V and Q to E), although in at least one case, a D to Q substitution can result in a marked reduction in antibody affinity for an epitope (Barnett *et al.*, 1990).

The three TbCRK proteins were detected in both dividing and non-dividing stages of the *T. brucei* life cycle. This, however, gives no clues to whether or not any of these proteins are involved in control of the cell cycle as the presence of the polypeptide does not necessarily mean that the kinase is active. In *L. mexicana,* LmmCRK1 is present in the amastigote stage, but immunoprecipitates show no histone H1 kinase activity (Mottram *et al.*, 1993). Therefore, to test whether or not any of the TbCRK proteins' kinase activities correlate to the division status of the life cycle stage, protein specific purification and activity assessment will have to be carried out. At present most of the antisera available are not specific enough for immunoprecipitation, and the interaction between CITAA and TbCRK1, unlike that between CITAA and the LmmCRK1 protein, does not allow immunoprecipitation (presumably due to amino acid changes within the recognised epitope/s).

It is also possible that the G1 cell cycle block in the short stumpy stage may be due to constitutive activity of a G1 specific CDK as opposed to a lack of activity. This would be analogous to the M phase block that occurs when CDC2 is not deactivated towards the end of mitosis. This can be caused by the expression of Mitotic cyclins that lack the N-terminal "destruction box" (see Chapter 1.2.3 and 1.2.6). When this occurs the cells are unable to exit from mitosis due to the continuous activity of the CDC2/cyclin complex, normally deactivated by proteolysis of the cyclin subunit (Draetta *et al.*, 1989). It is also possible that, due to the divergence between the kinetoplastids and other eukaryotes, the TbCRK proteins have no role in controlling the cell cycle. There is evidence that LmmCRK1 might not be involved in the control of the cell cycle (Mottram *et al.*, 1993), at least not in all life cycle stages. The protein phylogeny analysis implies that TbCRK2 and TbCRK3 at least are related to the G1/S phase activated CDK4 and CDK6 (see Figure 3.3.9). As noted though, this convergence may be an artifact, caused by the divergence of the TbCRK2/3 and the CDK4/6 proteins from the CDC2/CDK2 sub-family. Long branches within these analyses can cluster due to their differences from the conserved core sequence as opposed to clustering due to their similarity to each other.

The similar size of TbCRK1 and TbCRK3 on SDS-PAGE, as shown by the Western blots of His-tagged proteins (Figures 4.4.1 and 4.4.3) may explain the fact that only two PSTAIR mAb reactive proteins were detected in *T. brucei* protein extracts when using the mini-gel SDS-PAGE system. Another explanation is that, at least in the short stumpy stage, TbCRK3 is associated with the membrane fraction of an S100 protein extract (Figure 4.5.7). Most of the previous Western blots were performed using the supernatant of protein extracts (Mottram *et al.*, 1993), which would cause insoluble TbCRK3 to be lost. The *L. mexicana* homologue of TbCRK3 runs notably slower than TbCRK3/H on SDS-PAGE as a His-tagged protein (Figure 4.6.1), and so it should be easy to see separate bands for LmmCRK1 and LmmCRK3 in *L. mexicana* protein extracts. In fact only one PSTAIR mAb reacting band is seen in *L. mexicana* S100 protein extracts, although a 35 kDa band can be seen in S100 extracts from *L. major* promastigotes (Mottram *et al.*, 1993). There is now evidence that LmmCRK3 is soluble and expressed in promastigote *L. mexicana*, but at very low levels (J. Mottram, personal communication). If so then the expression, and solubility of the CRK3 proteins would appear to be another difference (to go with the lack of a leishmanial homologue of *tbcrk2*) between these two relatively closely related kinetoplastids in genes that would be thought to encode well conserved functions. The difference in CRK3 solubility when the proteins are extremely similar would imply that the association with the insoluble fraction is modulated either by protein modification or via association with another protein, because it is not purely to do with integral characteristics of the polypeptide primary sequence.

Figure 4.6.1 Detection of LmmCRK3/H by the TC3HG antiserum.

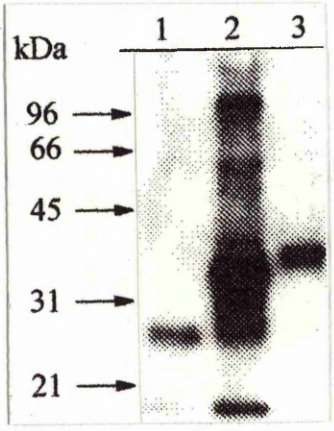


Figure 4.6.1 Extracts of proteins were run on 12 % SDS-PAGE and electroblotted to PVDF membrane. Lane 1, 10 ng of TbCRK1/H; lane 2, 10 ng of semipurified TbCRK3/H;lane 3, 2 ng of LmmCRK3/H. The membrane was incubated with TC3HG Immune antiserum (1:800).

One puzzling finding was the occasional detection of an approximately 26 kDa protein with various antisera in different life cycle stages. This was detected by TC3HG Immune serum in the supernatant of short stumpy form S100 extracts (see Figure 4.5.7, lane 3), CITAA in procyclic extracts (see Figure 4.5.2, lane 3), and also by EVREE in procyclic trypanosomes (Figure 4.6.2). These bands were not always detected, but the recognition of a similar sized band by the three antisera is striking. The CITAA and EVREE antisera are specific (at least among the CRK proteins) to CRK1 and TbCRK2 respectively. It is possible that TC3HG antiserum cross reacts with TbCRK2 as well as with TbCRK3 which adds another level of uncertainty as to whether the band seen in the short stumpy S100 supernatant is due to a partially digested TbCRK3 fragment. These bands would not be seen by PSTAIR box reactive antisera as the cleavage of the N-terminus to leave the C-terminal 230-240 amino acids (consistent with the mobility shift) would remove the PSTAIR region. It may be that there is a common pathway for the proteolytic destruction of the TbCRK proteins, presumably encoded by the primary sequence of the polypeptide chain. If the 26 kDa protein recognised by the TC3HG antiserum in short stumpy supernatant is a TbCRK3 fragment, and there is a common degradation pathway, then the region of TbCRK3 that results in the sub-cellular localisation must be contained within the N-terminal 90 residues.

Figure 4.6.2 A 26 kDa band detected by the EVREE antiserum.

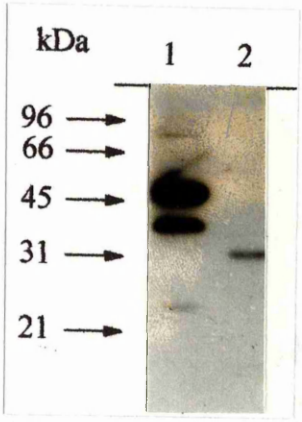


Figure 4.6.2 S100 protein extracts from procyclic *T. brucei* run on 12 % SDS-PAGE, electroblotted to PVDF membrane and probed with; lane1, EVREE antiserum (1:50); lane 2, CITAA antiserum (1:50).

CHAPTER FIVE
ANALYSIS OF TBCRK FUNCTION BY YEAST COMPLEMENTATION AND PROTEIN BINDING STUDIES

5.1 Introduction.

The inability to deduce the function of the TbCRK proteins based on amino acid sequence homology with CDK proteins with defined roles in other organisms (see Chapter 3.3), together with the expression data showing no clear variation between dividing and non-dividing form *T. brucei* (see Chapter 4.5), necessitated alternative approaches to the analysis of CRK function in trypanosomes. Two approaches were adopted.

(1) Genetic, using an assay based on complementation of a *S. pombe cdc2* temperature sensitive mutant. This method has been used to define *cdk* functional homology in other systems (Michaelis and Weeks, 1992a; Jimenez *et al.*, 1990; Lee and Nurse, 1987).

(2) Biochemical, by purification of active kinase complexes. It is possible to purify and characterise other proteins which interact with CDC2, and related proteins, by isolating the kinase complex itself.

(a) One method which has been used is to produce CDC2 related kinases in a heterologous system, e.g. *E. coli*, in a translational fusion with a protein sequence which allows purification, e.g. an antigen tag for which specific antibodies enable immunoprecipitation of the kinase (Fisher and Morgan, 1994), or a polyhistidine tag which enables affinity purification by metal-chelate chromatography. Tagged kinase then can be activated using a lysate from the cells of interest. The exogenous kinase can then be purified using the tag, and proteins co-purified characterised. In this study an analysis of proteins associating with the TbCRK kinase was attempted by activation of purified His-tagged TbCRK1.

(b) Another method used to try to purify CRK complexes, which had proved successful in other organisms, was to exploit the binding of the complex to the *S. pombe* protein, $p13^{suc1}$, or its homologues from other organisms (Azzi *et al.*, 1992; John *et al.*, 1991; Richardson *et al.*, 1990). This

protein can form multimeric complexes and binds CDC2 and closely related kinases with high affinity (see Chapter 1.2.4). A homologue of *S. pombe suc1* has been cloned from *L. mexicana* (*lmmcks1*) (Mottram and Grant, 1996). LmmCKS1 had been expressed and purified from *E. coli* and attached to an inert matrix to create an affinity column.

5.2 Complementation Assay of a *S. pombe cdc2* temperature sensitive mutant with *tbcrk3*.

5.2.1 Introduction.

A method previously used successfully to isolate *cdc2* homologues, and closely related proteins, from a wide variety of organisms was the expression of heterologous genes in yeast strains which contain mutations in the *cdc2/CDC28* genes conferring temperature sensitive phenotypes (Jimenez *et al.*, 1990; Lee and Nurse, 1987; Ninomiya-Tsuji *et al.*, 1991). These strains of yeast grow normally at low temperatures, but mis-sense mutations in the gene result in a protein which is unstable at higher temperatures. This results in the mutant cells being unable to complete the cell cycle when thay are incubated at the higher, restrictive, temperature. If the 'foreign' gene is able to restore growth at the restrictive temperature then it may be because it is homologous enough to the mutated gene to replace it. If this is the case then the heterologous protein is interacting with the components of the yeast cell cycle correctly, and it is therefore likely to be cell cycle regulating in the organism it was originally cloned from. It is also possible that genes restoring growth to the mutated yeast strains are suppressers of the mutation. In these cases the heterologous protein synthesised does not replace the mutated protein, instead it may bind to and stabilise the mutated cdc2/CDC28 protein at the restrictive temperature, resulting in a functional kinase. Therefore restoration of growth at the restrictive temperature has also been seen with

protein such as cyclins and $p13^{suc1}$ homologues (Surana *et al.*, 1991; Hayles *et al.*, 1986).

It had been previously shown that *lmmcrk1* could not complement a *S. pombe* *cdc2*-ts mutant (Mottram *et al.*, 1993). As LmmCRK1 and TbCRK1 share sequence motifs in most of the important CRK control regions (see Chapter 3.3), it was thought unlikely that *tbcrk1* would complement the yeast mutant. Also the *T. congolense* homologue, *tccrk1*, had been found to be unable to complement yeast mutants (N. Murphy, ILRAD, personal communication). *tbcrk2* had been shown to be unable to restore growth at the restrictive temperature in the same *S. pombe* mutant (J. Kinnaird, personal communication). It was decided to repeat the experiment with *tbcrk3* as it was thought possible that it was the *T. brucei cdc2* homologue. Although TbCRK3 lacked a well conserved PSTAIR box, the protein does have most of the other control regions relatively well conserved, e.g. the ATP binding region, the DSEI box and the region around Threonine-161 (see Chapter 3.3.5).

5.2.2 Cloning *tbcrk3* into a *S. pombe* expression vector, and transfection of *S. pombe* *cdc2-33*.

The full length *tbcrk3* ORF was generated using PCR with oligonucleotides designed to the region of the start ATG, and just after the Stop codon. Restriction enzyme sites were added to allow ligation of the gene into the *S. pombe* expression vector, pMNS21L (Figure 5.2.1). The vector encodes the *S. cerevisiae* LEU2 gene which can complement the *S. pombe leu1* gene, so that transfection into a *leu1*$^-$ strain of *S. pombe* allows selection of successfully transfected cells on plates lacking leucine. The promoter controlling the inserted gene's expression is repressed by the addition of 5 μg / ml thiamine to the growth medium (Maundrell, K. 1990). By plating on both repressing and non-repressing media, this theoretically enables the experiment to show that the rescue of mutant phenotypes is due to expression of the plasmid encoded gene, and not a reversion of the temperature sensitive *cdc2* nuclear copy to wild-type.

Figure 5.2.1 The *S. pombe* complementation vector and *tbcrk3* oligonucleotides.

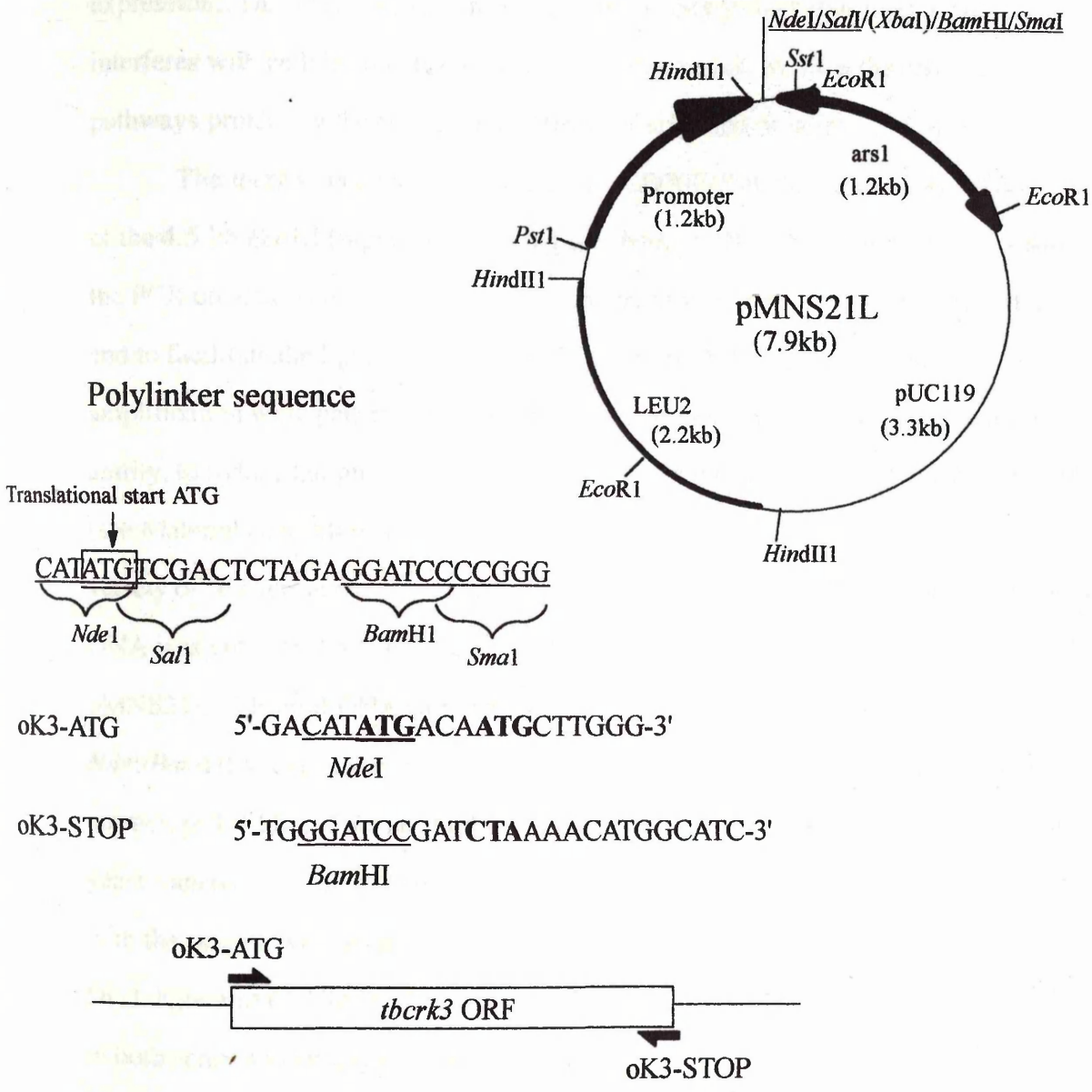


Figure 5.2.1. The *S. pombe* expression vector used for complementation studies was pMNS21L. It allows controlled expression of the gene of interest, regulated by the presence or absence of thiamine.

oK3-ATG and oK3-STOP are the oligonucleotides used to perform PCR so that *tbcrk3* could be cloned into pMNS21L. The underlined bases are the engineered restriction sites for correct insertion into pMNS21L. The bold bases are the probable initiating ATG's and the stop codon.

However, we subsequently discovered that the repressed state still results in low level expression of the gene, and that the unrepressed state should cause extremely high level expression. This may result in problems with toxicity if the heterologous protein interferes with cellular functions, membrane integrity, or swamps the chaperone pathways protecting the cell from the effects of unfolded proteins.

The *tbcrk3* gene was amplified from pCD70E(S)8, the insert of which consists of the 4.5 kb *Eco*RI fragment containing the *tbcrk3* ORF. The oligonucleotides used for the PCR created an *Nde*I site at the 5' end of the *tbcrk3* gene and a *Bam*HI site at the 3' end to facilitate the ligation of the *tbcrk3* ORF into pMNS21L. 10 rounds of amplification were performed using *pfu* DNA polymerase, which has proof reading ability, to reduce the probability of mutations being introduced into the *tbcrk3* sequence (see Materials and Methods). Portions of the amplified DNA were mapped with a variety of restriction enzymes as a partial check for mutations. The rest of the amplified DNA was cut with *Nde*I and *Bam*HI, followed by ligation into *Nde*I/*Bam*HI cut pMNS21L. Plasmid DNA from transformed colonies was mapped with *Hin*dIII and *Nde*I/*Bam*HI to check for insertion of the correct DNA. One plasmid was found to be correct, pCD144m. This plasmid was renamed pMNS/*tbcrk3*. Plasmid DNA for the yeast transfection was isolated using a large scale QIAGEN plasmid preparation with both the original vector and pMNS/*tbcrk3*. Other control plasmids were provided by Dr. J. Kinnaird (WUMP). These consisted of the human *cdc2* gene, also in pMNS21L, in both sense and antisense orientations (pMNS/*Hscdc2*+ and pMNS/*Hscdc2*- respectively), and the *S. pombe cdc2* gene, this time in another, non-repressible, vector (p*Spcdc2*). The mutant *S. pombe* strain, *cdc2*-33, *leu1*-32h$^-$, grew normally at 25 ^{o}C, and did not divide at 36 ^{o}C due to the *cdc2*-33 mutation. The transfection was carried out using the Lithium acetate method (see Materials and Methods). Aliquots of the cells were spread onto 6 plates of minimal medium with or without thiamine (+/-thi). After 12-16 hr at 25 ^{o}C, for recovery, 3 plates from each set of 6 were moved to 36 ^{o}C, the restrictive temperature, and the plates left to grow for 3 days.

5.2.3 Results of the complementation assay.

Table 5.2.1 shows the results of the complementation assay. The average number of colonies per plate, formed after 3 days at the indicated temperature are shown. The transfections using vectors based on pMNS21L all resulted in more than 100 colonies per plate at the non-restrictive temperature, with or without thiamine. Therefore, there is no toxicity associated with TbCRK3 expression. pMNS/*tbcrk3* was found to be unable to complement the yeast mutation at the restrictive temperature. However, both plasmids containing the human *CDC2* gene complemented the yeast mutation with limited penetration. The plasmid containing the *S. pombe cdc2* gene gave a very low level of transfection, but given that, complementation occurred in most cases. Equivalent results were obtained using the same control plasmids and testing for complementation with a *Theileria annulata cdc2* related gene, *tacrk1* (Kinnaird *et al*, 1996). The low level of complementation by both of the *Hscdc2* containing plasmids was due to a mistake in cloning the gene. The 5' primer for the PCR had been designed to be complementary to sequence upstream of the ORF instead of being complementary to the sequence around the initiator ATG. This may have resulted in the cloning of part of the promoter region, which might have caused low level expression of the human protein from both plasmids. This may explain the increase in the number of cells complemented when thiamine was present, as in its absence the gene would be predominantly transcribed either from further upstream (pMNS/*Hscdc2*+) resulting in translation initiating before the *in vivo* start ATG, or the opposite strand would be the major mRNA (pMNS/*Hscdc2*-), presumably resulting in low levels of the sense strand RNA being available for translation.

Table 5.2.1 Results of the complementation assay.

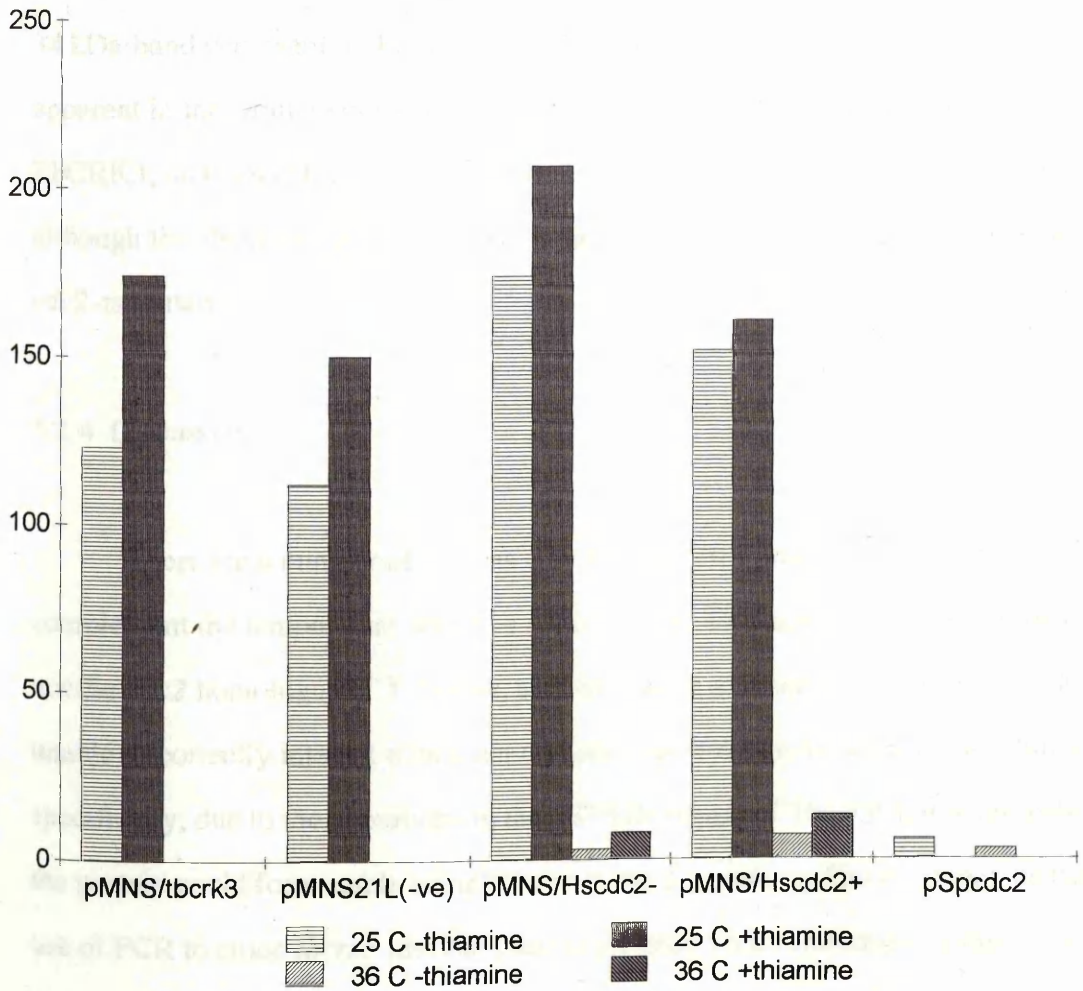
	25°C -thiamine	25°C +thiamine	36°C -thiamine	36°C +thiamine
pMNS/*tbcrk3*	123	174	0	0
pMNS21L(-ve)	112	150	0	0
pMNS/*Hscdc2-*	174	207	3	8
pMNS/*Hscdc2+*	152	161	7	13
p*Spcdc2*	6	ND	3*	ND

Table 5.2.1 The numbers in the table are the average number of colonies per plate formed after 3 bays at the indicated temperature.

ND - Not done as the plasmid promoter was not repressed by thiamine.

* - Only the result of one plate due to fungal contamination.

Figure 5.2.2 Bar chart showing the complementation results.



The vertical scale shows the average number of colonies per plate.

The fact that similar numbers of colonies containing pMNS/*tbcrk3* grew at 25 ^{o}C in the absence and presence of thiamine implies that expression of the *tbcrk3* gene is not toxic to the yeast cells. In order to show that the TbCRK3 protein was being expressed in the yeast and to prove that even though the protein was present the gene was unable to complement the *cdc2* mutation, Western blots were performed. Protein extracts of yeast cultures, grown at 25 ^{o}C, containing pMNS/*tbcrk3* or pMNS/*Hscdc2+* were made. These were then run on SDS-PAGE, blotted to nitro-cellulose membrane and probed with both the pre-immune and immune TC3H/G antiserum (Figure 5.2.3). Although both the pre-immune and the immune antisera recognise many different proteins in the yeast whole cell extracts, one significant difference is detected between the *tbcrk3* and *Hscdc2* containing strains when probed with the immune antiserum. A 34 kDa band is present in the strain containing pMNS/*tbcrk3* (lane 4) which is not apparent in the strain expressing HsCDC2 (lane 3). This band is the expected size of TbCRK3, and is most likely to correspond to this protein. The data suggest that although the *tbcrk3* gene is expressed in the yeast cells it is unable to complement the *cdc2*-ts mutant.

5.2.4 Discussion.

There are a number of reasons why the TbCRK3 protein could fail to complement the temperature sensitive *cdc2* mutant. The most obvious is that *tbcrk3* is not the *cdc2* homologue of *T. brucei*, and that due to sequence divergence TbCRK3 is unable to correctly interact with other components of the yeast cell cycle machinery; specifically, due to the alterations in the PSTAIR region of TbCRK3, it is unlikely that the protein could form stable complexes with the *S. pombe* cyclin(s). It may be that the use of PCR to clone *tbcrk3* into the yeast expression vector introduced a mutation into *tbcrk3*. The detection of the (approximately) correctly sized protein in the yeast extracts would appear to rule out a premature stop codon being introduced, but to

Figure 5.2.3 Western blot showing the expression of TbCRK3 in *S. pombe*.

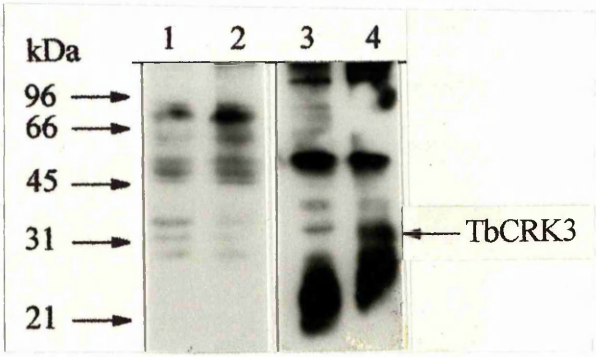


Figure 5.2.3 Protein extracts from *S. pombe* strains containing *tbcrk3* or human *cdc2* were probed using TbCRK3 reactive antiserum. Lanes 1 and 3 contain whole cell extracts from *S. pombe* expressing *Hscdc2*, lanes 2 and 4 are whole cell extracts from *S. pombe* containing *tbcrk3*. The cells were grown at 25 ^{o}C before lysis. The extracts were diluted into 1 x FSB and run on 12.5 % SDS-PAGE. The proteins were electroblotted onto nitro-cellulose and incubated with either pre-immune antiserum (lanes 1 and 2), or immune T3H/G antiserum (lanes 3 and 4), before detection with the ECL system.

absolutely rule out a missense mutation it would be necessary to resequence the gene. This was not achieved in the time available. Another possible explanation is suggested by the evidence that TbCRK3 is associated with insoluble components in the *T. brucei* cell (see Chapter 4.5). This could result in any complex formed in yeast being incorrectly targeted. There is also the possiblity that the *T. brucei* protein is unable to phosphorylate the correct yeast substrates. In addition the TbCRK3 protein may only be active in insect form trypanosomes, and therefore may be inactive at temperatures above those found in the fly (approximately 28 ^{o}C). One way to address this question would be to try complementation of a cold sensitive mutant.

The complementation data suggest that *tbcrk3* is not the *cdc2* homologue, however a full assessment of TbCRK3 activity during the cell cycle of the different life cycle stages is required to answer this question. Although *CDC2* genes (and closely related genes e.g. *CDK2*) from many organisms can complement yeast mutants, no protozoan *crk* genes have so far been shown to function in this assay e.g. *Theileria* (Kinnaird *et al.*, 1996), *Leishmania* (Mottram *et al.*, 1993) and *Plasmodium* (Ross-McDonald *et al.*, 1994), although the *Plasmodium* gene did not express, so the protein may still complement if expression could be achieved. Either none of these genes are the *cdc2* homologue from that organism or, more likely, the evolutionary divergence between yeast and the protozoans has resulted in cell cycle components that cannot interact with each other adequately. It is not possible, at present, to assay TbCRK3 activity through a typical cell cycle as trypanosomes cannot be successfully synchronised, but it may be possible to reach some conclusions from other experiments, such as the binding of kinases to p13^{suc1} (or its homologues) (see Chapter 5.4), and reverse genetic approaches (see Chapter 6).

5.3 The activation of histidine tagged TbCRK proteins *in vitro*.

5.3.1 Introduction.

One method previously used to characterise non-kinase components of the CDK complexes was to use tagged CDK proteins which could be purified from cell lysates. The tagged CDK proteins were either expressed *in vitro* (Fisher *et al*, 1994; Poon *et al*, 1993), or *in vivo* (Desai *et al*., 1992), and the complexes isolated by immunoprecipitation. Other components of the complexes could by analysed by SDS-PAGE, Western blotting and protein sequencing. These modified CDK's were apparently functional *in vivo*, and therefore, presumably, both associated with their normal regulatory subunits and were under the control of the same post-translational modifications as the untagged, wild-type proteins. The poly-histidine tag used in the previous chapter allows purification of the tagged protein by metal-chelator affinity chromatography. As the expression and purification of TbCRK1/H and TbCRK3/H had already been performed successfully these proteins were considered suitable for this experiment.

A second approach designed to produce histidine tagged TbCRK complexes was the use of homologous recombination to replace a wild type copy of the *tbcrk* gene with the histidine tagged version. This would then theoretically allow purification of the *in vivo* kinase complex directly from *T. brucei* cell extracts.

5.3.2 Activation of *E. coli* expressed TbCRK1/H.

TbCRK1/H was used for the attempted *in vitro* activation and purification as it was expressed at high level in *E. coli* (unlike TbCRK2/H) and could be isolated in a relatively pure form, and it was soluble (unlike TbCRK3/H) and so the activated complex could be purified using non-denaturing conditions which would not be expected to disrupt the kinase complex. The protocol used closely followed those

which resulted in the activation of HsCDC2, HsCDK2 and HsCDK4 by CAK in other systems (Fisher *et al*, 1994; Poon *et al*, 1993). Purified TbCRK1/H was incubated with *T. brucei* cell lysates made from log phase procyclic form cultures, using a number of different methods of cell lysis including sonication and Triton X-100. ATP and Mg^{2+} were also added to the incubations, following the protocols used for CDK activation in other systems (Solomon *et al.*, 1993; Desai *et al.*, 1992). Control incubations using either no *T. brucei* cell extract or no purified TbCRK1/H were performed in parallel with the attempted activation. Because of the histidine/Ni^{2+}/agarose purification system used, the solutions used for lysis and activation had to be EDTA free, as this chelator would strip the Ni^{2+} from the agarose support (see Chapter 4.3.1).

In the first experiment a cell extract was made by sonicating the procyclic *T. brucei* in Lysis Solution with protease inhibitors, without EDTA and without Triton X-100 (LSI-ET). The protease inhibitors used were; 1, 10 phenanthroline (inhibits Zn^{2+} metallo-proteases), pepstatin A (inhibits aspartic, and many acidic, proteases), leupeptin (inhibits some serine and cysteine proteases) and phenylmethylsulfonylfluoride (PMSF), which irreversibly inhibits serine proteases. Affinity purified TBCRK1/H was added to the whole cell extract and incubated for 30 minutes at either 4 or 25 ^{o}C. The incubated extracts were then centrifuged at 4 ^{o}C for 45 minutes at 15 000 rpm in a microfuge, and the supernatant transferred to another tube. One fifth of the supernatant (10 μl) was retained for a histone H1 kinase assay, while the rest was added to 10 μl of Ni^{2+}/agarose bead slurry, allowed to bind for 30 minutes at 4 ^{o}C before being centifuged and washed twice with 0.5 ml of Kinase Assay Buffer without EGTA (KAB-E). Protein bound to the beads was assayed for histone H1 kinase activity *in situ*. The pelleted cell debris was resuspended and an aliquot used in the kinase assay. For the assay, each sample was added to a Kinase Assay Mix (KAM) containing ^{32}P-γATP and histone H1. 20 μl of each 50 μl assay was run on 12 % SDS-PAGE, stained with coomassie blue 250, then dried down and exposed to X-ray film (see Materials and Methods).

Figure 5.3.1 shows the results of the experiment performed at 25 ^{o}C. The experiment performed at 4 ^{o}C gave identical results. The lack of radioactive histone H1 in lanes 1, 4 and 7 show that purified TbCRK1/H from *E. coli* has no, or very low, kinase activity on its own. The low level of signal in lane 1 is possibly due to spillage from the next lane. The histone H1 kinase activity in the *T. brucei* extract under these conditions is almost entirely in the fraction associated with the insoluble cell components (compare lanes 2 and 3 with 5 and 6). The other bands show phosphorylation of *T. brucei* proteins by cellular kinases. It may be that the predominantly insoluble nature of the kinase activity is contributed to by incomplete lysis of the cells. However, *T. brucei* lysate supernatant plus TbCRK1/H (lane 5), shows elevated histone H1 kinase activity compared to both the TbCRK1/H only (lane 4) and the *T. brucei* supernatant only (lane 6). It would appear that without the presence of detergent in the lysis buffer the TbCRK protein complexes are largely insoluble. The data suggest that TbCRK1/H is interacting with components of the lysate to give a low level of soluble active kinase. With the number of steps involved in activation of CDK kinases, it would seem unlikely that TbCRK1/H is being activated by a protein on the exterior of the procyclic trypanosomes. It may be that the added TbCRK1/H is forming active complexes with excess, soluble, regulatory subunits and being phosphorylated by kinases in the insoluble fraction. An alternative possibility is that the addition of an excess of TbCRK1/H may release the wild type, active TbCRK1 complex from the insoluble fraction by competing for whatever is binding to the TbCRK1. In this experiment no activity above background was recovered on the Ni^{2+}/agarose beads, which may be due to the low level of activation and inefficient binding of the Ni^{2+}/agarose to the histidine tag.

Other experiments were performed using *T. brucei* procyclic extracts lysed with Triton X-100 instead of sonication. These extracts were split into two. One portion was centrifuged for 30 minutes at 100 000 g (S100) prior to addition of the TbCRK1/H, the other portion was centrifuged after the 30 minute incubation with TbCRK1/H.

Figure 5.3.1 Activation of TbCRK1/H using sonicated *T. brucei* procyclic extract, without added Triton X-100

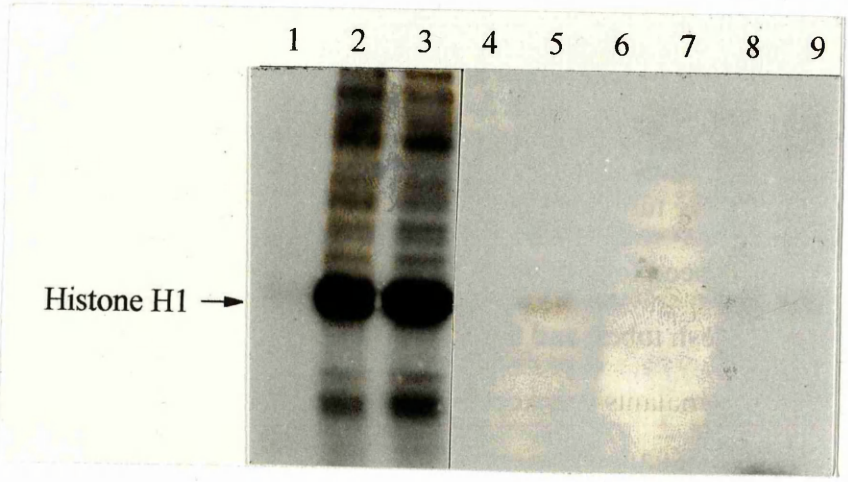


Figure 5.3.1 Autoradiograph showing histone H1 kinase activity of bacterially expressed TbCRK1/H incubated with *T. brucei* procyclic extract. The cell extract was prepared by pelleting 10 ml of log-phase (~8 x 10^6 cells / ml) procyclic *T. brucei*, washing with PBS, resuspending in 500 μl of Lysis Solution plus Inhibitors, without EDTA, without Triton X-100 (LSI-ET) and sonicating, on ice, for 3 x 20 seconds. To one 38 μl aliquot of cell extract was added 10 ng (in 5 μl) of *E. coli* expressed purified TbCRK1/H, to the another 38 μl aliquot was added 5 μl of the buffer used for TbCRK1/H purification. A third tube was prepared with 10 ng of TbCRK1/H and LSI-ET only. After the tubes were incubated for 30 minutes at 25 ^{o}C the mixtures were pelleted at full speed for 30 minutes in a microfuge at 4 ^{o}C. The supernatants were transferred to fresh tubes, and the pellets resuspended in LSI-ET. A 10 μl aliquot of each of the supernatants was kept aside for the kinase assays. The supernatant was then added to 10 μl of Ni^{2+}/agarose bead slurry, incubated for 30 minutes at 4 ^{o}C, and washed twice with 0.5 ml of Kinase Assay Buffer (KAB). The beads, the retained supernatant and 1 μl of the resuspended pellet fraction were used for the histone H1 kinase assays. Lanes 1, 4 and 7 contain kinase assays performed with the incubations containing only purified TbCRK1/H, lanes 3, 6 and 9 contain no added TbCRK1/H and lanes 2, 5 and 8 show the results of TbCRK1/H incubated with *T. brucei* extract. Lanes 1-3 are kinase assays performed with the resuspended pellet, lanes 4-6 used the supernatant fraction and 7-9 contained the Ni^{2+}/agarose beads.

In both cases the activation incubation was performed at room temperature. The selection and kinase assays were performed in the same manner as before.

The assays using the Triton X-100 lysed extracts are shown in Figure 5.3.2. Panel (A) shows the kinase assays using the whole cell extracts for the incubation with TbCRK1/H, while panel (B) shows the results of a parallel experiment using the S100 extracts for TbCRK1/H activation. The addition of Triton X-100 solublises much of the kinase activity [compare Figure 5.3.2(A), lanes 1 and 3, and Figure 5.3.1, lane 3]. Most of the histone H1 kinase activity is now present in the soluble fraction [Figure 5.3.2(A), lane 3], although considerable kinase activity is still detected in the assay with the insoluble fraction [Figure 5.3.2(A), lane 1]. It is plausible that some of this activity is due to TbCRK3, which is probably insoluble under these lysis conditions in bloodstream form *T. brucei* (see Chapter 4.5.2). Panel (A) lane 3 and Panel (B) lane 1 show phosphorylation of higher molecular weight *T. brucei* proteins, as well as a very high level of phosphorylation of low molecular weight proteins. The coomassie staining of the SDS-polyacrylamide gel showed that these lower bands were predominantly products of histone H1 degradation. The same degradation, though less complete, occurred in the assays using the Ni^{2+}/agarose selected extracts [Panel (A) lane 5, Panel (B) lane 3] showing that there was notable carry over of proteases, and therefore presumably other proteins, on the Ni^{2+}/agarose matrix. In both experiments the assay using the Ni^{2+}/agarose beads in the presence of TbCRK1/H [Panel (A) lane 6, Panel (B) lane 4] shows a slightly higher level of kinase activity than the control lanes [Panel (A) lane 5, Panel (B) lane 3]. Although the increase is not substantial it is consistent. It is therefore possible that activation of the TbCRK1/H protein is occuring, and it appears possible to bind the active kinase complex to the Ni^{2+}/agarose beads. If TbCRK1/H is being activated then the components responsible for this must be in the soluble fraction of the Triton X-100 lysed cells as S100 cell extracts are competent to activate the kinase. Due to the insoluble nature of TbCRK3 this experiment implies that TbCRK3 is not the *T. brucei* CDK activating kinase.

Figure 5.3.2 Activation of TbCRK1/H using a *T. brucei* Triton X-100 procyclic extract.

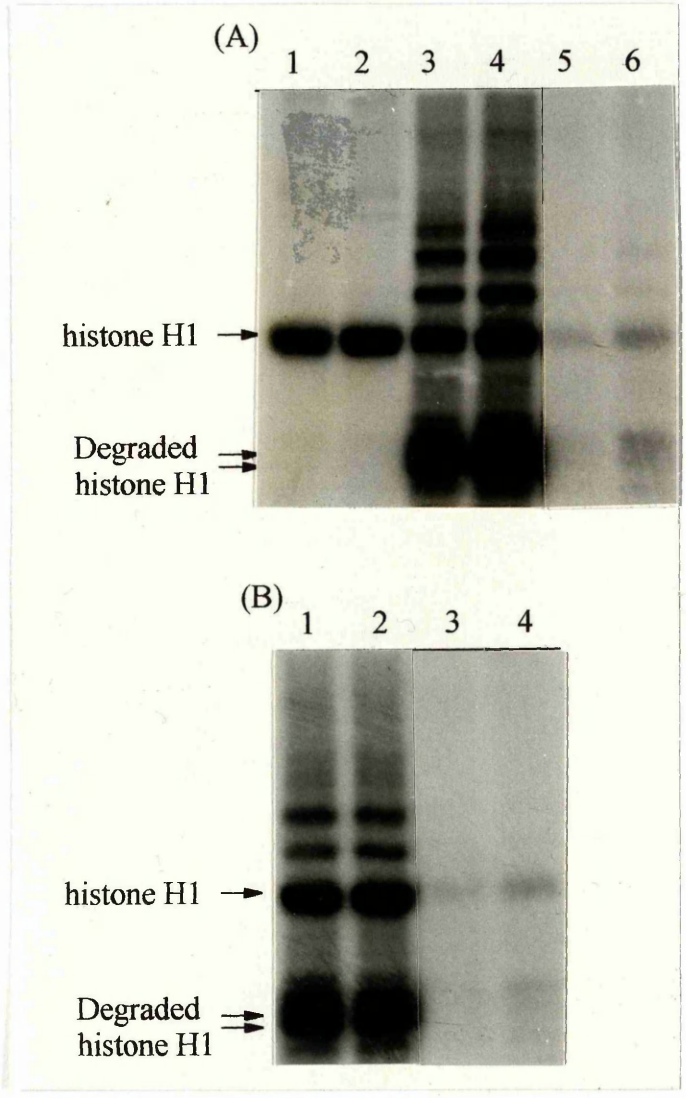


Figure 5.3.2 Histone H1 kinase assays of either bacterially expressed TbCRK1/H incubated with *T. brucei* procyclic extract or just *T. brucei* procyclic extract. The extract was prepared by pelleting log phase procyclic *T. brucei*, washing with PBS and resuspending in Lysis Solution plus Inhibitors, without EDTA (LSI-E) which included 0.5 % Triton X-100. The extract was divided into two aliquots. One aliquot was processed as described in Figure 5.3.1(A), except that 20 ng of TbCRK1/H were used [Panel (A)]. The other aliquot was centrifuged at 100 000 g and the supernatant (S100) used for the activation incubation [Panel (B)]. As no kinase activity had been detected with the purified TbCRK1/H in the previous experiment, the TbCRK1/H only control was not performed.

Panel (A), lanes 1, 3 and 5 contain no added TbCRK1/H, lanes 2, 4 and 6 show the results of TbCRK1/H incubated with whole cell *T. brucei* extract. Lanes 1 and 2 are kinase assays performed with the pellet resuspended after the activation incubation, lanes 3 and 4 used the supernatant fraction and the assays in lanes 5 and 6 contained the Ni^{2+}/agarose beads.

Panel (B), lanes 1 and 3 contain no added TbCRK1/H, lanes 2 and 4 show the results of TbCRK1/H incubated with S100 *T. brucei* extract. Lanes 1 and 2 contain kinase assays using the supernatant fraction and lanes 3 and 4 show the assays with the Ni^{2+}/agarose beads.

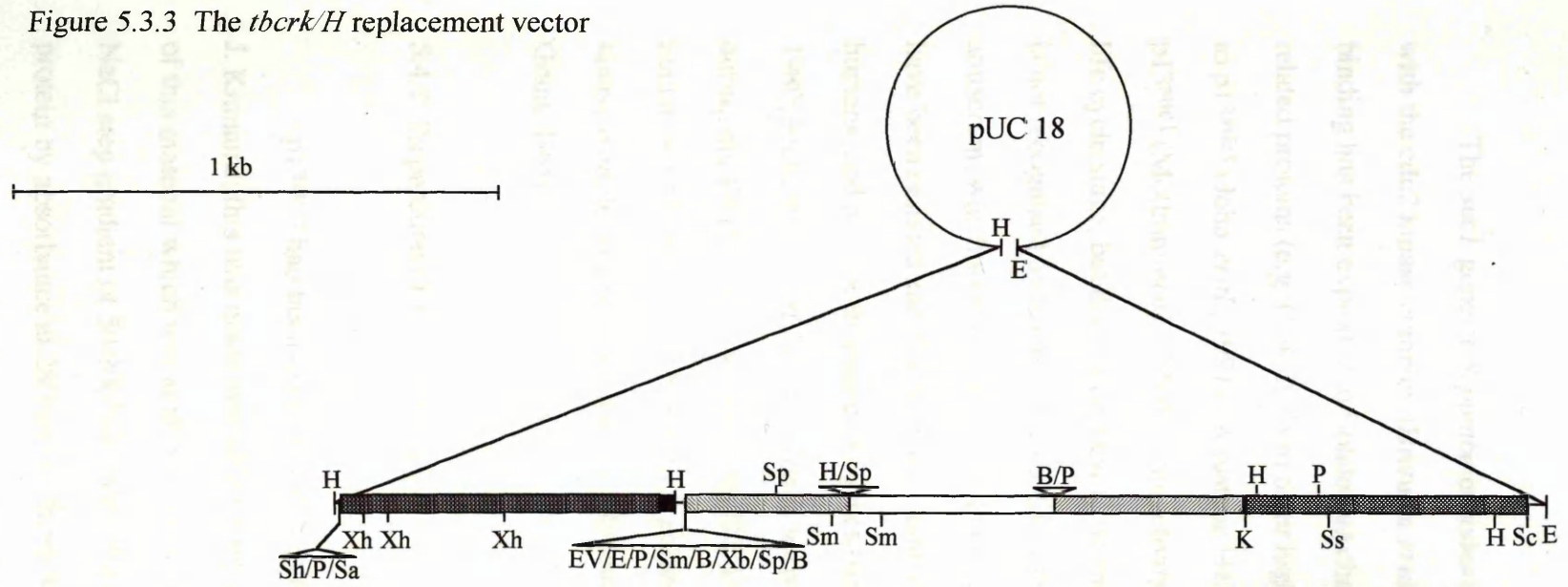
It was not possible to reduce the degradation of the histone H1 in this system (which lacked EDTA), even using a number of different protease inhibitors. Although degradation of *T. brucei* extracts was not seen when 10 mM EDTA was added to the Lysis Solution, this was not an option in this experiment as EDTA would have removed the Ni^{2+} from the agarose matrix. As the detected proteolysis occurs during the kinase assay, after the activation incubation, it seems likely that both the *T. brucei* proteins and the added TbCRK1/H would suffer degradation. This could have been checked by Western blotting with CITAA antiserum. Any degradation would explain the poor recovery of activity on the Ni^{2+}/agarose beads. It also would make analysis of the active kinase complex difficult. With considerable proteolysis occurring in the samples it seemed unlikely that characterisation of other components in the complex could be carried out. Due to the inability to inactivate what appears to be a high level of metallo-protease activity without the chelating effect of EDTA it was decided to attempt other methods of CRK complex purification (see Chapter 5.4).

5.3.3 Attempted *in vivo* histidine tagging of TbCRK3.

One approach to isolating active TbCRK kinase complexes was the integration of a tagged *tbcrk* gene into the genomic locus of the gene in place of a wild type allelle. It was thought that it may be possible to integrate a copy of *tbcrk3/H* into the *T. brucei* genome in place of one or both wild type alleles, and then use the poly histidine tagged protein to purify the kinase complex. This was prior to the knowledge that TbCRK3 is insoluble in the standard lysis solutions, and that proteolysis was difficult to inhibit without EDTA present. Although the vector was constructed (Figure 5.3.3), the replacement of one allele of the wild type gene was not successfully accomplished. It is possible that the C-terminal histidine tag may interfere in TbCRK3 function, for example giving a dominant negative phenotype in which case the integration event would not be detected. A number of dominant *cdc2* mutations have been found making the scenario of a single replacement causing non-viability possible (Fleig and Nurse,

1991). However, C-terminal epitope tags in other systems have had little or no effect on CDK protein function (Desai *et al.*, 1992), and a histidine tag on the C-terminus of the *L. mexicana* CRK1 protein caused no effect (Mottram *et al.*, 1996). As the transfection was only performed once, it may simply be due to a technical problem in the experiment causing a lack of transfection or integration. Once it became clear that TbCRK3 was insoluble it seemed likely that the use of *in vivo* tagged TbCRK3 to isolate the kinase complex would not be such a useful approach. Treatment to solubilise the protein is likely to destroy the protein/protein interactions which hold the complex together. Also it is evident from the failed attempt to purify TbCRK2/H that effective purification of poorly expressed histidine tagged proteins is difficult, and according to the manufacturers purification from eukaryotes is generally more difficult than from *E. coli* (QIAGEN). The degradation of added histone H1 during the attempted activation of TbCRK1/H showed that without EDTA in the lysis solution, *T. brucei* extracts contain active proteases, showing the importance of metallo-protease inhibition. It is therefore likely that using an epitope tagged version of the *tbcrk* genes would be a better approach to complex purification. This method has been used successfully in other organisms and recently in *T. brucei* (Bastin *et al.*, 1996) and does not require a lack of EDTA, so proteolysis could be limited by the addition of high concentrations of the chelator to the cell extract. The epitope tag approach may not solve the problem of TbCRK3's insolubility however, as immunoprecipitation of insoluble proteins is difficult. Assessment of the function and components of this kinase complex may have to occur by other methods, such as reverse genetics.

Figure 5.3.3 The *tbcrk/H* replacement vector



B -	*BamHI*	E -	*EcoRI*	EV-	*EcoRV*	H -	*HindIII*	K -	*KpnI*
P -	*PstI*	Sa -	*SalI*	Sc -	*SacI*	Sh-	*SphI*	Sm-	*SmaI*
Sp -	*SpeI*	Xb -	*XbaI*	Xh-	*XhoI*				

tbcrk3 ORF.

PARP promotor and Splice Acceptor site.

PARP 3' untranslated region.

ble resistance gene ORF.

PCR fragment containing 5' end of the *tbmefg* ORF (*Kpn*I and *Sac*I cloning sites were engineered as part of the PCR oligonucleotides).

DNA coding for a poly-histidine tag.

Figure 5.3.3 The *tbcrk3* Histidine Integration Vector (HIV) was constructed using the tagged *tbcrk3* gene previously used to express, purify and raise antibodies to TbCRK3/H, the *ble* expression cassette which gives resistance to the antibiotic phleomycin in procyclic *T. brucei*, and a PCR product containing the 5' end of the *tbmefg* gene amplified from pCD70E(S)8 with oligonucleotides which had been designed with *Kpn*I and *Sac*I restriction enzyme sites added to enable cloning into the HIV vector. The integration cassette was cut from the vector with *Xho*I and *Sac*I and gel purified before transfection into procyclic *T. brucei*.

5.4 Isolation of TbCRK proteins by binding to p12^{LmmCKS1}

5.4.1 Introduction

The *suc1* gene of *S. pombe* encodes a 13 kDa protein which associates tightly with the cdc2 kinase complex (Brizuela *et al.*, 1987; Hindley *et al.*, 1987). This tight binding has been exploited to isolate and characterise cdc2 homologues, and closely related proteins (e.g. CDK2), from other higher eukaryotes, as these proteins also bind to p13^{suc1} (John *et al.*, 1991). A histone H1 kinase activity from *L. mexicana* binds to p13^{suc1} (Mottram *et al.*, 1993). This activity correlates with the division status of the life cycle stage, but does not appear to be encoded by *lmmcrk1* or *lmmcrk3* as the kinase is not recognised by LmmCRK1 specific antisera or the PSTAIR monoclonal antiserum, which would be expected to recognise LmmCRK3. Homologues of *suc1* have been isolated and cloned from a number of organisms, including *S. cerevisiae* and humans, and have been named *cks* genes (for cdc2 kinase subunit)(Hadwiger *et al.*, 1989; Richardson *et al.*, 1990). The *L. mexicana* homologue, *lmmcks1* was isolated during my Ph.D. The predicted LmmCKS1 protein is 11 kDa with 72 % identity to human p9^{CKS1} and 50 % identity to *S. pombe* p13^{suc1}. p12^{LmmCKS1} binds yeast cdc2 kinase complexes implying that it is a functional homologue of p13^{suc1} (Mottram and Grant, 1996).

5.4.2 Preparation of p13^{suc1} beads

p13^{suc1} had been expressed in *E. coli* and semi-purified by gel filtration by Dr. J. Kinnaird; this was made available for my studies. HPLC was performed with 3 mg of this material which was applied to an anion exchange column and eluted using a NaCl step gradient of 50/200/500 mM. Fractions were assessed for the presence of protein by absorbance at 280nm and those with high readings were run on 17.5 % SDS-PAGE and stained with Coomassie blue R-250. Fractions 75 to 81 inclusive were

pooled and dialysed into 0.1 M phosphate buffer suitable for binding the protein onto amino-link gel matrix (Pierce). The dialysed sample was then concentrated by covering the dialysis tubing with PEG-6000 for approximately 1 hour. The concentration of the solution was then assayed by 280 nm readings and Bradford assays, which gave the value of approximately 2 mg / ml in 0.75 ml of buffer. 0.5 ml of $p13^{suc1}$ was then bound to 0.5 ml of the amino-link matrix to give a 2 mg / ml matrix (see Materials and Methods). Determination of the protein concentration left in solution after the binding reaction showed that 87 % of the $p13^{suc1}$ was successfully bound to the matrix. 2 ml of control beads, with Tris-HCl used as the coupling reagent instead of $p13^{suc1}$ protein, were made in parallel. Similar beads, at a protein concentration of 5 mg / ml, were made using histidine tagged $p12^{LmmCKS1}$, expressed in *E. coli*, and purified using Ni^{2+}/agarose (Mottram and Grant, 1996).

5.4.3 Binding histone H1 kinase activity to $p13^{suc1}$/$p12^{LmmCKS1}$ beads

As a $p13^{suc1}$ binding kinase had been described in *L. mexicana* (Mottram *et al.*, 1993) we were interested in whether there was a similar activity in *T. brucei* extracts. S100 protein extracts were prepared from frozen pellets of long slender, short stumpy and procyclic form *T. brucei*. These were incubated with $p13^{suc1}$ and control beads at 4 ^{o}C for 2 hours, washed extensively with Lysis buffer plus Inhibitors and then assayed for histone H1 kinase activity (see Materials and Methods). The phosphorylated proteins were often kept at -20 ^{o}C overnight. 20 μl of the 50 μl kinase assay was run on 15 % SDS-PAGE, the gel stained with Coomasie blue R-250, and then dried onto 3MM paper. The gel was stained with coomasie to check if the added histone H1 (typically the only visible bands) had been degraded during the assay. The dried gel was then exposed to X-ray film overnight. Figure 5.4.1 shows the result of histone H1 kinase assays performed with extracts from long slender (lanes 1&2), short stumpy (lanes 3&4), and procyclic (lanes 5&6) form *T. brucei* that had been bound to either control (odd lanes) or $p13^{suc1}$ (even lanes) beads. A low level of kinase activity above

background was detected in the long slender, and procyclic lanes (lanes 2&6) but this was not repeatable and indeed in some later assays the control beads were found to have higher activities than the p13^{suc1} beads. To check that the p13^{suc1} beads and the other components of the assay system were functioning correctly the later experiments included assays performed with protein extracts from *S. pombe* which had been bound to the p13^{suc1} beads in parallel with the *T. brucei* extracts. Figure 5.4.2 shows one such experiment. High levels of kinase activity were found to bind to the p13^{suc1} beads using the *S. pombe* protein extracts (lanes 3&4), thus implying no fundamental problem with the components of the assay system.

Similar experiments were carried out with the p12^{LmmCKS1} beads and a procyclic extract. A significantly higher histone H1 kinase activity was detected bound to the p12^{LmmCKS1} beads in comparison to the control or the p13^{suc1} beads (Figure 5.4.3). This is presumably due to the putative *cks* gene of *T. brucei* encoding a protein which is considerably more homologous to p12^{LmmCKS1} than to p13^{suc1}. A number of other proteins, presumably from the procyclic extract, were also phosphorylated during the kinase assay. The 23 kDa band is most likely to be degraded histone H1 as it could be seen on the Coomasie stained gel, and proteolysis was occasionally seen when the protease inhibitors had not been made up immediately prior to the experiment. The higher molecular weight bands can be seen in other kinase assays performed with procyclic *T. brucei* extracts (see Figures 5.3.1, 5.3.2 and 5.4.2), but the marked lower 2 bands (21 kDa and 18 kDa) appear specific to the extract purified on the p12^{LmmCKS1} beads, and may therefore be components of a kinase complex. To assess whether it was possibly TbCRK protein kinase activity that was being detected with this assay, proteins bound to the beads were eluted, run on 12.5 % SDS-PAGE, blotted onto nitro-cellulose and probed with antisera tested for TbCRK reactivity.

Figure 5.4.1 Histone H1 kinase assay of *T. brucei* extracts bound to p13^{suc1} beads.

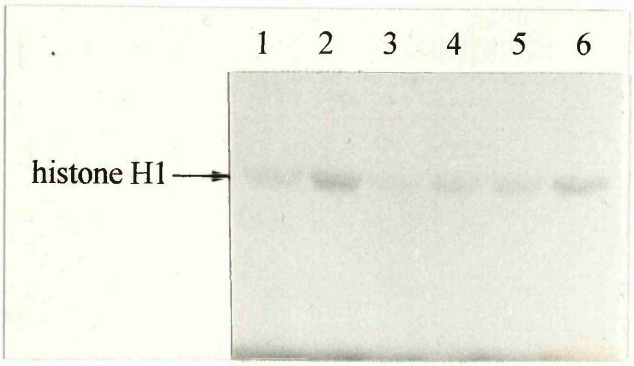


Figure 5.4.1 Histone H1 kinase assays using trypanosome extract bound to either p13^{suc1} beads (lanes 2, 4 and 6) or control amino-link beads (lanes 1, 3 and 5). S100 extracts were made from procyclic form (lanes 1 and 2), short stumpy form (lanes 3 and 4) and long slender form (lanes 5 and 6) *T. brucei.* 250 μl of 2 mg / ml extract were incubated with 50 μl of the 50 % bead slurry at 4 ^{o}C for 2 hours with constant agitation. After repeated 250 μl washes (1 x LSI, 2 x LSI+1M NaCl, 1 x LSI, 1 x Kinase Assay Buffer) the beads were transferred to eppendorf tubes for kinase assays. 20 μl of each 50 μl reaction were then run on 15 % SDS-PAGE, stained with Coomassie blue to check if there had been histone degradation, dried down and exposed to X-ray film.

Figure 5.4.2 Histone H1 kinase assay of *T. brucei* procyclic and *S. pombe* extracts bound to $p13^{suc1}$ beads.

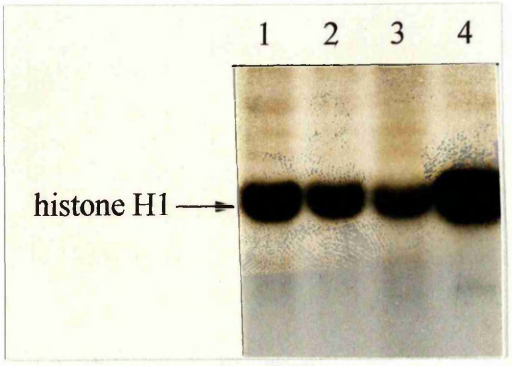


Figure 5.4.2 Histone H1 kinase assays performed with freshly centrifuged cultured *T. brucei* procyclic form extracts (lanes 1 and 2) or *S. pombe* extracts (lanes 3 and 4). The S100 extracts were incubated with $p13^{suc1}$ (lanes 2 and 4) or control beads (lanes 1 and 3) before being washed and used for the kinase assays as described in Figure 5.4.1.

Figure 5.4.3 Histone H1 kinase assay of *T. brucei* procyclic extracts bound to p13^{suc1} or p12^{LmmCKS1} beads.

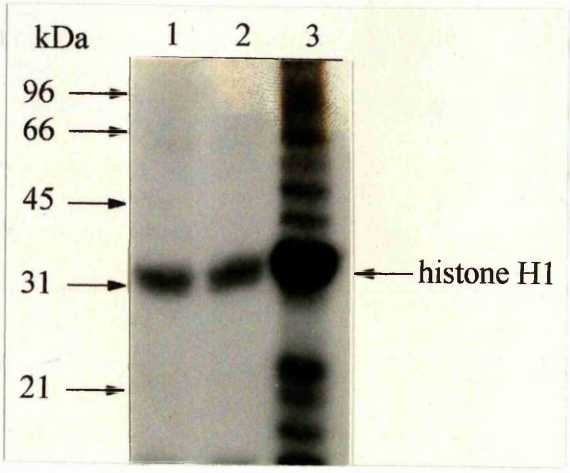


Figure 5.4.3 Histone H1 kinase assays using S100 extracts made from procyclic form *T. brucei.* The extract was incubated with either control amino-link beads (lane 1), p13 ^{suc1} beads (lane 2) or p12^{LmmCKS1} beads (lane 3) at 4 ^{o}C for 2 hours with constant agitation. After repeated washes with LSI the beads were transferred to eppendorf tubes for kinase assays. The products of the assay were then separated by 15 % SDS-PAGE, stained with coomassie to check if there had been histone degradation, dried down and exposed to X-ray film.

5.4.4 Detection of TbCRK proteins bound to p12^{LmmCKS1} beads by Western blotting

T. brucei S100 protein extracts were made from either freshly cultured procyclic forms or frozen pellets of mixed bloodstream *T. brucei* (i.e. containing both long slender and short stumpy forms). The extracts were incubated with p12^{LmmCKS1} or control beads, washed repeatedly, then treated with 5 x FSB and boiled for 5 minutes. After a 1 minute centrifugation in a microfuge 10 μl of each extract was loaded onto a 12.5 % SDS-PAGE, along with 5 μl of the original cell extract. After electrophoresis these gels were electroblotted onto nitro-cellulose membrane and probed with CRK1, TbCRK2 and PSTAIR reactive antisera. As TbCRK3 is not present in S100 extracts the T3H/G and T3H/S antisera were not used.

CITAA antiserum, previously shown to recognise TbCRK1/H (see Figure 4.4.1) detected a 33 kDa protein in the procyclic S100 extract (see Figure 5.4.4, lane 1). This protein was not bound to the control beads (lane 2), but did bind to the p12^{LmmCKS1} beads (lane 3). Therefore TbCRK1 either binds to p12^{LmmCKS1} directly or is part of a complex that does.

Figure 5.4.4, lanes 4-6, shows the same extracts probed with the EVREE antiserum, which was raised to a peptide corresponding to the TbCRK2 C-terminal 16 amino acids. The 39 kDa band in lane 6 is indication that TbCRK2 was bound to p12^{LmmCKS1}. Other proteins in the procyclic cell extract (lane 4) which were recognised by the EVREE antiserum, notably the intense 50 kDa band, did not bind to the p12^{LmmCKS1} beads, implying that the association of TbCRK1 and TbCRK2 with p12^{LmmCKS1} is specific, and not a general protein/protein interaction between the *T. brucei* proteins and p12^{LmmCKS1}.

On a Western blot of procyclic cell extracts (Figure 5.4.4, lanes 7-9), 4 bands were recognised in the crude extract at 33, 40, 48 and 65 kDa by the PSTAIR mAb. The 33 and 40 kDa bands are presumably TbCRK1 and TbCRK2 respectively, and as predicted, they bind to the p12^{LmmCKS1} beads specifically. The 48 kDa band also binds

Figure 5.4.4 Western blot of *T. brucei* procyclic extract bound to p12^{LmmCKS1} or control beads.

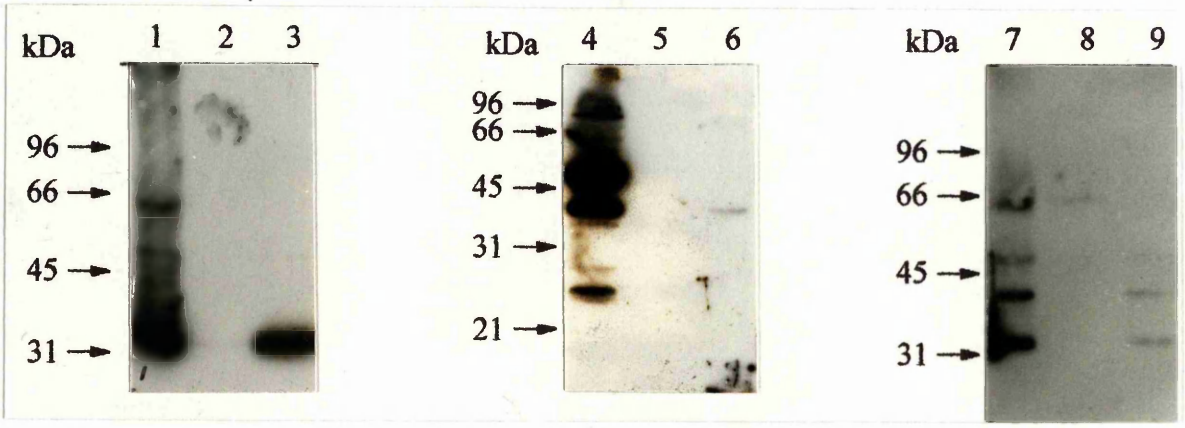


Figure 5.4.4 A *T. brucei* S100 procyclic form extract was incubated with either control amino-link beads or p12^{LmmCKS1} beads at 4 ^{o}C for 2 hours with constant agitation. After repeated washes with LSI the beads were transferred to eppendorf tubes, 1 x FSB was added and the extracts boiled. The samples, together with an aliquot of the starting cell extract, were then run on 15 % SDS-PAGE, electroblotted onto nitro cellullose membrane and used for a Western blot using TbCRK reactive antisera. Lanes 1/4/7 - S100 extract, lanes 2/5/8 - eluate from control beads, lanes 3/6/9 - eluate from p12^{LmmCKS1} beads. Lanes 1-3 probed with CITAA (TbCRK1 reactive) antiserum (1:50), lanes 4-6 probed with EVREE (TbCRK2 reactive) antiserum (1:50), lanes 7-9 probed with the affinity purified PSTAIR mAb (1:50).

Figure 5.4.5 Western blot of *T. brucei* mixed bloodstream form extract bound to p12^{LmmCKS1} or control beads.

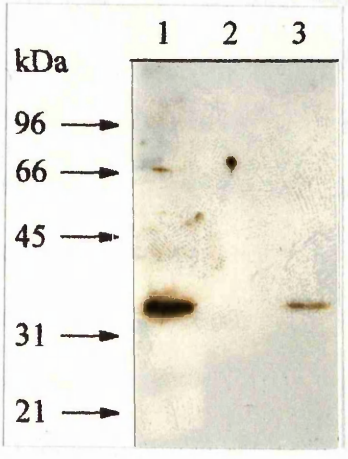


Figure 5.4.5 A *T. brucei* S100 mixed bloodstream form extract was incubated with either control amino-link beads or p12^{LmmCKS1} beads at 4 ^{O}C for 2 hours with constant agitation. After repeated washes with LSI the beads were transferred to eppendorf tubes, 1 x FSB was added and the extracts boiled. The samples, together with an aliquot of the starting cell extract, were then run on 15 % SDS-PAGE, electroblotted onto nitro cellullose membrane and a Western blot performed using CITAA (TbCRK1 reactive) antiserum (1:50). Lane 1 - original extract, lane 2 - eluate from control beads, lane 3 - eluate from p12^{LmmCKS1} beads.

to the p12^{LmmCKS1} beads, while the 65 kDa band appears, surprisingly, to specifically bind to the control beads.

Figure 5.4.5 shows an experiment using the S100 protein extracts from mixed bloodstream form *T. brucei*, with crude extract (lane 1), control beads (lane 2) and p12^{LmmCKS1} beads (lane 3), probed with the CITAA antiserum. As was found with the procyclic extracts, a 33 kDa band was bound specifically to the p12^{LmmCKS1} beads and not to the control beads, therefore TbCRK1 is also in a complex which can bind to p12^{LmmCKS1} in bloodstream form trypanosomes.

The Western blots indicate that TbCRK1 from both procyclic and bloodstream form trypanosomes binds to p12^{LmmCKS1}. TbCRK2 in procyclic extracts binds to p12^{LmmCKS1}, and it is thought likely that the same protein in bloodstream form *T. brucei* will act in the same way, although this binding has not been shown. It is likely that both TbCRK1 and TbCRK2 contribute to the kinase activity detected in the histone H1 kinase assay. It is possible however that another kinase also contributes to the activity. The histone H1 kinase in *Leishmania* that binds p13^{suc1}, named Suc1 Binding CDC2 Related Kinase (SBCRK), is not LmmCRK1. Therefore it is possible that there is a *T. brucei* homologue of SBCRK bound to the p12^{LmmCSK1} beads. SBCRK is unlikely to be encoded by a leishmanial homologue of *tbcrk2*. There is no evidence for a *crk2* homologous gene and the TbCRK2 reactive antisera recognise no likely proteins in *Leishmania* protein extracts.

5.4.5 Metabolic labelling of *T. brucei* proteins to assess components of the complexes binding p12^{LmmCKS1}.

The low level of homology between cyclins (either classes within an organism or within the same class between organisms) has , unlike the situation for CDC2 related kinases, produced antisera that are either protein or species specific. Therefore these heterologous antisera could not be used to detect trypanosome cyclins. To determine if the CRKs which bind to p12^{LmmCSK1} were part of complexes possibly containing

cyclins, procyclic *T. brucei* were metabolically labelled and extracts selected on $p12^{LmmCSK1}$ beads. Procyclic *T. brucei* were pelleted, washed, and resuspended in Eagles medium lacking methionine. SDM-79, the normal growth medium for procyclic trypanosomes, lacking methionine was unavailable, and for the short time needed for labelling, the trypanosomes appeared to continue division and remained motile in Eagles medium. After 30 minutes in culture, to deplete cellular stores of methionine, 100 μCi of ^{35}S-methionine was added to the medium and the culture incubated for 6 hours. The cells were washed, pelleted and an S100 extract made. The S100 extracts were incubated with either control beads or $p12^{LmmCKS1}$ beads. After repeated washing the beads, the original extract and the unselected flow through from the column were diluted into 1 x FSB and boiled. These samples were then run on 12.5 % SDS-PAGE in duplicate. One of the gels was treated with Intensify (see Materials and Methods) before both were dried onto 3MM paper and exposed to X-ray film. Intensify amplifies the radioactive signal by converting the low energy beta particle into light. This results in a greater proportion of decay events interacting with the X-ray film, but does cause an increase in band width, and resulting loss of definition. Figure 5.4.6 shows lanes from the X-ray film either treated [panel (B)] or not treated [panel (A)] with Intensify. The S100 extract prior to adding to the beads is shown in (A) lane 1. Panel (B), lanes 1 and 2 are the flow through from the $p12^{LmmCKS1}$ and control beads respectively. There are no visible differences between the original extract and the flow throughs. No depletion of any of the original bands can be detected. The proteins which bound to the $p12^{LmmCKS1}$ beads, plus or minus Intensify, are shown in (B)4 and (A)2. Intensify treated gel showing the proteins bound to the control beads is shown in (B)3. The different gels are used because, as noted before, although the Intensify causes the images of the labelled bands to become darker, and thereby makes it possible to see proteins not otherwise visible, it also has the effect of making the bands notably more diffuse.

Most, if not all, of the proteins over 45 kDa in size which bind to the $p12^{LmmCKS1}$ beads (panel (A), lane 2) do so non-specifically and can be seen, albeit at

Figure 5.4.6 ^{35}S-Methionine labelling of procyclic *T. brucei* protein extracts selected on p12^{LmmCSK1} beads.

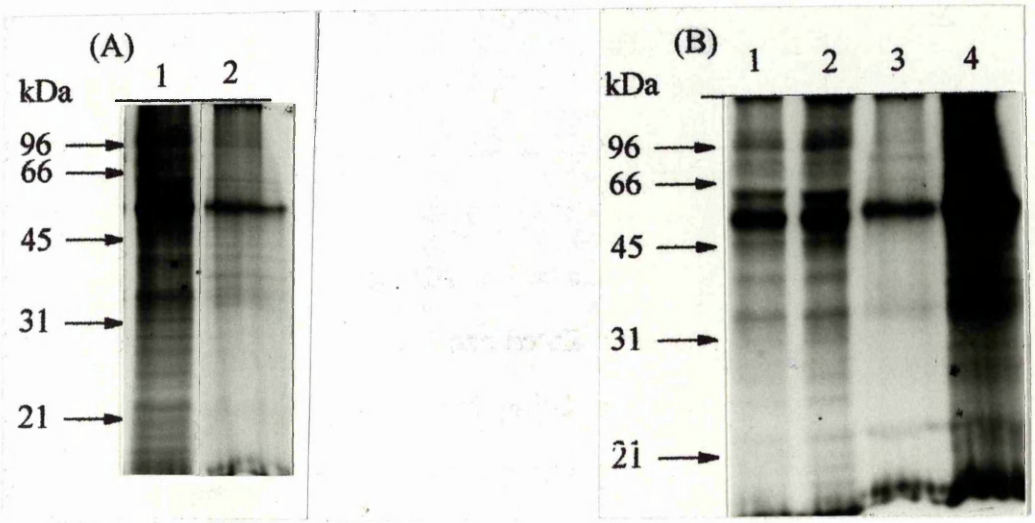


Figure 5.4.6 10 ml of log phase (~7 x 10^6 cells / ml) procyclic *T. brucei* cultures were pelleted, washed twice with PBS, and resuspended in 10 ml of Eagles media lacking methionine. After 30 minutes 100 μCi of ^{35}S-methionine were added to the medium and the culture incubated for 6 hours. The cells were pelleted and an S100 extract made. To bind the extract to control and p12^{LmmCSK1} beads, 250 μl of 2 mg / ml extract were incubated with 50 μl of the 50 % bead slurry at 4 ^{o}C for 2 hours with constant agitation. After repeated 250 μl washes (1 x LSI, 2 x LSI+1M NaCl, 1 x LSI) 1 x FSB was added and the extracts boiled. Aliquots of the original extract and the flow through from both columns were diluted into 1 x FSB and boiled. Duplicate 12.5 % SDS-PAGE gels were run. Before being dried onto 3MM paper, one gel was treated with Intensify which can increase the signal detected by the X-ray film.

Panel (A) shows film from the untreated gel, panel (B) shows the signal from the gel treated with Intensify. S100 protein extract (A)1. Flow through from the control beads [(B)1] and the p12^{LmmCKS1} beads [(B)2]. Proteins bound to the control beads [(B)3]. Proteins bound to the p12^{LmmCKS1} beads [(A)2 and (B)4].

a lower intensity, on the control beads (Panel B, lane 3). No large, mitotic cyclin-like, proteins could be distinguished from other proteins which bind to the matrix non-specifically. The bands below the intense, non-specific, band at 50 kDa are predominantly specific. There are labelled proteins in Panel A, lane 2 of approximately 45 kDa, 44 kDa, 43 kDa, 40 kDa, 36 kDa and 34 kDa which do not have corresponding bands in the control lane. The two bands below these, at 33 kDa and 32 kDa, are possibly present in the control lane (B)3. It is possible that the ~45 kDa protein might be the 48 kDa PSTAIR mAb reactive band seen bound to the $p12^{LmmCKS1}$ column in Figure 5.4.4 lane 9. It may be however that this protein's signal would be obscured by the intense, non-specific, 50 kDa band The 40 kDa protein is close in size to TbCRK2, which was shown by Western blotting to bind specifically to the $p12^{LmmCKS1}$ beads (see Figure 5.4.4, lane 3). The relatively well labelled 36 kDa protein detected binding to the $p12^{LmmCKS1}$ beads is the same size as the putative cyclin previously isolated from *T. brucei* (Affranchino *et al.*, 1993) which was shown by immunoprecipitation and Western blotting to be part of a complex with a 34 kDa 'PSTAIR' containing protein. Of the triplet around 33 kDa, the lower two proteins may be present in the control extracts, while the higher, 34 kDa band appears specific to the $p12^{LmmCKS1}$ beads. It is possible that this 34 kDa protein is TbCRK1, which was also shown to bind to the $p12^{LmmCKS1}$ beads using Western blotting (see Figure 5.4.4, lane 6). There are three proteins with a Molecular weight lower than 30 kDa which appear to bind specifically to the $p12^{LmmCKS1}$ beads [compare panel (B), lanes 3 and 4]. These proteins have an estimated mass of 28 kDa, 26 kDa and 21 kDa. The 21 kDa protein is the same size as some inhibitor proteins of CDK activity seen in other organisms, but there is no further evidence for this possibility. Recently two other *T. brucei* cyclins have been isolated (J. Mottram personal communication) with predicted sizes of 45 kDa (CYC3) and 24 kDa (CYC2) respectively. These predicted sizes are similar to the estimated mass of two of the proteins bound to the $p12^{LmmCKS1}$ beads.

5.4.6 Discussion of $p13^{suc1}$/$p12^{LmmCKS1}$ binding studies

There is a histone H1 kinase activity in procyclic *T. brucei* protein extracts which binds to $p12^{LmmCKS1}$, but not to $p13^{suc1}$ (see Figure 5.4.3). Immunoblotting experiments with CRK1 and TbCRK2 specific antisera revealed the binding of TbCRK1 and TbCRK2 to the $p12^{LmmCKS1}$ beads (see Figure 5.4.4). Therefore the histone H1 kinase activity is likely to be, at least partially, due to TbCRK1 and TbCRK2. Evidence from metabolic labelling experiments suggests that these kinases are part of multi-protein complexes.

A histone H1 kinase activity was detected in procyclic *T. brucei* which binds to $p12^{LmmCKS1}$ (see Figure 5.4.3), but no such activity was demonstrated binding to $p13^{suc1}$ in either procyclic or bloodstream trypanosomes (see Figure 5.4.1). This is unlike the situation in *L. mexicana* which contains SBCRK, a $p13^{suc1}$ binding kinase (Mottram *et al.* 1993; Mottram and Grant, 1996). Histone H1 kinase activity from *S. pombe* was selected on $p13^{suc1}$ beads thereby showing no fundamental problem with the experimental procedure. It may be that there is a *T. brucei* homologue of SBCRK, but that it has different $p13^{suc1}$ binding characteristics. Another explanation for this difference would be that SBCRK is specific to *Leishmania*, and not present in *T. brucei*. SBCRK could be one of the many CDC2 related kinases in protozoa (Mottram, 1994), and may not be the functional CDC2 homologue, even though its activity appears related to the division status of the life cycle stage (Mottram *et al.* 1993; Mottram and Grant, 1996). The fact that SBCRK is not recognised by the PSTAIR antisera also provides some evidence that it is not as closely related to CDC2 as the TbCRK proteins. This is further support for the idea that many of the protozoan CDC2 related proteins are highly divergent, possibly evolved for tasks specific to the organism, and unlikely to be even functionally analogous to each other.

The kinase assays show a number of phosphorylated proteins that are not derived from histone H1 (the only exogenously added protein), and therefore are components of the *T. brucei* extracts (see Figure 5.4.3). It is possible that the low

molecular weight proteins could be CKI related proteins. These inhibitory subunits are effective when bound to the CDC2/cyclin complex with a 1:1 stoichiometry. Therefore there could still be kinase activity if only a subpopulation of the kinase complexes contain the inhibitory subunits. The smallest protein at 18 kDa could be the *T. brucei* homologue of $p12^{LmmCKS1}$. The *S. cerevisiae* $p13^{suc1}$ homologue is 18 kDa (Hadwiger *et al.*, 1989). In some organisms suc1/CKS proteins form dimers or hexamers, and so it is possible that hetero-complexes of leishmanial and trypanosome CKS proteins could form on the beads during the incubation with cell extracts. It is possible that the 21 kDa protein could be the CYC2 protein. The cyclins would be expected to be found in the $p12^{LmmCKS1}$ bound extract, and there is evidence for phosphorylation of cyclins by CDK complexes (Lanker *et al.*, 1996). These phosphorylated proteins could also be binding non-specifically to the $p12^{LmmCKS1}$ beads, and contain consensus sites for phosphorylation by the CRK complexes.

The Western blot of *T. brucei* extracts bound to $p12^{LmmCKS1}$ beads using the CITAA (CRK1 reactive) antisera showed that a complex containing TbCRK1 interacts with the *L. mexicana* CKS1 protein (see Figure 5.4.4, lanes 1-3). TbCRK2 containing complexes were detected bound to $p12^{LmmCKS1}$ beads using the EVREE (TbCRK2 reactive) antisera (see Figure 5.4.4, lanes 4-6). Therefore it is likely that these proteins would interact with a *T. brucei* CKS1 homologue. The *suc1* gene is essential in *S. pombe* and well conserved homologues have been isolated from *S. cerevisiae*, *Leishmania* and *H. sapiens*, making it extremely likely that there is a *T. brucei* homologue. The PSTAIR mAb also detected the binding of a 48 kDa protein to the $p12^{LmmCKS1}$ beads (see Figure 5.4.4, lanes 7-9). This possible kinase may be involved in the phosphorylation of histone H1, but this is unlikely to be the sole H1 kinase as the activation experiments using TbCRK1/H did show some kinase activity which was correlated to selected His-tagged TbCRK1 (see Figure 5.3.2). Subsequently histone H1 was shown to be a relatively poor substrate for the LmmCRK1/H protein kinase so other substrates may be more suitable for continuations of these experiments (J. Mottram, personal communication).

Using ^{35}S-methionine labelling, several proteins were detected that specifically bound to the p12^{LmmCKS1} beads (see Figure 5.4.6). The 36 kDa protein could be the CYC1 protein isolated previously (Affranchino *et al.*, 1993). CYC1 was shown, by immunoprecipitation experiments, to interact with a 34 kDa protein recognised by the PSTAIR mAb. As TbCRK1 is approximately 34 kDa and reacts with the PSTAIR mAb, it is possible that TbCRK1 is a kinase subunit for CYC1. A 39 kDa PSTAIR reactive band was not seen in CYC1 immunoprecipitates (Affranchino *et al.*, 1993), implying that TbCRK2 has different complex components. These would probably include one or more of the other proteins detected bound to the p12^{LmmCKS1} beads, two of which could be the CYC2 or CYC3 proteins. The lower molecular weight protein (21 kDa) specifically bound to the p12^{LmmCKS1} beads in the ^{35}S-methionine labelling experiment (see Figure 5.4.6) may be the same as the 21 kDa phospho-protein detected previously in the p12^{LmmCKS1} bound kinase assays (see Figure 5.4.3).

TbCRK3 was found in the insoluble fraction after lysis in Triton X-100 and so it was not possible to determine if TbCRK3 could bind to the p12^{LmmCKS1} beads using this protocol. It may be possible to solublise TbCRK3 using other buffers such as high salt, or different detergents, but these treatments could not be too stringent if the complex is to remain intact.

5.5 Discussion

Various methods of analysis were used for the characterisation of the TbCRK family. As with *lmmcrk1* and *tbcrk2* , *tbcrk3* was found to be unable to complement a *cdc2*-ts mutant of *S. pombe*. It is possible that there is no *cdc2* complementing gene in *T. brucei*, although the equivalent experiments in *S. cerevisiae* may be tried. Attempts to complement a *S. cerevisiae CDC28*-ts mutant using a *T. brucei* cDNA library proved unsuccessful (Neuville and Mottram, unpublished). This is further evidence for the lack of a complementing *cdc2* related kinase. There is evidence that there are other *cdc2* related genes in *T. brucei* (see Figure 5.4.4, lane 7), but due to the methods used to

clone *tbcrk1-3* (degenerate oligonucleotide PCR and library screening), it seems likely that any other *tbcrk* genes will be less conserved than those already isolated. It is possible that *T. brucei* contains a homologue of the *Crithidia fasciculata crk* gene (Brown *et al.*, 1992). This gene encodes a CDC2-related protein (CfaCRK) with a degenerate PSTAIR box (12 out of 16 amino acids conserved) and two large insertions resulting in a 53 kDa protein, a size not too dissimilar to the 48 kDa band recognised by the PSTAIR mAb. The PSTAIR mAb used in this paper did not recognise the CfaCRK protein, but it is possible that this mAb recognises a different epitope to the mAb used during my Ph.D.

The activation of TbCRK1/H, and the purification of the activated kinase, showed that this method was a viable means of isolating active kinase complexes if proteolysis could be controlled. The dependance of the kinase activity on the exposure to the cell extract implies that the TbCRK1 protein requires the binding of other proteins and/or modifications (such as phosphorylation) for correct function. This accords with what is known from other cdc2-related proteins. The *Plasmodium falciparum* protein PfPK5 is associated with a weak kinase activity when purified from *E. coli* (Ross-MacDonald *et al.*, 1994), but this activity is considerably stimulated by replacement of the equivalent of the Thr-161 residue by an acidic amino acid, simulating the phosphorylated state (Graeser *et al.*, 1996). Similar results have been found with other CDK subunits where phosphorylation of the Thr-161 equivalent is necessary for kinase activity, while cyclin binding is required for optimal activity (Desai *et al.*, 1992). One exception to this is CDK5 which has kinase activity when complexed with its p35 subunit without needing phosphorylation (Qi *et al.*, 1995).

The *in vitro* activation result also implies that, at least with this protein, the histidine tag does not seriously interfere with the protein function. This has been confirmed *in vivo* in *Leishmania* where LmmCRK1-his is functional (Mottram *et al.*, 1996). It seems probable that modification of this technique, possibly by using an epitope tagged protein allowing immunoprecipitation, would allow analysis of the protein factors and modifications involved with the activation. For this to happen

though, it would be best to use a specific antiserum that does not cross react with *T. brucei* proteins, and so far this has been difficult to obtain in antisera with high enough affinity to allow immunoprecipitation. The fact that the activation took place when the TbCRK1/H was added to S100 supernatant from the lysed cultures implies that TbCRK3 is not involved in the activation, as under these conditions TbCRK3 is insoluble and remains in the pellet fraction. If epitope tagged proteins can be isolated efficiently from *T. brucei* then the targeted replacement of the wild type genes with the tagged version would also allow assessment of the components of the kinase complexes, although due to its insoluble nature this still might not work as well for TbCRK3. Although immunoprecipitation of TbCRK3 may be difficult, the epitope tagged version would allow easy localisation of the protein in fixed cells using fluorescent antibodies.

The lack of a histone H1 kinase activity binding to the $p13^{suc1}$ beads is another indication of the evolutionary distance between *T. brucei*, yeast and metazoans. This lack of an association could be one reason why the *tbcrk* genes failed to complement *S. pombe cdc2*-ts mutants. It would appear, however, that *T. brucei* does contain a $p13^{suc1}$ related protein. A homologous gene (*lmmcks1*) has been isolated from *L. mexicana* and the binding of both TbCRK1 and TbCRK2 to the $p12^{LmmCKS1}$ beads suggests that there will be a related gene in trypanosomes. As a number of trypanosome CRKs were found to bind to the $p12^{LmmCKS1}$ beads it is difficult to assess which of the proteins contributed to the kinase activity detected. The TbCRK1 protein bound to the $p12^{LmmCKS1}$ column may contribute to the isolated kinase activity, but this is not definite as the form bound to $p12^{LmmCKS1}$ may be inactive. Equally, there is no evidence that the TbCRK2 isolated is active. The 48 kDa PSTAIR reactive band in the procyclic extract (see Figure 5.4.4, lanes 7-9), which bound to the $p12^{LmmCKS1}$ beads, may also contribute to the histone H1 kinase activity.

Labelling procyclic form *T. brucei* cultures with ^{35}S methionine showed several proteins which specifically bound to the $p12^{LmmCKS1}$ beads, although others may have been obscured by non specific bands. Of the specific bands, one was the approximate

size of CYC1, a cyclin like protein previously isolated from *T. brucei* (Affranchino *et al.*, 1993) which according to immunoprecipitation experiments interacted with a 34 kDa PSTAIR containing protein, presumably TbCRK1. There is also the possibility of TbCRK1 and TbCRK2 being detected.

CHAPTER SIX
ASSESSMENT OF *TBCRK3* FUNCTION BY REVERSE GENETICS

6.1 Introduction.

As it was not readily possible to analyse TbCRK3 function either by *in vitro* expression or *in vivo* purification due to the insoluble nature of the protein combined with the need to retain an intact protein complex, the assessment of TbCRK3 protein function by *in vivo* mutagenesis was attempted. The creation of mutants by homologous recombination using constructs with antibiotic resistance markers has previously been performed with a number of different genes in the kinetoplastids (see Chapter 1.1.3(ii)). As procyclic form *T. brucei* are considerably easier to grow *in vitro* than bloodstream form, procyclic cultures were used. Dependent on whether *tbcrk3* was essential for trypanosome growth or not, different results could be expected:

(1) if the gene was non-essential in the procyclic form, null mutant *T. brucei* would be isolated, and it would then be necessary to analyse the cells to assess any phenotypic effect of the knockout, such as early or delayed division.

(2) if *tbcrk3* was essential in the procyclic form, either no mutants would be isolated or parasistes resistant to both antibiotic markers used for gene targetting would be isolated but at low numbers, and as described in Chapter 1.1.3(ii), the cells would retain a copy of the wild type gene through recombination, duplication, retention of an episomal copy or changes in ploidy.

(3) the gene might be essential in some life cycle stages and not others, thus it would be stage specific lethal. Although the protein is expressed in all the stages tested (see Chapter 4.5) it is possible that in some forms there would be functional redundancy, as found with the CLN cyclin genes of *S. cerevisiae* (Rowley *et al.*, 1992). Null mutants may be generated in procyclic form *T. brucei* that would be unable to complete the life-cycle if the gene was essential in the bloodstream form.

This approach was not attempted with *tbcrk1* because an attempt to create null mutants in the single copy *lmmcrk1* gene was unsuccessful, although tetraploid *Leishmania* were isolated following the second round of transfection (J. Mottram, personal communication). This implies that the gene is essential for the growth of

promastigote form *L. mexicana*. The closely related gene from *T. brucei* (*tbcrk1*) is able to complement *lmmcrk1* mutants in *Leishmania* following the deletion of both copies of the *lmmcrk1* gene. It is therefore likely that *tbcrk1* is functionally homologous to *lmmcrk1* and an essential gene in the *T. brucei* procyclic form.

6.2 Creation of putative null mutants of *tbcrk3*.

6.2.1 Construction of the plasmid background for resistance cassette insertion

No *cis*-introns in protein coding genes have been found in *T. brucei*. Therefore it would be very unlikely for a resistance cassette integrated into a genomic locus to be spliced out of the transcribed RNA thus resulting in a functional gene product from the disrupted locus. However, the gene knockout vectors were designed to not only integrate a resistance cassette into the middle of the gene but also to remove part of the *tbcrk3* ORF when integrating into the genomic loci. This approach would ensure the removal of TbCRK3 function. The CDK genes are usually transcribed at relatively low levels in other organisms (Welch and Wang, 1992) and therefore low levels of drug resistance may occur at this locus. As the *ble* resistance gene product provides resistance in a non-catalytic manner (Jefferies *et al.*, 1993) by binding to and sequestering the drug, it therefore might need a high level of expression to achieve good resistance. For this reason the drug resistance genes were placed under the control of the highly active PARP/procyclin promoter, rather than relying on read through transcription of the presumably polycistronic RNA from the *tbcrk3* region. The resistance cassettes were inserted between the *Xba*I site and the most 3' *Xho*I site within the *tbcrk3* ORF (Figure 6.2.1). This results in the deletion of 388 bp (over one third of the ORF), from the region just upstream of the PSTAIR box, to just downstream of the DSEI region (amino acids 88 to 215 inclusive). pBlueScript (pBS) contains both *Xba*I and *Xho*I sites in the MCS, and so these plasmid sites had to be destroyed to allow the subcloning of the resistance cassettes into the *tbcrk3* ORF. To do this pBS SK+ was

first digested with *Xba*I and *Spe*I. The sites for these enzymes have compatible overhangs, and are adjacent in the pBS MCS. The digest was precipitated with iso-propanol, which is less efficient than ethanol at precipitating small DNA molecules, thereby removing the 6 bp *XbaI/SpeI* DNA fragment. The DNA was resuspended and ligated with T4 DNA ligase. After transformation of *E. coli*, colonies were picked at random, plasmid DNA prepared, and the DNA incubated separately with *Xba*I (to check for the loss of the site) and *Xho*I (as a control for digestion). A plasmid negative for the *Xba*I site were called pBS(-S/X). This plasmid was then treated with *Xho*I and *Sal*I. These enzymes also have compatible overhangs, and are adjacent in the pBS MCS. In this case it was found necessary to re-precipitate the ligation reaction, resuspend the DNA and incubate it again with *Xho*I before transformation. This procedure linearised molecules which had not been digested in the first reaction, or in which the MCS fragment had religated, and so reduced the background of the pBS(-S/X) plasmid to a low level. Colonies were picked at random after the transformation and plasmid DNA prepared from overnight cultures. The DNA was tested with *Xba*I, *Xho*I and *Eco*RI in separate reactions. The plasmid which failed to digest with either of *Xba*I or *Xho*I, but which was linearised by *Eco*RI was named pBS(-2S/2X). Both digestion/ligation reactions retained the original *lacZ* open reading frame. This plasmid was the backbone for the knockout vectors.

The *tbcrk3* ORF was located on a 2.6 kb *Hin*dIII fragment of pCD70E(S)8. As a *Hin*dIII site was present within the *tbcrk3* gene a partial digest was performed in order to isolate the complete gene on a 2.6 kb *Hin*dIII fragment. A 100 μl digest containing 10 μg of pCD70E(S)8 plasmid DNA was performed with 10 U of *Hin*dIII. 20 μl samples were taken at 5, 10, 20, 30, and 45 minutes. 4 μl of each sample were run on a 0.7 % agarose gel containing EtBr. The sample showing the highest proportion of the 2.6 kb band was then run on another 0.7 % agarose gel, followed by excision of the band and purification of the DNA using Geneclean (see Materials and Methods). This was then ligated into pBS(-2S/2X) cut with *Hin*dIII and transformed into competent XL1-Blue cells. After plating on X-gal/IPTG containing plates, white colonies were

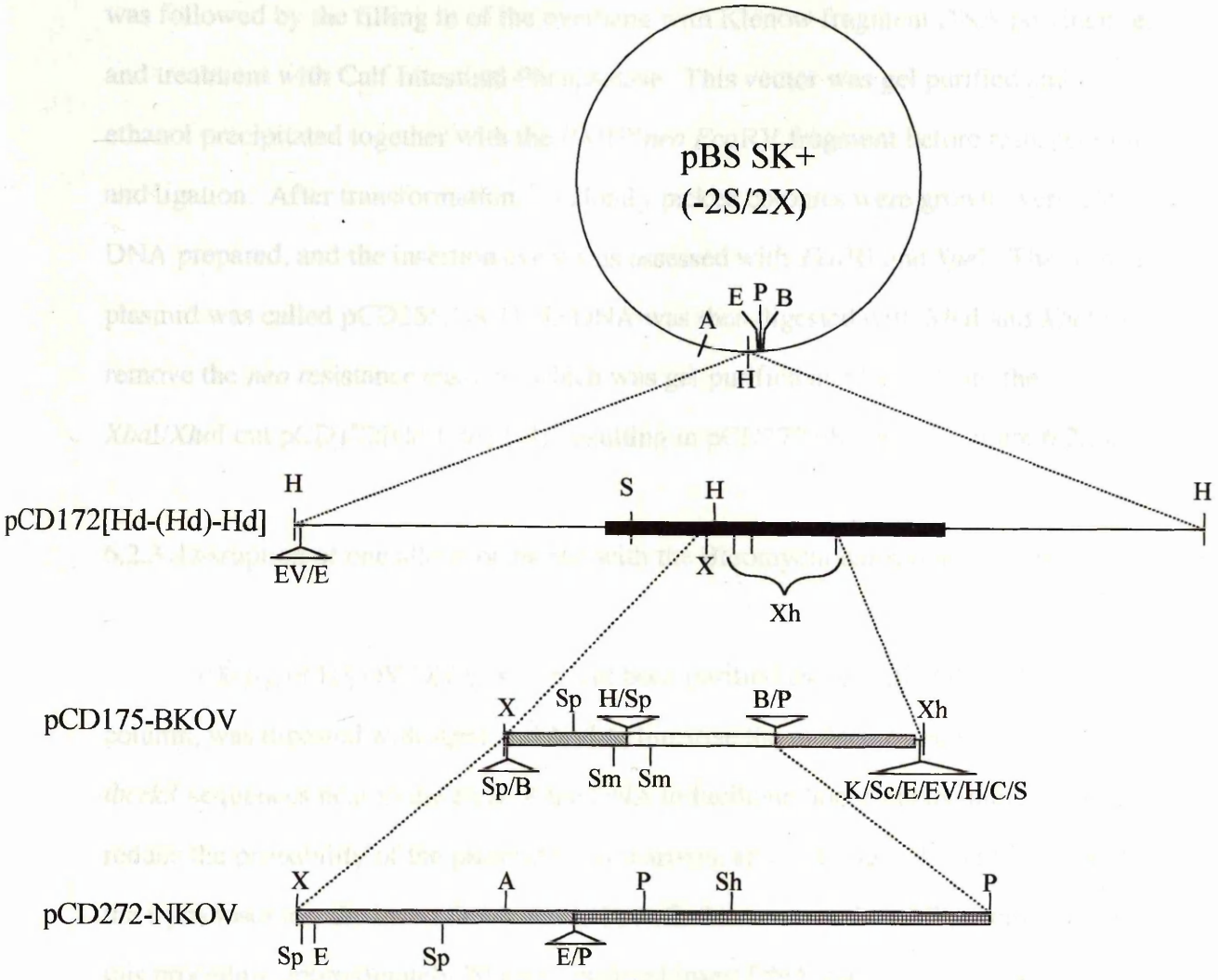
selected and grown in LB medium overnight, and the prepared plasmid DNA was treated with a variety of restriction enzymes, including *Hin*dIII, *Xho*I with *Xba*I, *Eco*RI and *Sal*I to determine if the correct cloning had been achieved. The correct plasmid was named pCD172[Hd-(Hd)-Hd] (see Figure 6.2.1).

6.2.2 Construction of the replacement vectors.

The plasmid containing the Bleomycin resistance cassette (pBle) was a gift of Dr. S. Graham. The cassette contained the *ble* resistance gene under the control of the PARP promoter, with a splice acceptor site for correct *trans*-splicing, and the PARP 3' untranslated region for polyadenylation (see Figure 6.2.1). pBle had a pUC18 background, and did not have a *Xho*I site. The resistance cassette was removed from the vector with *Eco*RI and *Xba*I, and ligated into pBS SK+ which had been cut with *Eco*RI and *Xba*I. This resulted in a plasmid [designated pBS(Ble)] containing a *ble* resistance cassette which had a *Xba*I site at the 5' end, and a *Xho*I site at the 3' end. After digestion with *Xba*I and *Xho*I, the 1.1 kb resistance cassette was ligated into *Xba*I/*Xho*I cut pCD172[Hd-(Hd)-Hd]. This plasmid was named pCD175BKOV (Bleomycin KnockOut Vector) (see Figure 6.2.1).

A plasmid containing a Neomycin resistance cassette was the gift of Dr. D. Jeffries. The plasmid (pNT) contained the *neo* gene (which conveys resistance to the antibiotic G418) under the control of a region containing the PARP promoter, with the 3' untranslated region of the *T. brucei* alpha-tubulin gene. The downstream region of this construct did not contain restriction sites usable for the insertion strategy and so it was necessary to move the promoter and *neo* gene into another plasmid. The pNT DNA was digested with *Eco*RV which removed a 2.0 kb fragment that contained both the PARP promoter and the *neo* resistance gene. The 2.0 kb fragment was gel purified using a spin-X column (Materials and methods). The pBS(Ble) plasmid, containing the *ble* resistance cassette ligated into the *Eco*RI and *Xba*I sites of pBS SK+, was digested with *Bam*HI, which cut out the PARP promoter and the *ble* resistance gene. The digest

Figure 6.2.1 The *tbcrk3* knockout vectors.



A -	*Apa*I	E -	*Eco*RI	K -	*Kpn*I	Sc -	*Sac*I	Sh-	*Sph*I
B -	*Bam*HI	EV-	*Eco*RV	P -	*Pst*I	Sm-	*Sma*I	X -	*Xba*I
C -	*Cla*I	H -	*Hin*dIII	S -	*Sal*I	Sp -	*Spe*I	Xh-	*Xho*I

tbcrk3 flanking DNA

tbcrk3 ORF

PARP promotor and Splice Acceptor site

PARP 3' untranslated region

ble resistance gene ORF

neo resistance gene ORF

1 kb

was followed by the filling in of the overhang with Klenow fragment DNA polymerase, and treatment with Calf Intestinal Phosphatase. This vector was gel purified and ethanol precipitated together with the PARP/*neo* *Eco*RV fragment before resuspension and ligation. After transformation, randomly picked colonies were grown overnight, DNA prepared, and the insertion event was assessed with *Eco*RI and *Spe*I. The correct plasmid was called pCD255. pCD255 DNA was then digested with *Xba*I and *Xho*I to remove the *neo* resistance cassette, which was gel purified and ligated into the *Xba*I/*Xho*I cut pCD172[Hd-(Hd)-Hd], resulting in pCD272NKOV (see Figure 6.2.1).

6.2.3 Disruption of one allelle of *tbcrk3* with the Bleomycin KnockOut Vector.

100 μg of BKOV DNA, which had been purified using a QIAGEN Midi-column, was digested with *Apa*I and *Not*I to linearise the molecule and to leave the *tbcrk3* sequences near to the ends of the DNA to facilitate homologous integration. To reduce the probability of the plasmid recircularising and forming stable episomes within the trypanosomes, the insert DNA was gel purified to remove the pBS plasmid. After this procedure approximately 20 μg of purified insert DNA was left, which was ethanol precipitated, dried in a sterile hood, and resuspended in 10 μl of sterile water. Approximately $1x10^8$ cells of STIB247 procyclic form *T. brucei* in log phase growth were pelleted and resuspended in 2 ml of ZPFM electroporation buffer. 10 μg of the purified insert DNA was added to 1 ml of the resuspended cells and electroporated at 1500 V and 25 μF (see Materials and Methods). After electroporation the cells were split into 2 flasks, each containing 5 ml of SDM-79 medium with no antibiotic selection. A control was performed in which cells were treated in an identical manner except that instead of the DNA solution, 5 μl of sterile water was added to the 1 ml of resuspended cells. After approximately 16 hours (overnight) the cell density was counted using a haemocytometer, the cultures were passaged to a level of 1×10^6 cells / ml into SDM-79 medium containing 20 μg / ml of phleomycin, an antibiotic to which the *ble* gene conveys resistance. The transfected cells were split into two flasks. After

less than one week no phleomycin resistant trypanosomes were observed in the control flasks, whereas all of the DNA transfected cultures resulted in populations of resistant trypanosomes. However, three of the flasks became contaminated and so only one line could be analysed. Although none of the cultures were clonal, the rapid growth supported the idea that each flask had contained cells from multiple independent integration events and so each flask was probably representative of the others. Therefore, this one culture [designated the Ble(M) line] was analysed.

Genomic DNA was prepared from the Ble(M) line and from the wild type STIB247 using the Nucleon protocol (see Materials and Methods). The DNA was digested with *Eco*RI or *Pst*I and two lanes of each were run on a 0.7 % TBE/agarose gel, followed by Southern blotting. The duplicate blots were either hybridised to a mixture of the *Hin*dIII insert fragments from pCD172[Hd-(Hd)-Hd], or to the *ble* gene, labelled by random priming (see Materials and Methods). From the previous Southern blots performed with STIB 247 DNA (see Figure 4.1.2) the expected sizes of the *tbcrk3* hybridising bands from wild type DNA were 4.4 kb in the *Eco*RI digest and 2.8 kb in the *Pst*I digest. Following the integration of BKOV, *Eco*RI and *Pst*I sites would be inserted into the *tbcrk3* ORF, and so two extra bands hybridising to *tbcrk3* would be expected in both digests. These would be expected at 2.3 kb and 2.9 kb for the *Eco*RI digest, and 2.0 kb and 1.7 kb in the *Pst*I digest. The 2.3 kb band from the *Eco*RI digest and the 2.0 kb band from the *Pst*I digest would be expected to hybridise to the *ble* gene probe. If the BKOV had not integrated and was being maintained as an episome then the *tbcrk3* probe would recognise the wild type bands and two extra bands at 2.3 kb and 1.1 kb for the *Eco*RI digest and 4.8 kb and 1.4 kb in the *Pst*I digest. Of these bands, the 2.3 kb (*Eco*RI) and the 4.8 kb (*Pst*I) bands would also hybridise to the *ble* gene probe. Figure 6.2.2 shows the resultant autoradiograph. The odd lanes contain DNA from STIB247, the even lanes from Ble(M). The *ble* gene probe hybridised to Ble(M) DNA (lanes 6 and 8) but not to DNA from STIB247 (lanes 5 and 7). The sizes of the new bands in lanes 2 and 4 are those predicted for the insertion of the *ble* resistance cassette into the *tbcrk3* ORF. The ratio of intensity of the 3 bands in lanes 2 and 4 might be

Figure 6.2.2 Southern blot of DNA from the phleomycin resistant *T. brucei* culture showing disruption of one *tbcrk3* allelle.

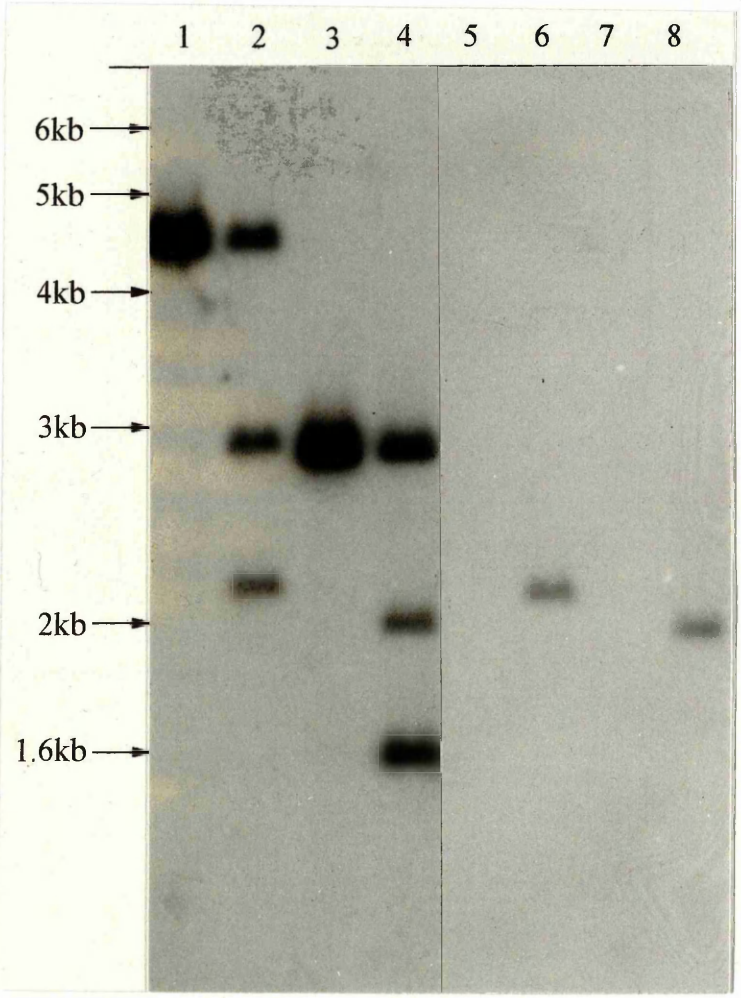


Figure 6.2.2. Genomic DNA was prepared from procyclic cultures of STIB247 or Ble(M) (see Materials and Methods). 20 μg of DNA was digested in a total volume of 78 μl containing 30 U of either *Eco*RI or *Pst*I. After 2 hours a further 20 U of enzyme were added. After 2 more hours Loading Buffer was added to a final volume of 100 μl. 50 μl of the digest was loaded into each lane of a 0.7% TBE/agarose gel and run overnight at 30 V. The gel was then stained with Ethidium Bromide and photographed before being treated for Southern blotting. The DNA was fixed to the Hybond-N filter by baking at 80 ^{o}C for 2 hours before prehybridisation. Hybridisation was performed overnight at 65 ^{o}C.

Odd number lanes contain DNA prepared from the STIB247 cell culture, even lanes contain DNA from the Ble(M) culture. Lanes 1, 2, 5 and 6 were digested with *Eco*RI, lanes 3, 4, 7 and 8 were digested with *Pst*I. Lanes 1-4 were hybridised to the *tbcrk3*-containing fragments from pCD172[Hd-(Hd)-Hd], lanes 5-8 were hybridised to the *ble* ORF.

expected to be approximately 2:1:1 if each cell had had one copy of *tbcrk3* disrupted. This is not the case, as the bands corresponding to the insertion event are less intense than expected. This may be due to the loss of 388 bp of hybridising sequence from the two lower bands due to the integration event, and the increased diffusion of smaller molecules during electrophoresis. Also the molecule used for the labelling was dCTP, and the region of *tbcrk3* deleted by the insertion event has a base composition of 46 % GC, while those regions of the flanks that have been sequenced have only 41 % GC, therefore the labelled molecules hybridising to the wild type locus are likely to be of a higher specific activity. The lack of Restriction Fragment Length Polymorphisms (RFLPs) detected at the *tbcrk3* locus (see Figure 4.1.2) meant that integrations at the two loci could not be distinguished, and the inability to separate the two homologous chromosomes (see Figure 4.1.3) meant that they could not be distinguished using pulse field gel electrophoresis. Therefore it was unknown whether the integration events occurred preferentially in one chromosome or randomly.

6.2.4 Integration of the Neomycin resistance gene into *tbcrk3*.

Several results were theoretically possible following transfection of the neomycin KOV into the phleomycin resistant procyclic line, Ble(M), dependent on whether TbCRK3 function was essential for cell viability. If *tbcrk3* is not essential then doubly resistant *T. brucei* would be recovered if both antibiotic selection pressures were applied. If only G418 selection is applied then it should be possible to isolate trypanosomes in which the NKOV had replaced the BKOV, as well as trypanosomes in which the NKOV had inserted into the other copy of *tbcrk3*.

If *tbcrk3* was essential then either no viable cultures would grow in medium containing both antibiotics, or the cultures would contain cells which had undergone rearrangements or changes in ploidy, which resulted in the maintenance of at least one wild type copy of *tbcrk3*. If the medium only contained G418 then the resistant cultures

would only contain cells in which the neomycin knockout vector had replaced the bleomycin resistance cassette.

As the neomycin resistance cassette contains an *Apa*I site, the pCD272 (NKOV) plasmid was digested with *Not*I and *Pvu*I, for which there is a site approximately 250 bp upstream of the start ATG for *tbcrk3*. QIAGEN purified NKOV DNA was digested, separated by agarose gel electrophoresis, purified by Geneclean, precipitated and resuspended in sterile water. 10 μg of the DNA was transfected into the Ble(M) trypanosomes (treated as the STIB247 cells in Chapter 6.2.3), which after the overnight recovery were passaged to 1 x 10^6 cells / ml in medium containing 20 μg / ml of phleomycin and 8 μg / ml of G418. Of four such flasks, one gave rise to a slow growing culture which appeared resistant. No such culture was seen in the four control flasks which contained Ble(M) trypanosomes electroporated in the presence of sterile water. A stabilate was prepared of the entire culture in parallel with STIB247 and Ble(M) cultures, due to an enforced absence from the laboratory. When the stabilates were defrosted a month later and placed into medium free from antibiotics, the STIB247 and Ble(M) stocks recovered rapidly, but the putative doubly resistant line failed to recover.

The transfection was repeated with 2.5 x 10^8 mid-log phase Ble(M) cells resuspended in 4 ml of ZPFM. 2 ml were transfected with NKOV insert DNA in two batches of 1 ml (batch A and B), while 2 ml were electroporated without DNA present. Each 1 ml was immediately split into 2 flasks for the overnight recovery, so that each of the four experimental flasks transfected with NKOV insert DNA [designated A(1/2), A(3/4), B(1/2) and B(3/4)] would contain independent transfection events. Each of these four cultures were then passaged into ten flasks consisting of two sets of five different G418 concentrations (0/5/7.5/10 and 15 μg / ml). i.e. A(1/2) was passaged into flasks labelled A1(0), A1(5), A1(7.5), A1(10), A1(15) and A2(0/5/7.5/10 and 15) and so forth. Therefore the four groups of flasks, A1&2, A3&4, B1&2 and B3&4, contain independent events. Each of the four control cultures were passaged into one set of five flasks with the same range of G418 concentrations. Both controls and transfected cell

lines grew at 5 μg / ml of G418, though at a reduced rate. At 7.5 μg / ml of G418, no antibiotic resistant cells were recovered from the four control flasks, whereas six of the eight NKOV transfected lines gave rise to G418 resistant cultures after two weeks (NA1/3/4, NB1/2/4). There was no G418 resistant growth in the flasks containing 10 or 15 μg / ml of G418. The six G418 resistant cultures (which were non-clonal) were grown up, DNA purified, digested with *Eco*RI, and a Southern blot performed (Figue 6.2.3). As controls, *Eco*RI digested DNA from STIB247 and Ble(M) lines was run on the same gel. The blot was hybridised to the *tbcrk3* containing fragments from *Hin*dIII digested pCD172[Hd-(Hd)-Hd], labelled by random priming. The bands expected to hybridise to *tbcrk3* after a NKOV integration event were 2.9 kb (the equivalent DNA fragment to that seen in the *ble* integration [see Figure 6.2.1]) and 1.2 kb. If *tbcrk3* was essential, or if the integration event could not occur at the second allelle due to differences in the nucleotide sequence, then the cultures would only contain cells in which the *neo* resistance cassette had replaced the *ble* cassette and the second BKOV associated band at 2.3 kb should be missing from the Southern blots. If cells lacking *tbcrk3* were viable then mixed populations of both double knockout events and replacement events would be detected assuming that null mutants could multiply at a similar rate to the *tbcrk3* containing trypanosomes. DNA from such a mixed population would show four bands, the wild type (4.4 kb), the Ble(M) bands (2.9 kb and 2.3 kb), and the new NKOV band at 1.2 kb. If the NKOV was retained as an episome then the hybridising bands would be at 1.0 kb and 1.2 kb. Figure 6.2.3 shows the resultant autoradiograph. Lanes 1 and 2 are STIB247 and Ble(M) DNA respectively, while lanes 3-8 contain DNA from each of the G418 resistant lines (NA1, 3 and 4, NB1, 2 and 4). The 2.3 kb band specific to the *ble* insertion event is not present in any of the lanes containing DNA from the G418 resistant cultures (compare lane 2 with lanes 3-8). The 1.2 kb band can be seen in five of the six G418 resistant lines (lanes 3-7) and may not be visible in lane 8 due to the underloading of this sample. Therefore all of the integration events appeared to be replacements of the *ble* cassette by the *neo* containing vector. This implied that *tbcrk3* is an essential gene, or that its loss greatly impairs the

Figure 6.2.3 Southern blot of DNA from the G418 resistant *T. brucei* cultures showing replacement of the *ble* resistance cassette by the *neo* resistance cassette.

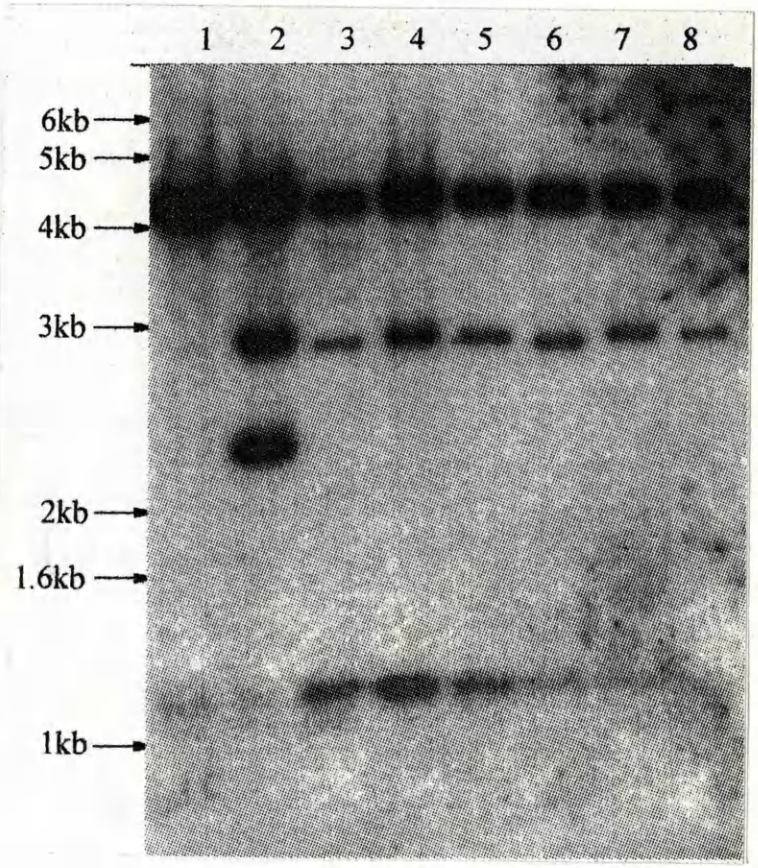


Figure 6.2.3. Genomic DNA was prepared from G418 resistant cultures (Scotlab). DNA (up to 10 μg) was digested in a total volume of 39 μl containing 15 U of *Eco*RI. After 2 hours a further 10 U of enzyme were added. After 2 more hours 5 x Loading Buffer was added to a final volume of 50 μl and this was loaded onto a 0.7 % TBE/agarose gel and run overnight at 30 V. The gel was then stained with Ethidium Bromide and photographed before being treated for Southern blotting. The DNA was fixed to the Hybond-N filter by baking at 80 ^{o}C for 2 hours before prehybridisation. Hybridisation to labelled *tbcrk3*-containing fragments from *Hin*dIII digested pCD172[Hd-(Hd)-Hd], was performed overnight at 65 ^{o}C. Lanes 1 and 2 contain DNA previously prepared from the STIB247 and the Ble(M) cell culture respectively, lanes 3-8 contain DNA from G418 resistant cultures: lane 3 - NA1, lane 4 - NA3, lane 5 - NA4, lane 6 - NB1, lane 7 - NB2, lane 8 - NB4.

growth of *T. brucei* such that null mutants are outgrown by those cells undergoing replacement events. It was possible, however, that the copy of *tbcrk3* which had already been disrupted may have had a predisposition to integration events and the sensitivity of the Southern blot was not good enough to spot a small number of null mutants. To assess this possibility it was decided to perform PCR, using oligonucleotides homologous to the 5' and 3' ends of *tbcrk3*, on the G418 resistant cultures when they were still at a low density, soon after the transfection event. This approach could detect such rare events. The preferred option of cloning individual cell lines from the population and assaying their genotype and phenotype was curtailed due to lack of time.

6.3 Analysis of the putative *tbcrk3* null mutants.

6.3.1 PCR analysis of *tbcrk3* mutants

PCR was performed using oligonucleotides previously employed in the cloning of *tbcrk3* into the yeast complementation vector, and were homologous to the regions of the *tbcrk3* initiation ATG and the termination codon (see Chapter 5.2). As the PCR was to be performed on cultures immediately after selection, with low cell density, it was decided to utilise a crude boiled cell extract as the starting template without further DNA purification. Template DNA was prepared by pelleting 100 μl of culture, washing with 1 ml of 1 x Phosphate Buffered Saline (PBS), resuspending the cells in 100 μl of 5 mM EDTA and then boiling for 10 minutes. Each 50 μl PCR contained 1 μl of the template solution.

The first PCR was performed on cells from the NB1 culture that had undergone 2 weeks of G418 selection only. Figure 6.3.1 shows the EtBr stained agarose gel on which 10 μl of the PCR had been loaded (lane 1), along with control lanes containing 5 μl of PCR reactions which used purified pCD172[Hd-(Hd)-Hd], pCD175BKOV or pCD272NKOV as template (lanes 2-4). The wild type gene was expected to result in a

PCR product of ~1 kb, the *ble* resistance cassette should give a product of 1.6 kb, and the *neo* resistance cassette should result in a band of 3 kb. The pCD172[Hd-(Hd)-Hd] plasmid (lane 2) and the pCD272NKOV plasmid (lane 4) gave the correct size fragments of 1 kb and 3 kb respectively. An unexpected result however, was obtained for the pCD175BKOV template (lane 3) which gave a 700 bp PCR product instead of the 1.6 kb product expected. Mapping this DNA fragment with various restriction enzymes (including *Bam*HI, *Hin*dIII, *Pst*I, *Spe*I, *Xba*I and *Xho*I), showed that the *tbcrk3* flanking sequences have amplified correctly, but the *ble* cassette was almost entirely deleted. The reaction using template prepared from the NB1 culture (lane 1) shows 3 bands of 700 bp, 1 kb and 3 kb, each the same size as a product in one of the control lanes. The 700 bp PCR product correlating to the *ble* resistance cassette is considerably less intense than the other 2 bands from the NB1 template. Although PCR is difficult to analyse quantitatively without careful controls, this low intensity band implied the presence of trypanosomes carrying both *ble* and *neo* resistance markers in this culture. It was not possible from this experiment to distinguish whether they were null mutants or if they had retained a wild type copy of *tbcrk3* by increasing *tbcrk3* copy number, either through ploidy changes or episome formation. Each of the G418 resistant cell lines was split in half, with one half being added to medium containing both G418 and phleomycin while the remaining culture was only exposed to G418. Of the six G418 resistant lines, only two (NB2 and NB4) gave rise to cultures which were resistant to both antibiotics (called B2D and B4D respectively). Suprisingly, NB1, the culture used for the PCR and which showed the presence of both *ble* and *neo* resistance markers, did not give rise to doubly resistant trypanosomes. Both B2D and B4D grew extremely slowly. The cultures under phleomycin and G418 selection barely increased in cell density over several weeks, but unlike other lines under the same selection, they did not die. The cells were regularly spun down, and resuspended in fresh medium containing G418 and phleomycin to ensure that the medium did not become depleted of nutrients. As the trypanosomes did not increase in number, there were not enough cells to prepare DNA for Southern blots, or protein extracts for Western blots. Eight weeks after the

Figure 6.3.1 PCR of *tbcrk3* mutant cell line NB1 with *tbcrk3* specific oligonucleotides.

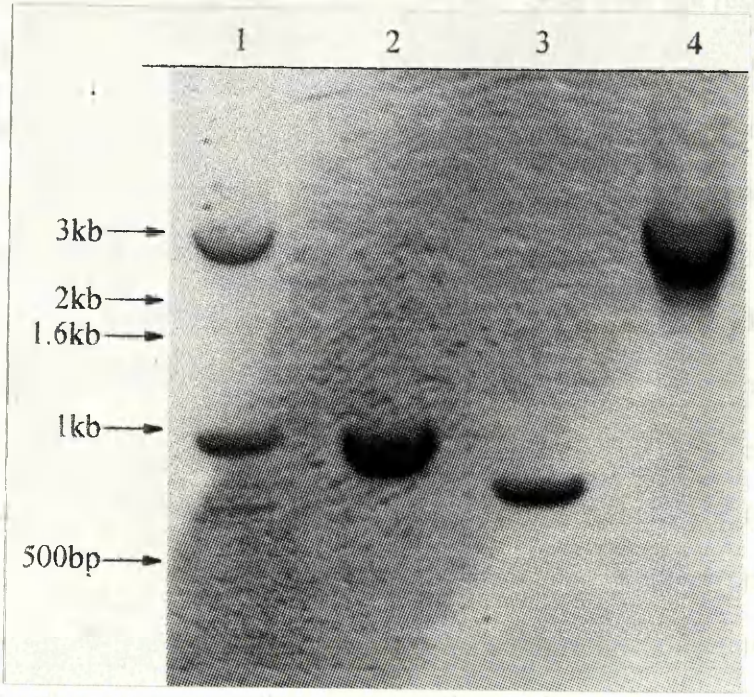


Figure 6.3.1 PCR was performed on template from the NB1 culture (lane 1), pCD172[Hd-(Hd)-Hd] (lane 2), pCD175BKOV (lane 3), and pCD272NKOV (lane 4). 100 μl of cells from the NB1 culture were pelleted, washed in 1 ml of PBS and then boiled in 100 μl of 5 mM EDTA. All control plasmids had been prepared using QIAGEN miniprep columns and mapped to ensure they were correct. The final control template concentration was 0.1 μg / ml. The oligonucleotides used were homologous to the 5' and 3' ends of the *tbcrk3* ORF. The PCR was performed using *Taq* DNA Polymerase in a total reaction volume of 50 μl. After an initial denaturation at 95^{o}C for 5 minutes, 30 cycles were performed with annealing at 60 ^{o}C for 1 minute, extension at 72 ^{o}C for 2 minutes 30s, and denaturation at 95 ^{o}C for 45s. 10 μl of the NB1 culture PCR and 5 μl of each control PCR was loaded in each lane.

double selection procedure the cell density of the cultures began to decrease. Light microscopy of the cultures did not show noticeable levels of cellular debris, unlike that seen in cultures undergoing antibiotic selection where cells were not resistant. The light microscopy also appeared to show a higher ratio of cells in the early/medium stages of cytokinesis, when the cell bodies remain at 45-90^{o} to each other, compared to other cell lines, and comparatively few in the final stages where the cells are joined at the most posterior point (see Figure 1.2.2 for diagram of cell division). PCR was used to characterise the *tbcrk3* loci in these populations (see Chapter 6.3.2), and Giemsa and DAPI staining of fixed cells was used to assess the phenotype (see Chapter 6.3.3). The cultures continued to decrease in cell density, even after the transfer of cells into non-selective medium, until no cells could be seen in repeated observations (approximately 11 and 12 weeks after selection for B2D and B4D respectively). The single replacement line, NB4, from which B4D had been derived, was passaged into phleomycin/G418 containing medium to assess the resultant effects on the cells, and it was also passaged into medium containing G418 which was supplemented with 25 % conditioned medium from the dead B4D culture (to assess if the loss of viability was due to contamination of the B4D cell line with a virus, or other organism invisible under the microscope). Neither of these conditions resulted in the same phenotype observed with the B2D and B4D lines (see Chapter 6.3.3). The NB4 cells treated with phleomycin died rapidly, with high levels of debris apparent and no obvious signs of delayed or altered cytokinesis, while the cells passaged into B4D medium grew as well as those in fresh medium.

6.3.2 PCR analysis of the *T. brucei* B4D cell line: null mutants or multiple *tbcrk3* loci?

The two cell cultures to be used for the PCR reactions (B2D and B4D) were prepared in the same manner as previously, except that the medium containing the cells was treated with DNAseI for 30 minutes before the cells were spun down, washed, resuspended and boiled. This treatment was used to avoid any contamination by

plasmid or genomic DNA left in the culture medium after transfection and selection (specifically the integration plasmid). The two sets of oligonucleotides used for amplification were specific to the *tbcrk3* ORF (see Chapter 5.2) and the *ble* ORF (gift of Dr. D. Jeffries). PCR using the template prepared from the B2D culture did not give reproduceable PCR products, but as the B2D culture acted in a very similar manner to the B4D line (B2D was maintained at a slightly lower cell density after selection than the B4D culture, and reached zero approximately one week earlier) the genomic events which created the two lines could be expected to be similar. Although the wild type *tbcrk3* ORF was amplified from unpurified *T. brucei* genomic template routinely, the loci with *ble* and *neo* cassette insertions only amplified occasionally even though amplification from plasmid template occurred without difficulty. It may be that, for differing reasons, these two PCR's had a relatively low efficiency. The *ble* cassette always resulted in a much smaller band than expected (see Figure 6.3.1), indicating that the PCR was not performing as predicted, and the *neo* cassette results in a 3 kb band using the *tbcrk3* oligonucleotides, which is towards the limits of the PCR protocol used in these experiments.

The result of a PCR reaction using the *tbcrk3* specific oligonucleotides is shown in Figure 6.3.2. Only the wild type loci have amplified from the phleomycin and G418 singly resistant cultures (lanes 3 and 4), and no such band is present in the reaction using B4D DNA as template (lane 5). Lane 6 shows the result of a PCR using B4D template purposefully contaminated with pCD172[Hd-(Hd)-Hd] plasmid, which contains the *tbcrk3* ORF. This results in the correct size band, and therefore shows no general inhibition of the *taq* DNA polymerase activity by the B4D extract. The lack of the 930 bp band in lane 5, which was repeated on several occasions, indicates that the BD4 culture does not contain a wild type *tbcrk3* allelle.

PCR was also performed using the *ble* 5' and 3' oligonucleotides (Figure 6.3.3). These oligonucleotides would not be expected to amplify any products from wild type *T. brucei* DNA, but the reaction using STIB247 template resulted in a large number of non-specific bands. However, the reaction did not contain the expected 380 bp product

(lane 1). Both Ble(M) and B4D template containing reactions did amplify the correct

Figures 6.3.2 PCR of wild type and *tbcrk3* mutant cell lines using *tbcrk3* specific oligonucleotides

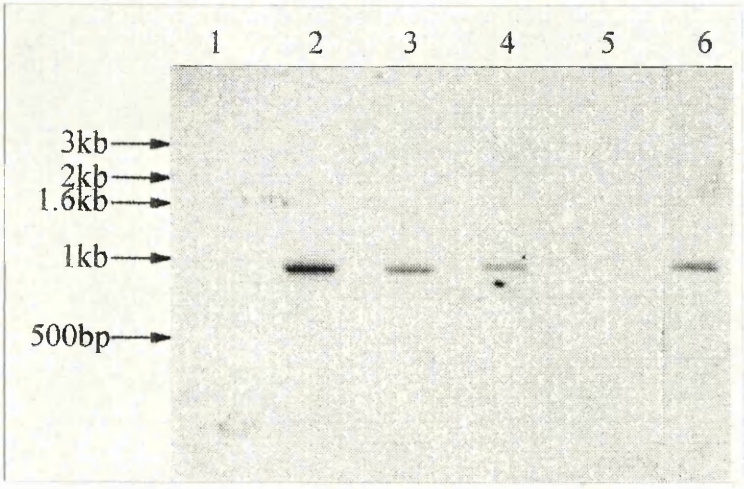


Figure 6.3.2 The template from *T. brucei* procyclic cultures was derived from 500 μl of cells. The samples were treated with DNAseI at a final concentration of 40 μg / ml, at 28 ^{O}C for 1 hour. EDTA was then added to 5 mM before the cells were pelleted. The supernatant was removed to leave ~15 μl of solution. The pellet was resuspended in this and boiled for 15 minutes. The PCR was performed using *Taq* DNA Polymerase in a total reaction volume of 50 μl. The primers were those previously used to amplify *tbcrk3* for subcloning into the *S. pombe* expression vector, pMNS21L (see Chapter 5.2). After an initial denaturation at 95 ^{O}C for 5 minutes, 30 cycles were performed with annealing at 60 ^{O}C for 1 minute, extension at 72 ^{O}C for 2 minutes 30s, and denaturation at 95 ^{O}C for 45s. Lane 1 was a no template DNA control, lane 2 - STIB247 wild type DNA, lane 3 - Ble(M) DNA, lane 4 - NB4 DNA, lane 5 - B4D DNA, lane 6 - B4D DNA plus 0.1 μg / ml pCD172[Hd-(Hd)-Hd].

Figure 6.3.3 PCR of wild type and *tbcrk3* mutant cell lines using *ble* amplifying oligonucleotides.

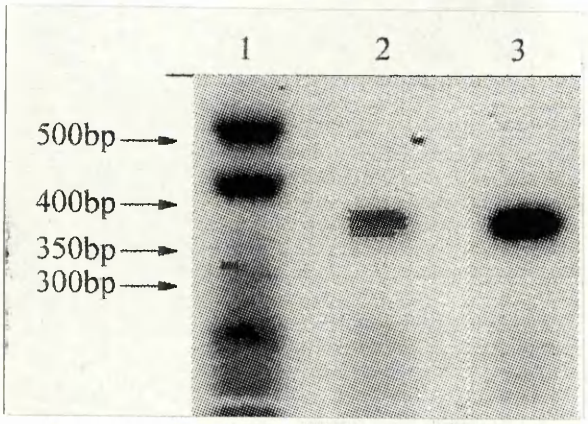


Figure 6.3.3 The template from *T. brucei* procyclic cultures was derived from 500 μl of cells. The samples were treated with DNAseI at a final concentration of 40 μg / ml, at 28 ^{O}C for 1 hour. EDTA was then added to 5 mM before the cells were pelleted. The supernatant was removed to leave ~15 μl of solution. The pellet was resuspended in this and boiled for 15 minutes. The PCR was performed using *Taq* DNA Polymerase in a total reaction volume of 50 μl. The pimers used annealed to the 5' and 3' ends of the *ble* ORF. After an initial denaturation at 95 ^{O}C for 5 minutes, 30 cycles were performed with annealing at 60 ^{O}C for 1 minute, extension at 72 ^{O}C for 2 minutes 30s, and denaturation at 95 ^{O}C for 45s. Lane 1 - STIB247 wild type DNA, lane 2 - Ble (M) DNA, lane 3 - B4D DNA.

Figure 6.3.4 PCR of wild type and *tbcrk3* mutant cell lines using *tbcrk3* amplifying oligonucleotides with a longer extension time.

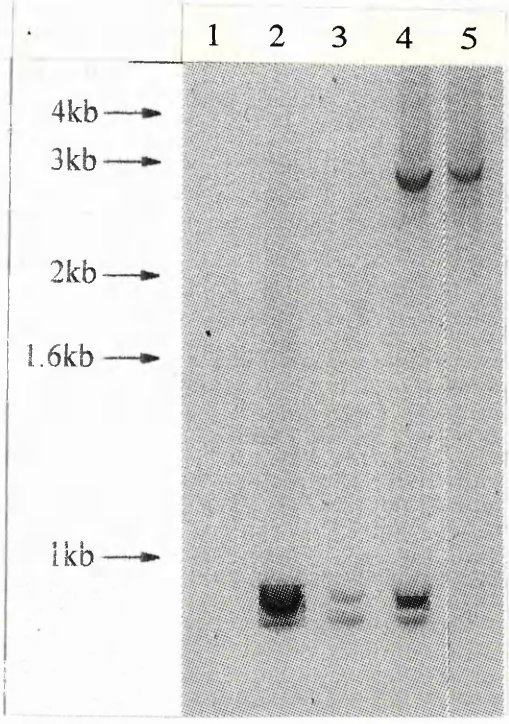


Figure 6.3.4 The template from *T. brucei* procyclic cultures was derived from 500 μl of cells. The samples were treated with DNAseI at a final concentration of 40 μg / ml, at 28 ^{O}C for 1 hour. EDTA was then added to 5 mM before the cells were pelleted. The supernatant was removed to leave ~15 μl of solution. The pellet was resuspended in this and boiled for 15 minutes. The PCR was performed using *Taq* DNA Polymerase in a total reaction volume of 50 μl. The pimers used annealed to the 5' and 3' ends of the *tbcrk3* gene. After an initial denaturation at 95 ^{O}C for 5 minutes, 30 cycles were performed with annealing at 60 ^{O}C for 1 minute, extension at 72 ^{O}C for 3 minutes 30s, and denaturation at 95 ^{O}C for 45s. Lane 1 - no template DNA, lane 2 - STIB247 wild type DNA, lane 3 - Ble(M) DNA, lane 4 - NB4 DNA, lane 5 - B4D DNA.

size 380 bp product (lanes 2 and 3), indicating that the Ble(M) and B4D cultures contain the *ble* resistance gene.

The products of another PCR performed with *tbcrk3* specific oligonucleotides is shown in Figure 6.3.4. The wild type *tbcrk3* gene is amplified as a 930 bp product, and is present in STIB247 (lane 2), Ble(M) (lane 3), and NB4 (lane 4) templated reactions, but not in the B4D reaction (lane 5). In both NB4 and B4D PCR reactions (lanes 4 and 5) a product of 2.9 kb was detected, the expected size of the amplification product of the *neo* resistance cassette. This indicates that the B4D cells contain the *neo* gene inserted into a *tbcrk3* ORF. It is not known why the *neo* resistance cassette was amplified in these reactions but not the *ble* resistance cassette.

Evidence from the PCR experiments suggest that the doubly resistant cultures, B2D and B4D, contain both antibiotic resistance cassettes, and are lacking any wild type *tbcrk3* gene copies. The phenotype of these cultures should, therefore, show evidence of the function of the TbCRK3 protein.

6.3.3 Analysis of *tbcrk3* mutants using DAPI staining and fluorescence microscopy.

Samples of the B2D and B4D cultures, and the NB4 culture as a control, were pipetted onto slides and allowed to dry in a sterile hood. Once dried, the cells were fixed with methanol, washed with PBS and again allowed to dry. The slides were either stained with Geimsa or DAPI (see Materials and Methods) and examined under the microscope. DAPI is a fluorescent dye which intercalates DNA allowing the visualisation of both the nucleus and kinetoplast. It was difficult to see the kinetoplast with Geimsa staining and so the assessment of phenotype was performed with DAPI stained cells, scoring each cell for the number of nuclei and kinetoplasts present. The microscope used had a moveable stage with a vernier scale. To ensure cells were only counted once each, the count started at the back, left corner of the slide. The stage was then gradually moved rightwards, scoring the phenotype of each cell within the field of vision. At the right-hand edge of the slide, the stage was moved forwards across 3

fields of vision before moving right, back across the slide, scoring more cells. The NB4 culture was used as a control because it had been treated in an identical fashion to the B2D cell line, and it was identical to the B4D cell line, up to their selection with medium containing both phleomycin and G418. It therefore controls for the fact that these cultures have undergone two rounds of transfection, integration and selection including prolonged growth in the presence of phleomycin, an antibiotic that damages DNA (Xu and Johnston, 1994).

Both B2D and B4D showed elevated numbers of cells with abnormal kinetoplast/nuclear ratios (Table 6.3.1, shown graphically in Figure 6.3.5). There are 125% and 50% more cells without nuclei (0N XK) respectively, reduced numbers of cells in the G1/S phases of the cell cycle (1K 1N) even ignoring the fact that many of these cells in the null mutants had abnormal morphology, and a large increase in the numbers of cells with two nuclei; both with the correct number of kinetoplasts (2N 2K) and with incorrect N/K ratios. The control sample using the NB4 line does show a higher level of unusual forms than another such survey (Das *et al.*, 1994), which may be due to several reasons. Firstly, the rate of imperfect cytokinesis may vary between various laboratory strains. The study by Das *et al* was performed with the TREU667 strain as while these mutant cultures were derived from the STIB247 line. It is also possible that the cells are in some way affected by the G418 in the medium, even though they are resistant to the drug. There is also the possibility that the single replacement event results in lower levels of TbCRK3 in the cell, and that this causes a mild version of the phenotype seen in the null mutants. The data suggest that the null mutant cell lines had a defect in cell division, although as cells from all points in the cell cycle were detected there appeared to be no particular restriction point in the cell cycle that the cells could not pass through.

Table 6.3.1 Analysis of the number of nuclei and kinetoplasts in *tbcrk3* mutant cell lines.

	NB4 (Controls)	B2D	B4D
0N XK	8% (9)	18% (23)	12% (18)
1N 0K	4% (5)	2% (2)	3% (5)
1N 1K	**81% (93)**	**30% (37a)**	**56% (85b)**
1N 2K	**4% (5)**	**4% (5)**	**1% (2)**
1N >2K	0	0	1% (1)
2N 0K	0	4% (5)	5% (7)
2N 1K	2% (2)	30% (37)	17% (26)
2N 2K	**1% (1)**	**8% (10)**	**3% (4)**
>2N XK	0	4% (5)	2% (3)

The first column shows the numbers of nuclei (N) and kinetoplasts (K) present in each cell. The percentage of the total population in each category is shown in each column, with the figures in brackets being the actual number of cells scored for each category. The rows in bold type are cell morphologies expected to be seen in a normal *T. brucei* cell cycle (Gull, 1985).

a - Of the 37 cells in this set, 20 were malformed in some manner. 3 had two or three flagella, although the most common abnormality was that the kinetoplast and /or the nucleus were not located in the expected position in the cell.

b - 26 of the 85 cells in this set were malformed, in a similar manner to those in the B2D cell line.

Figure 6.3.5 The percentage of cells in each category for each cell line (Data taken from Table 6.3.1).

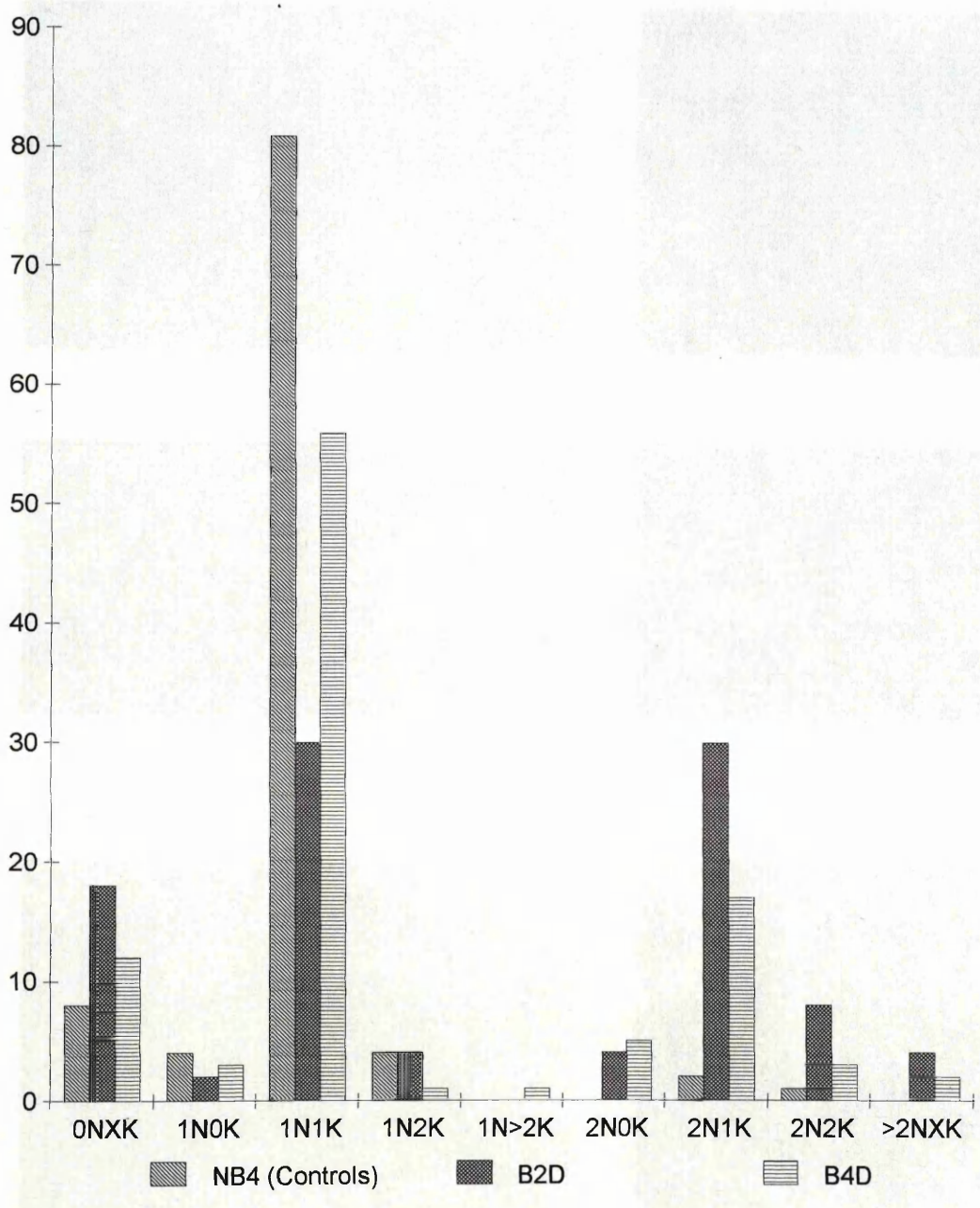
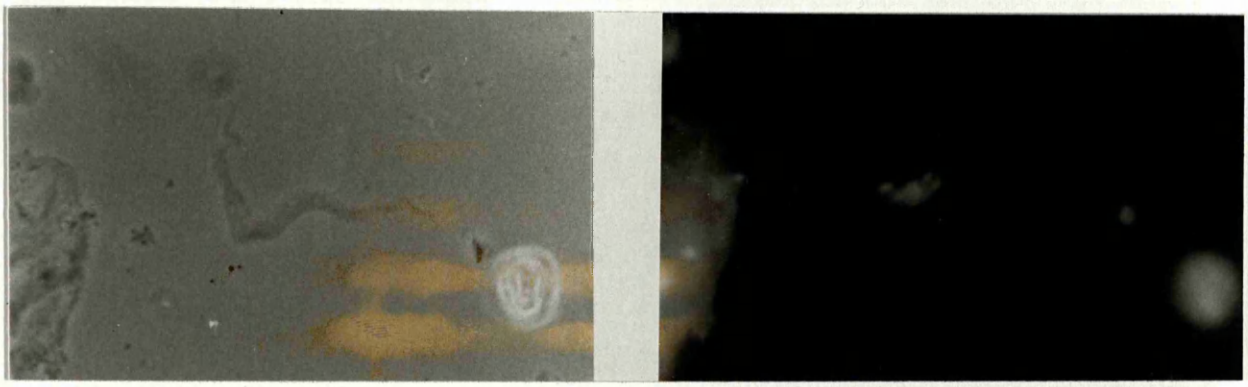
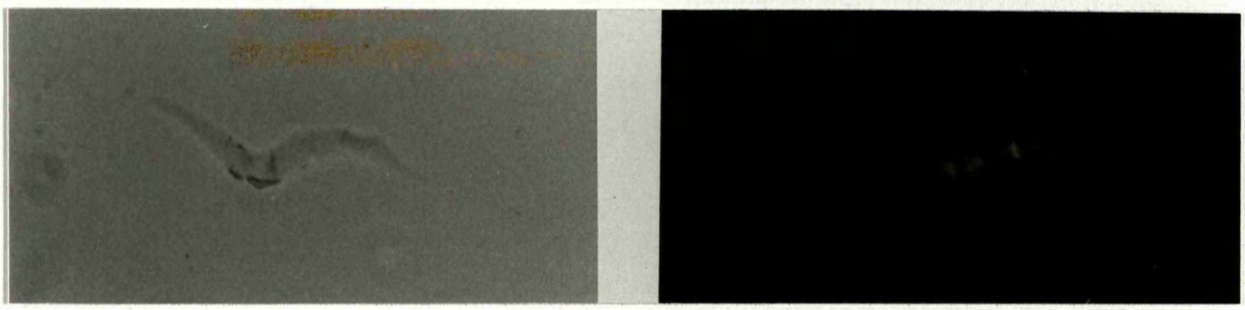


Figure 6.3.6(A) Phase contrast and fluorescence photographs of DAPI stained cells from the NB4 culture treated with phleomycin.

(1) (2)



(3) (4)



(5) (6)

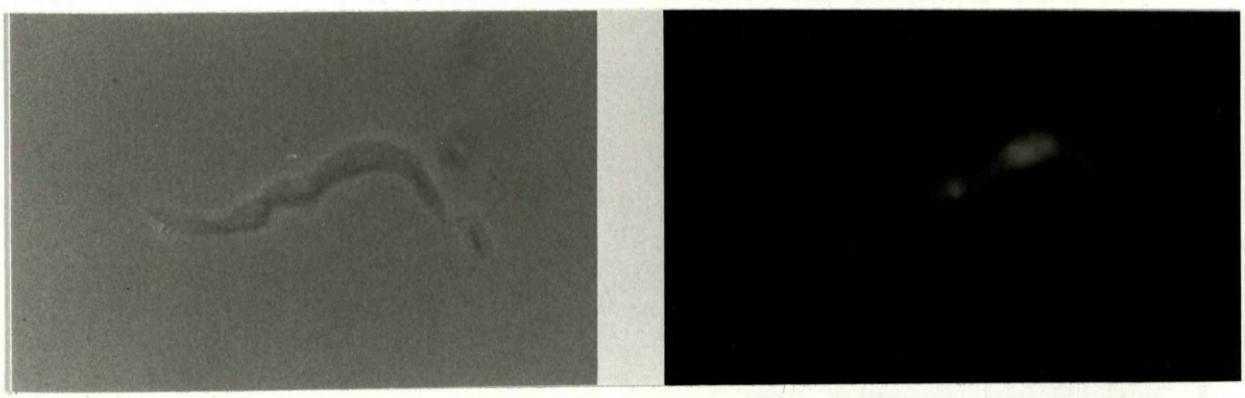


Figure 6.3.6(A) 100 μl of NB4 culture, 6 days after the start of treatment with 20 μg / ml phleomycin was spun down at 1000 rpm for 5 minutes. The cells were then gently resuspended in the supernatant. The centrifugation was performed so that the cells were treated in the same manner as the putative null mutants which needed concentration to make the analysis easier. 20 μl of the culture was then aliquoted onto a slide and allowed to dry in a tissue culture hood before fixing and staining with DAPI. Photographs 1, 3 and 5 - phase contrast microscopy, photographs 2, 4 and 6 - the same fields of view under fluorescence.

Figure 6.3.6(B) Phase contrast and fluorescence photographs of DAPI stained cells from the NB4 culture grown in the presence of medium from the B4D culture.

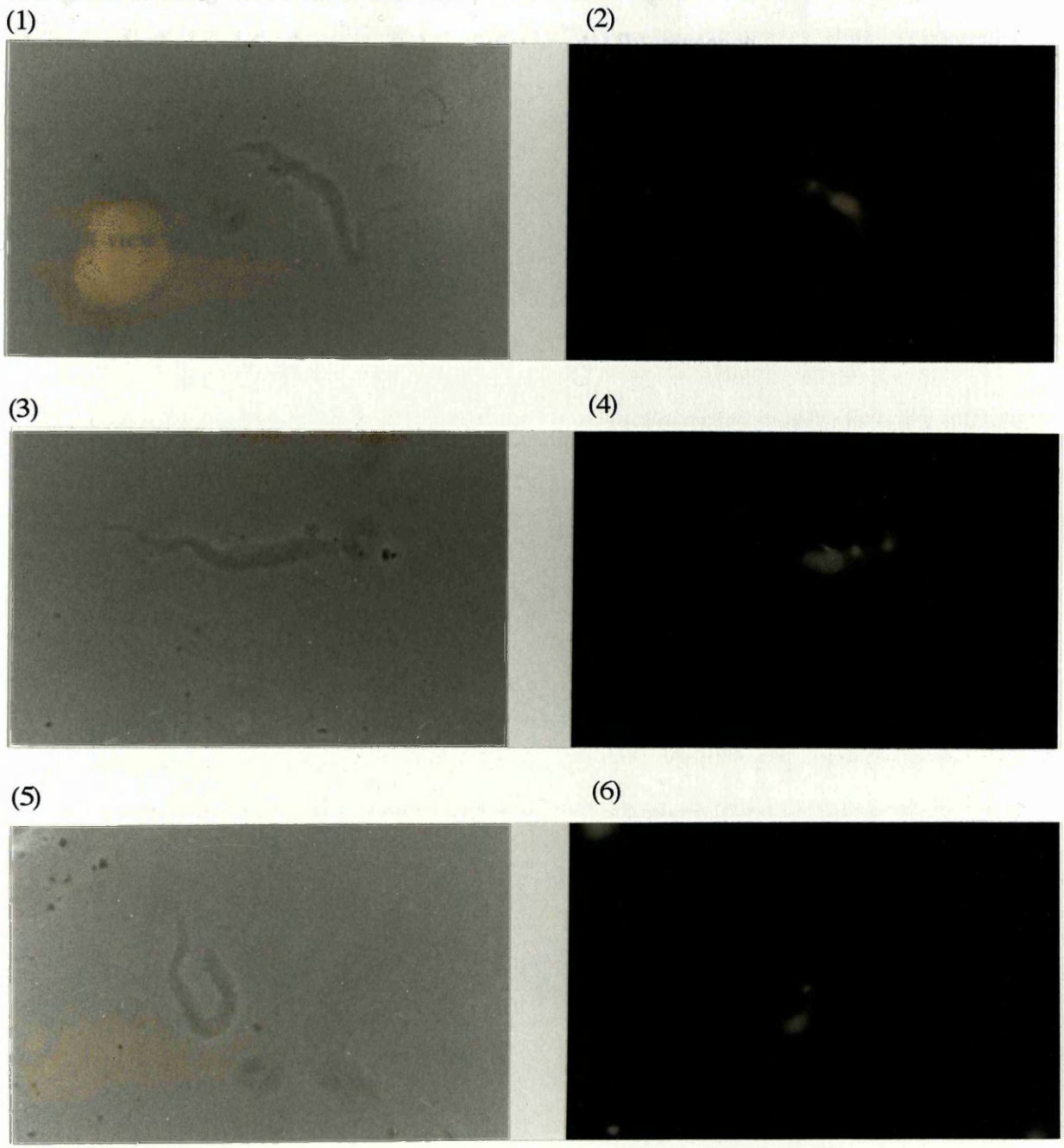


Figure 6.3.6(B) 100 μl of mid log phase NB4 culture was spun down at 1000 rpm for 5 minutes. The cells were then gently resuspended in the supernatant. 20 μl of this was then aliquoted onto a slide and allowed to dry in a tissue culture hood before fixing and staining with DAPI.. Photographs 1, 3 and 5 - phase contrast microscopy, photographs 2, 4 and 6 - the same fields of view under fluorescence.

Figure 6.3.6(C) (1-6) Phase contrast and fluorescence photographs of DAPI stained cells from the B2D putative null mutant culture

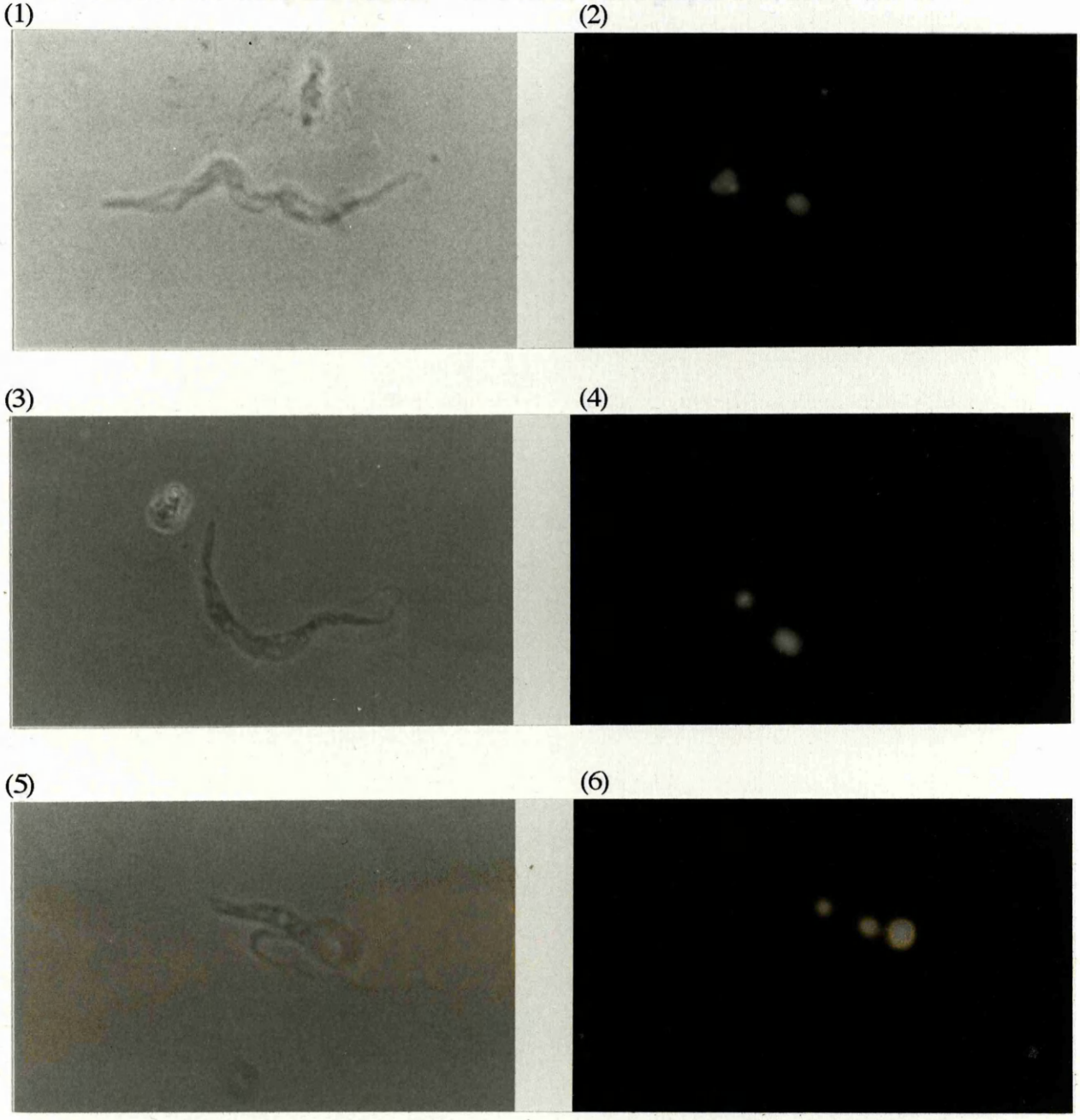


Figure 6.3.6(C) 100 μl of the B2D culture was spun down at 1000 rpm for 5 minutes. 80 μl of the supernatant was removed and the cells gently resuspended in the remaining liquid. This was then aliquoted onto a slide and allowed to dry in a tissue culture hood before fixing and staining with DAPI.. Photographs 1, 3, 5, 7, 9 and 11 - phase contrast microscopy, photographs 2, 4, 6, 8, 10, and 12 - the same fields of view under fluorescence.

Figure 6.3.6(C) (7-12) Phase contrast and fluorescence photographs of DAPI stained cells from the B2D putative null mutant culture

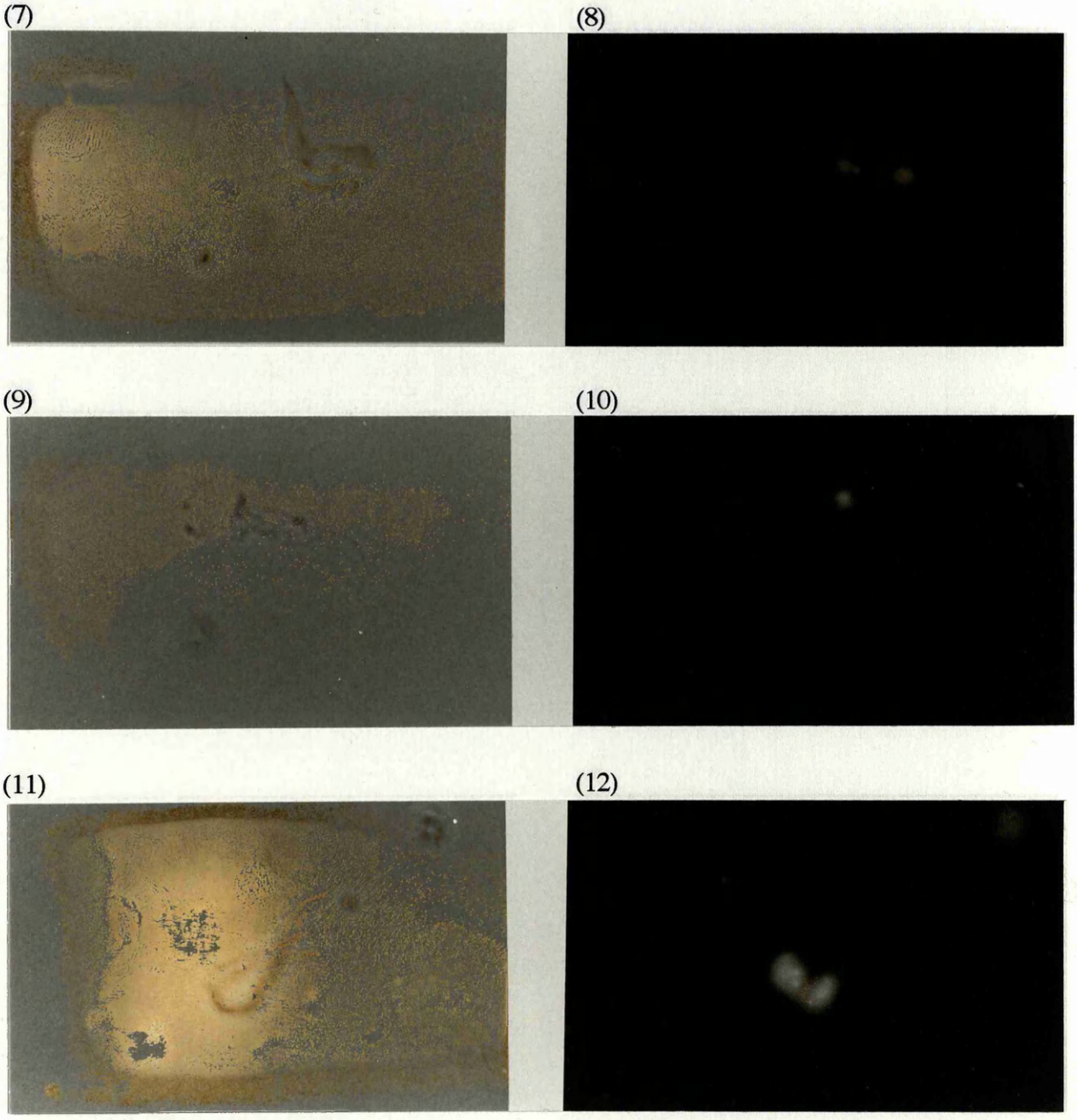


Figure 6.3.6(D) (1-6) Phase contrast and fluorescence photographs of DAPI stained cells from the B4D putative null mutant culture.

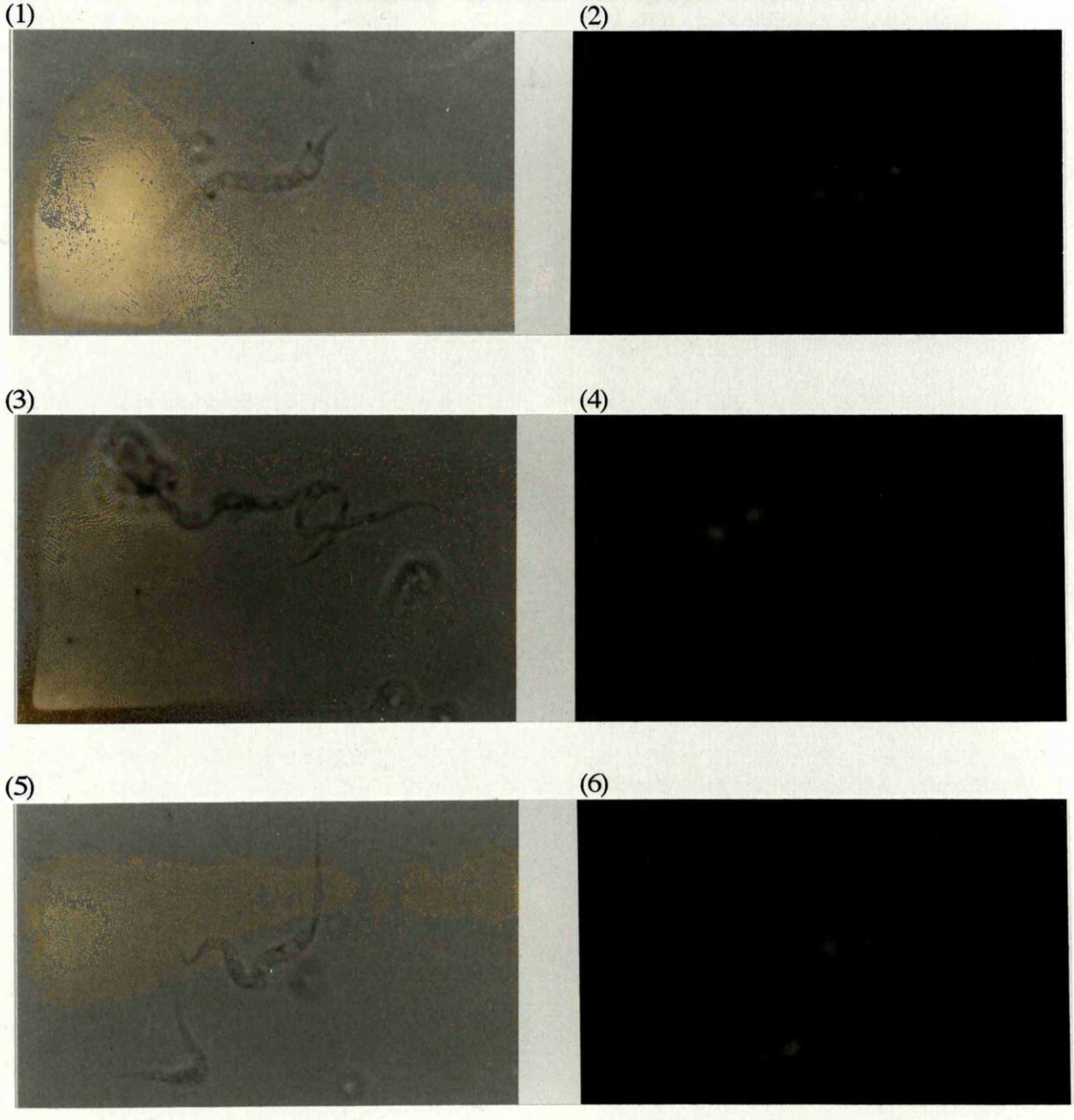
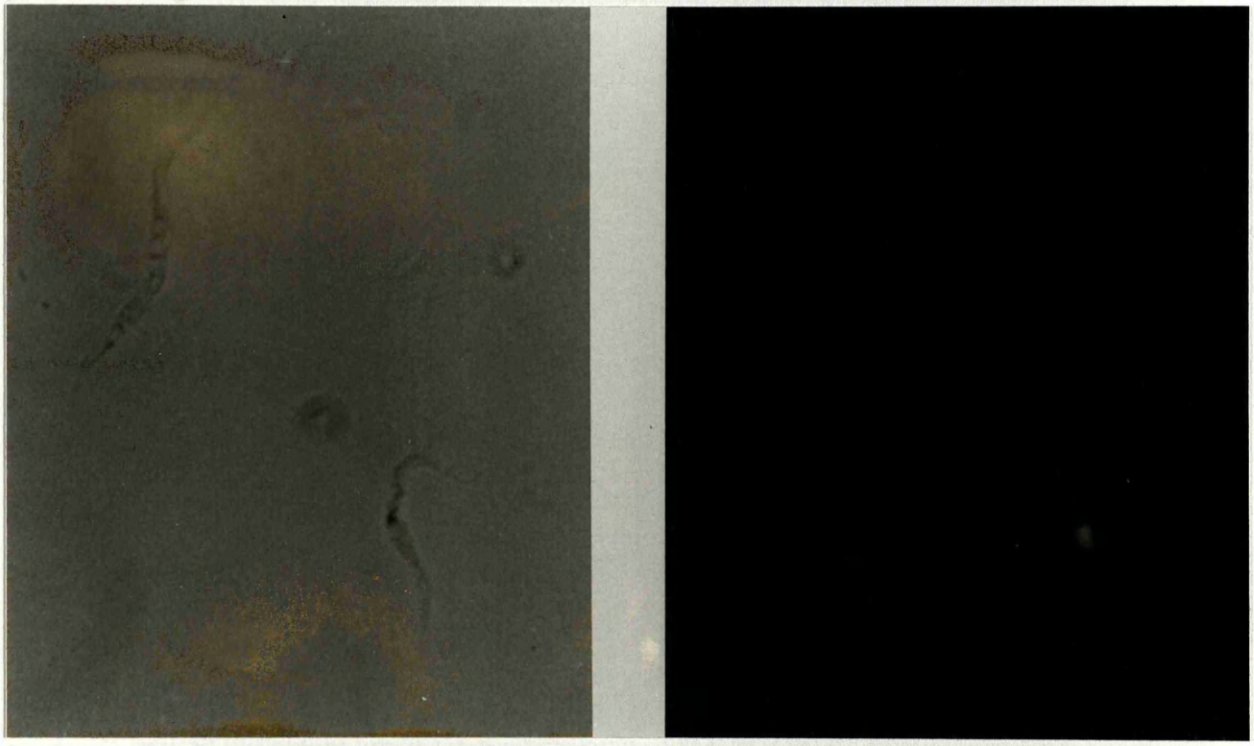


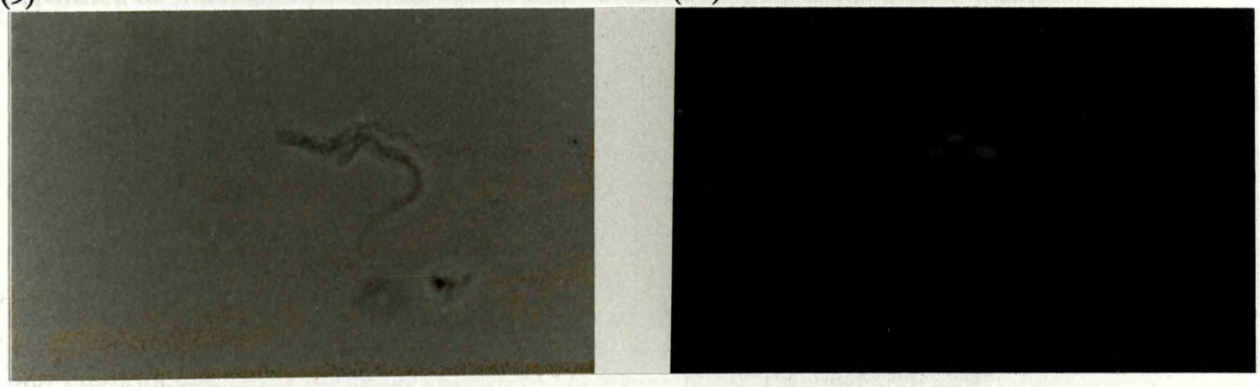
Figure 6.3.6(D) 100 μl of the B4D culture was spun down at 1000 rpm for 5 minutes. 80 μl of the supernatant was removed and the cells gently resuspended in the remaining liquid. This was then aliquoted onto a slide and allowed to dry in a tissue culture hood before fixing and staining with DAPI. Photographs 1, 3, 5, 7, and 9 - phase contrast microscopy, photographs 2, 4, 6, 8 and 10 - the same fields of view under fluorescence.

Figure 6.3.6(D) (7-10) Phase contrast and fluorescence photographs of DAPI stained cells from the B4D putative null mutant culture

(7) (8)



(9) (10)



Photographs of DAPI stained cells, representative of the various cell lines, were taken. Figure 6.3.6 shows the photographs, both phase contrast, showing cell morphology, and fluorescent, showing the kinetoplast and nucleus number, and their positions. Figure 6.3.6(A) shows cells from NB4, the G418 resistant line, 6 days after treatment with 20 μg / ml phleomycin. In each case the cells contain one kinetoplast, one nucleus and one flagellum. The patchy nature of the staining in the nucleus of these cells (particularly in 2 and 4) is presumably due to DNA damage caused by the phleomycin. Figure 6.3.6(B) shows cells from the NB4 line, grown in medium containing 25 % medium from a BD4 culture. As with Figure 6.3.6(A), the cells appear normal. Photographs (B)3 and 4 show a cell which has two kinetoplasts, which have separated prior to nuclear division, as expected for a cell in G2.

Figures 6.3.6(C) and (D) show cells from the two null mutant cultures B2D and B4D respectively. In (C)2 DAPI staining shows a cell with 2 nuclei but either no, or possibly one (at the right hand edge of the left hand nucleus), kinetoplast. (C)3/4 shows a normal appearing cell, although the kinetoplast does appear relatively large, perhaps having undergone replication without division. The cell in (C)5/6 appears to have separated its kinetoplasts, though again they are considerably larger than is typical. (C)8 clearly shows a cell which has two nuclei and only one kinetoplast, which is positioned between the two nuclei. This perhaps indicates that the cell has recently undergone an incorrect cytokinesis which resulted in the posterior kinetoplast being separated as a 'zoid'. In (C)10 there is a cell which has no nucleus, one kinetoplast and two flagella [see (C)9]. (C)11/12 show a cell with an unquantifiable number of nuclei and kinetoplasts. In this case division is clearly not occurring correctly. The kinetoplasts have not separated correctly, and they are not in the expected positions for a cell undergoing cytokinesis. The cell also appears to be starting cytokinesis from the wrong end. The cells in the B4D culture show similar phenotypes. (D)1/2 show a cell in which there is either one huge kinetoplast with more than the normal DNA content, and two nuclei, or there are three nuclei. The cell in (D)3/4 appears to be undergoing cytokinesis. Both nuclei are in what would become one of the daughter cells, while the

other cell only contains a kinetoplast. Neither of the cells visible in (D)5/6 are aberrant, and one is apparently progressing through the cell cycle, containing two, separated, kinetoplasts. The two cells in (D)7/8 however are both unusual. The lower cell has no kinetoplast, although the flagellum appears present, while the other has the kinetoplast incorrectly positioned, anterior to the nucleus. The cell in (D)9/10 is dividing. It contains the correct number of nuclei and kinetoplasts for a dividing cell (two of each), but as above, the kinetoplasts are not in the correct positions. They do not appear to have separated properly, and are both posterior to the more posterior nucleus, instead of one being between the two nuclei, as would be expected (Gull *et al.*, 1990).

6.4 Discussion

An analysis of TbCRK3 function was performed using homologous recombination to create procyclic form *tbcrk3* mutants. After one allelle was disrupted by the insertion of, and selection for, a bleomycin resistance cassette, the culture was again transfected, this time with a neomycin resistance cassette. Selection with both phleomycin and G418 antibiotics gave rise to a slowly growing population of cells. Analysis of these lines by PCR indicates that the cultures do not contain a wild type *tbcrk3* gene, and do contain both the *ble* and *neo* resistance genes inserted into a *tbcrk3* ORF. These mutants displayed a very slow rate of growth before the population died. This phenotype displayed by the null mutants of *tbcrk3* was unexpected. It was thought that a protein controlling the cell cycle would be essential, and if it was not essential, e.g. due to redundancy such that another TbCRK would function in place of the TbCRK3 protein, then it was probable that no phenotype would be seen. As the cultures did die it seems that the TbCRK3 protein is essential for cell viability. The relative longevity of the null mutants therefore has to be explained, as well as the lack of selection of tetraploid or rearranged genomes seen in other cases.

One possibility is that the mRNA, or the TbCRK3 protein, or both, are relatively stable with a long half life. If this is the case then the selection pressure against changes

in ploidy must be enough to remove those cells during the null mutants' early growth. It may be that aneuploid cells could not be isolated because other genes on the *tbcrk3* containing chromosome are under dosage control, but the large variations in karyotype, post-transcriptional control shown after gene deletion (de Lafaille and Wirth, 1992) and especially the isolation of triploid trypanosomes after genetic crosses (Gibson *et al.*, 1992; Wells *et al.*, 1987) makes this unlikely.

The phenotype displayed by the null mutant cell lines varied from cell to cell, both in the abnormality seen and in the severity of the condition. By light microscopy many cells appeared to be normal, even during the final stages of the cultures. In abnormal cells, commonly the cell organelles were incorrectly positioned, notably the kinetoplast/s were often misplaced, or not separated after replication. There was often an incorrect number of nuclei and kinetoplasts, and many of the kinetoplasts were unexpectedly large by DAPI staining. This may be due to the cells replicating their kDNA but not dividing the mitochondrion. Due to the longitudinal arrangement of the *T. brucei* cell, cytokinesis in *T. brucei* demands the accurate positioning of several of the cell's components, including the nucleus, kinetoplast and basal body/flagellum. The failure to accurately align these cellular structures would cause aberrant divisions, leading to the abnormal ratios of nuclei to kinetoplasts. There are other notable differences as well. Strikingly, the linkage between the kinetoplast and the flagellum was lost in a number of cases (see Figure 6.3.6(C) 9/10 and (D)7/8). During normal division there is a physical link between the kinetoplast and the basal body (Robinson and Gull, 1991) and there is one flagellum for each kinetoplast. However in the two mutant cultures, cells could be seen with more flagella than kinetoplasts, and vice versa. Possibly the most extreme alteration of cytokinesis was shown by the cells where cytokinesis appeared to be starting at entirely the wrong end of the organism (See Figure 6.3.6(C) 11/12).

A linking factor in these events is a role for the cytoskeleton. TbCRK3 may be involved in regulation of the cytoskeleton and its re-organisation during the cell cycle. Its loss results in a phenotype reminiscent of the mitotic catastrophe seen in yeast, with

the number of viable cells in a culture dropping as mistakes in division accumulate. This association could also explain the insoluble nature of TbCRK3 in Triton X-100, as this detergent keeps the cytoskeleton essentially intact. However, the cells predominantly retained their characteristic shape showing that TbCRK3 is unlikely to play a role in the organisation of the sub-pellicular microtubule 'basket' which runs underneath the cell membrane.

A role for TbCRK3 in cytoskeletal organisation though does not explain why tetraploid, and other forms of *tbcrk3*, *ble* and *neo* gene containing cells, were not found, since *tbcrk3* is essential. When *lmmcrk1* was targeted in a similar experiment (Mottram *et al.*, 1996), null mutants could not be obtained, which is the expected outcome for an essential gene. One theory which would explain these results is that one role of TbCRK3 may be to phosphorylate a stable protein which is part of a cellular structure involved in the control of organellar positioning. If this cellular structure is conservatively reproduced during the cell cycle, then one daughter cell would receive the old, already phosphorylated, structure while the other cell inherits a new one. If TbCRK3 and its mRNA are both relatively stable then after the second integration event the cell cycle could continue relatively unaffected for several generations until TbCRK3 is diluted out by subsequent cell divisions. As the levels of TbCRK3 drop the two daughter cells would become non-equivalent, as one would inherit an adequately phosphorylated structure while the other would inherit a structure consisting of newly sythesized protein lacking complete modification. While there was enough TbCRK3 in the cells it would be possible for the new structure to be phosphorylated before the next cell division occurred. However, as the levels of TbCRK3 decrease this would no longer be possible, and at this point aberrant division of the daughter cell with the unphosphorylated structure would begin. As the levels of TbCRK3 decreased still further then the cells would not keep up with the turnover of the phosphorylated protein and therefore increasing numbers of cells would start to exhibit the mutant phenotype. This theory would explain the observed population dynamics of the null mutants, consisting of a growth phase (when both daughter cells could successfully divide

again), a plateau (when one daughter cell could no longer divide) and the population crash (when every cell undergoing cytokinesis is dysfunctional).

One possibility for a cell structure which is likely to be conservatively replicated within procyclic *T. brucei* is the basal body. This structure is found in a precise position in the cell and is involved in the connection between the flagellum and the kinetoplast, a connection that is disrupted in the null mutant cell lines. After division, each cell contains one basal body and one pro-basal body. During the early G1 phase of the cell cycle, the pro-basal body elongates to become a full basal body. The new flagellum starts to form on the new basal body, and two new pro-basal bodies are formed. Interestingly, the two basal bodies are not equivalent as when they are separated, the old basal body with the older, longer, flagellum, always moves to become the more anterior of the basal bodies.

A single round of transfection with a *tbcrk3* deletion construct allowed stable integration of a resistance gene into the *tbcrk3* locus, with concomitant elimination of part of the *tbcrk3* ORF, creating cells haploid for *tbcrk3*. Following a second round of transfection with a construct containing a second drug resistance marker, a population of cells resistant to both drugs could be isolated, but these cultures were not viable over the long term. This lack of viability appeared due to an increasing proportion of cell division events being aberrant, resulting in *T. brucei* with incorrect numbers of nuclei and/or kinetoplasts. It seems likely that the mutant phenotype is due to a failure to regulate cytoskeletal components correctly, although gross morphological changes were only rarely seen, usually in cells lacking a nucleus. These cases appeared similar to that seen in non-resistant control cells and so were likely to be caused by the cell losing the resistance gene for one of the antibiotics due to incorrect separation of the nuclear DNA. The lack of morphological change implies that TbCRK3 does not play an important role in the organisation of the microtubular cytoskeleton.

The cause of the aberrant divisions was a loss of organisation at the organellar level, resulting in misplaced kinetoplasts, duplicated kinetoplasts that did not separate

correctly, cytokinesis occuring in an incorrect plane, and cytokinesis starting at the wrong end of the cell. There was also a loss of the connection between the flagellum and the kinetoplast with many cells no longer showing a 1:1 ratio. In the context of abnormal cytokinesis, it is striking that the cells continued to attempt to divide even when there were gross organisational changes. It may be that the fact that all three TbCRK proteins lack residues, conserved in other organisms, that affect S and M phase checkpoint control (Basi and Enoch, 1996) means that selection for parasitic rapid growth has resulted in an organism with few such checkpoint controls. This could be a reason for the lack of success in synchronisation of *T. brucei* cultures.

CHAPTER SEVEN
DISCUSSION OF THE DATA AND FURTHER WORK

7.1 Conclusions.

In this study a cdc2 related protein kinase (*tbcrk3*) was cloned from a *T. brucei* DNA lambda library and the open reading frame sequenced . Two other *T. brucei* genes from this family, *tbcrk1* and *tbcrk2* (Mottram and Smith, 1995) have also been characterised. Each of the three TbCRK proteins have the same level of similarity to CDC2 kinases from other organisms (~50 % identity). None of the three *T. brucei* proteins was predicted to contain all of the conserved domains found in the cdc2/CDK2 sub-family of CDKs (see Figure 3.3.8), leaving the question of which is likely to be the functional homologue unclear. TbCRK1 and TbCRK2 are poorly conserved in regions involved in the control of the activating Threonine-161 phosphorylation (the DSEI box and the region around Thr-161 itself), while the lack of a well conserved PSTAIR box in TbCRK2 and TbCRK3 (even though these proteins are recognised by the PSTAIR monoclonal antiserum) suggests that they will not interact correctly/adequately with mitotic cyclin type proteins from other organisms, making complementation assays unlikely to work. Alignments of the predicted protein sequences with sequence data from the family of cdc2 related kinases in higher eukaryotes and phylogeny analysis fail to convincingly place any of the *T. brucei* proteins in any of the known classes of Cyclin Dependant Kinases (CDKs). The inability to find a $p13^{suc1}$ binding kinase activity in *T. brucei* protein extracts is evidence that the soluble TbCRK proteins are divergent enough that they do not interact with this conserved component of the yeast cdc2/cyclin complex. The related trypanosomatid, *Leishmania mexicana*, contains homologues of two of the *T. brucei* genes (*lmmcrk1* and *lmmcrk3*) but no equivalent of *tbcrk2* has been found to date. *tbcrk2* was isolated by heterologous hybridisation of a *lmmcrk1* fragment to a *T. brucei* genomic library whereas *tbcrk1* and *tbcrk3* were isolated by PCR with degenerate primers designed to anneal to conserved domains. The heterologous hybridisation approach has not yet been tried with *Leishmania*, but the lack of detection of a protein of similar size to TbCRK2 in *Leishmania* protein extracts by either TbCRK2 or PSTAIR reactive antisera would imply that a leishmanial

homologue does not exist.. Previous evidence suggests that *lmmcrk1* is not the master regulator of the cell cycle in *L. mexicana*, although it does appear to be essential for cell growth in cultured promastigote forms. *lmmcrk1* was unable to complement a *S. pombe cdc2-ts* mutant (Mottram *et al.*, 1993). Amastigote form *L. mexicana* which are slowly dividing do not show LmmCRK1 kinase activity, while the non-dividing metacyclic stage do contain LmCRK1 kinase activity, although as previously noted, constitutive activity of a CDK can result in a cell cycle block. It is possible that the G1 cell cycle block in the pre-adapted life cycle stages of the kinetoplastids may be due to the constitutive activity of a G1 specific CDK as opposed to a lack of activity. This would be analogous to the M phase block that occurs when cdc2 is not inactivated. When mitotic cyclins that lack the N-terminal "destruction box" are transiently expressed the cells are unable to exit from mitosis due to the continuous activity of the cdc2/Cyclin complex which would normally be deactivated by proteolysis of the cyclin subunit (Draetta *et al.*, 1989). It is also possible that due to the extreme divergence between the Kinetoplastida and other eukaryotes the TbCRK proteins do not control the cell cycle. Given the sequence conservation between the CRKs and other cdc2-related kinases this would seem unlikely. Because of the sequence divergence (see Chapter 3.5) the failure of the *T. brucei* genes to complement a yeast *cdc2-ts* mutant was not unexpected.

The protein phylogeny analysis (Figure 3.3.9) implies that TbCRK2 and 3 play a role in cell cycle control as they cluster near the CDK4/6 proteins which have been shown to be involved in the control of G1/S phase transitions (Xiong *et al.*, 1992; Lucas *et al.*, 1995). This convergence may be artefactual however, due to the divergence between the CRK and the CDK4/6 proteins, and the cdc2/CDK2 protein sub-family. Diverged branches can cluster due to their differences from the conserved sequence, as opposed to clustering due to their similarity. The TbCRK1 protein, although containing the least degenerate PSTAIR box of the TbCRK proteins, is placed on an outlying branch of the analysis.

The *tbcrk3* gene was found to be closely linked to the gene for the mitochondrial elongation factor G (*tbmefg*), a link which was found to be conserved in the *lmmcrk3* gene locus in *L. mexicana* (J. Mottram, unpublished). Although the genes are tightly linked, and TbCRK3 does contain a N-terminal extension, there is no evidence for TbCRK3 being targeted to the mitochondrion. The TbCRK3 extension has no homology with the mitochondrial protein extensions found in the kinetoplastids, but as these mitochondrial targeting signals have no clear consensus in other better characterised organisms this cannot be ruled out.

When TbCRK3 was expressed in *E. coli*, with a poly-histidine tag to allow purification by metal-chelator chromatography, it was found to be insoluble. This was not found for TbCRK1, and surprisingly LmmCRK3 was soluble as well, even though the CRK3 proteins are ~80 % identical. The purification of tagged TbCRK3/H enabled the raising of polyclonal antibodies in rabbits. Western blots using crude *E. coli* lyssates expressing TbCRK proteins or proteins purified from these lysates allowed the testing of the available antisera for cross-reaction between epitopes on the CRKs. The affinity purified polyclonal CITAA and PSTAVR antisera were both shown to recognise, not only the protein which contained the sequence of the peptide to which they were raised (LmmCRK1 and TbCRK2 respectively), but also other members of the kinetoplastid CRK family. CITAA recognised TbCRK1 (with 11/16 residues identical), and PSTAVR also recognised both TbCRK1 (10/16) and TbCRK3 (10/16). The PSTAIR monoclonal antiserum was also shown to recognise related sequences even when they contained multiple substitutions. TbCRK1 (14/16 identical residues), TbCRK2 (12/16) and TbCRK3 (10/16) were all detected when expressed in *E. coli*. Western blots with *T. brucei* protein extracts from different life cycle stages showed that the three TbCRK proteins are expressed in the non-dividing short stumpy stage as well as in the replicating procyclic and long slender forms. The PSTAIR monoclonal antiserum also detects a 48 kDa and an 80 kDa band in some extracts suggesting the presence of at least two more genes with a similarity to *cdc2*. A cdc2-related kinase protein (CfCRK4) of 53 kDa which is not homologous to any of the three TbCRK

proteins studied in this thesis has been isolated from *Crithidia fasciculata* (Bran *et al*, 1992). The 48 kDa *T. brucei* protein may be the trypanosome CRK4 homologue. The evidence from *T. brucei* protein extracts is that TbCRK3 is insoluble in 1 % Triton X-100 *in vivo*, unlike TbCRK1 and 2. The insoluble nature of TbCRK3 could be another possible reason for the failure of *tbcrk3* to complement a *S. pombe cdc2-ts* mutation.

There was evidence on the Western blots for a conserved degradation pathway for the TbCRK proteins with antibodies specific for the three proteins all detecting a 26 kDa band in some extracts. The antisera which detected these bands were all raised either to the C-terminus of the respective proteins (CITAA vs LmmCRK1, EVREE vs TbCRK2) or against the entire protein (TC3H/G). The antisera raised to the region of the PSTAIR box detected no such fragments. This implies a common C-terminal fragment can be protealytically cleaved from the TbCRK protein under certain conditions. The 26 kDa putative TbCRK3 fragment was detected in the soluble fraction of the S100 protein extracts implying that the insoluble nature of TbCRK3 may be modulated by the N-terminal 80-90 amino acids that would be removed from the TbCRK3 protein to leave a 26 kDa fragment.

The TbCRK proteins were detected by Western blotting in the different life cycle stages, however this gives no evidence as to whether the kinases are active or not. In the absence of high quality antisera capable of immunoprecipitation, assays using protein bound to $p13^{suc1}$ or a homologue ($p12^{LmmCKS1}$) were used to begin to answer this question. The $p13^{suc1}$ failed to reproducibly bind a histone H1 kinase activity in *T. brucei* extracts, unlike the result from *L. mexicana* where a kinase activity related to the division status of the life cycle stage was observed (Mottram *et al.*, 1993; Mottram and Grant, 1996). It is now believed that the kinase subunit of the Suc1 Binding Kinase (SBCRK) may be encoded by *lmmcrk3* (Mottram and Grant, unpublished). LmmCRK3 is expressed at very low levels and has proved difficult to purify. If LmmCRK3 is the SBCRK then one would expect that the TbCRK3 protein would also be capable of interacting with $p13^{suc1}$, but its insoluble nature in the buffers used for lysis caused it to remain in the pellet fraction of the protein extracts used to isolate the activity. When *T.*

brucei extracts were bound to the p12^{LmmCKS1} column significant levels of histone H1 kinase activity could be recovered. Protein was eluted from these columns for Western blotting. Both TbCRK1 and TbCRK2 were detected binding specifically to the beads, as was another, 48 kDa, PSTAIR mAb reactive protein. Therefore the recovered kinase activity could be due to any, or all, of these three proteins, or even to another protein not detected by any of the antisera used. To begin to assess the expected other components of the TbCRK complexes ^{35}S methionine labelled procyclic cell extracts were bound to the p12^{LmmCKS1} column before elution, SDS-PAGE and autoradiography. although several proteins bound to the column in a non-specific manner, many proteins could be seen to bind only to the p12^{LmmCKS1} beads. By the estimated molecular weights of these proteins it would seem possible that the column is binding TbCRK complexes containing putative *T. brucei* cyclins.

The *tbcrk* genes are single copy (but diploid) making it relatively easy to create null mutants by two rounds of homologous recombination using two antibiotic resistance genes flanked by DNA from the genomic locus of interest. When this was performed in *Leishmania* with *lmmcrk1*, null mutants could not be isolated. Doubly resistant clones could be selected for, but analysis showed the cells to have retained a wild type *lmmcrk1* gene either through aneuploidy or by the retention of episomal resistance genes. This has been seen previously with gene knockouts in the kinetoplastids and has been assessed as evidence for the gene being essential to cell survival. When this approach was attempted with *tbcrk3*, doubly resistant cells could be isolated, but on both occasions the cells grew slowly and eventually the cultures died. Because of the small number of cells available in the cultures PCR was used to analyse the genotype of the phleomycin/G418 resistant cultures. PCR using primers which correctly amplified the *tbcrk3* ORF from wild type STIB247 and from single replacement mutants, failed to display the correct 1 kb product when B4D was used as template. The results indicated that these cells were null mutants containing both *ble* and *neo* resistance genes within the *tbcrk3* ORF. As the B2D and B4D cultures aged it became clear that the cells were displaying a phenotype of slow growth with a possible

defect in cytokinesis. These cells were not dying due to necrosis initiated by antibiotic selection as control cultures did not exhibit the same phenotype. In control cultures treated with G418, the procyclic cells were killed by the antibiotic over a ten day period, treatment with phleomycin killed the cells considerably faster than this. In the putative null mutant cultures most cells died in the seven days after transfer to double selection medium, but the cultures then slowly increased in cell density for two/three weeks. The cultures remained at this plateau for over a month before slowly dying over a period of three to four weeks. To further evaluate this phenotype, aliquots of cells from the culture were fixed and stained with DAPI which allows the visualisation of nuclei and kinetoplasts. Light microscopy showed that the cells displayed a number of different phenotypes, with many cells having incorrect ratios of nuclei to kinetoplasts. It was also obvious that the positioning of the kinetoplasts in most cells was improperly regulated, with kinetoplasts often not separating correctly after replication. Nuclear positioning was also affected with some dividing cells partitioning both nuclei into one daughter cell, creating large numbers of so called "zoids" with a flagellum and kinetoplast but no nucleus. It was also possible to find cells where the linkage between the kinetoplast and the flagellum appeared to have broken down with some cells having a flagellum without a kinetoplast. There were also, occasionally, more severe cases where the cell polarity had appeared to have broken down. In these cells it could be seen that cytokinesis had started from the opposite end to normal.

Although the phenotype initially resembled the mitotic catastrophe seen in some yeast mutants, where the cells divide whether or not the nucleus is ready , the later disassociation of the kinetoplast from the flagellum, and the loss of cell polarity implies roles for TbCRK3 other than the timing of cell division. These phenotypes, along with the insolubility of TbCRK3 in 1 % Triton X-100 suggest that the TbCRK3 kinase is involved in regulating cytoskeletal functions possibly including; organellar movement (both of the nucleus and kinetoplast), the attachment of the kinetoplast to the basal body, and the organisation of the anterior/posterior axis within the cell.

7.2 Future work.

7.2.1 *T. brucei* Elongation Factor-G.

To obtain the entire open reading frame of the *tbmefg* gene will either require further subcloning from the lambda clone previously isolated when screening for *tbcrk3* or further screening of a library using an probe consisting of the 3' end of the available *tbmefg* sequence. To check for cellular localisation it will be necessary to raise antibodies either to fusion proteins or synthesised oligopeptides. Another option would be to express an epitope tagged protein in procyclic form *T. brucei* by DNA transfection. In combination with antibodies raised to the N-terminal extension it should be possible to show whether the putative signal sequence is cleaved on translocation into the mitochondrion, as with the kinetoplast associated proteins from *Crithidia* (Xu and Ray, 1993), or not. Null mutants could be constructed to assess if *tbmefg* is essential and to assess the relevance of mitochondrial translation to trypanosome survival both in procyclic culture and, by passage through Tsetse flies, in the mammalian bloodstream.

7.2.2 The TbCRK family

Northern blots using total cellular RNA were attempted but without achieving a significant signal. It is likely that the *tbcrk* genes are expressed at low levels and poly[A]$^+$ selection of mRNA would be needed to visualise the transcripts by Northern blotting. It could be possible to use Reverse Transcriptase-PCR to assess expression at the RNA level. It would be interesting also to examine the expression of *tbmefg* in relation to *tbcrk3* and also in the different life cycle stages.

In an attempt to solublise TbCRK3 the composition of the lysis buffer used to extract *T. brucei* proteins could be altered. It may be useful to try other detergents and other salt concentrations. If TbCRK3 is binding to the cytoskeleton then it may be possible to release the protein into solution by the use of microtubule or actin

depolymerising drugs. The sub-pellicular array of microtubules can be disrupted by calcium ions (Robinson and Gull, 1991). This would be a test for whether these are the insoluble components that TbCRK3 is associated with, and if so could be a relatively mild way of solublising TbCRK3. If solublisation could be achieved then $p12^{LmmCKS1}$ binding could be assayed, although if more stringent conditions are used then this might result in CRK complex disassociation, and in order to be active the kinase is likely to require a cyclin partner.

It should be possible to use specific antisera, such as the TbCRK1 reactive CITAA, to localise the protein to specific areas of the cell by immunolabelling of fixed *T. brucei* cells. Analysis of a dividing population may allow assessment of whether the protein undergoes positional regulation during the cell cycle as has been reported with cdc2 in other organisms. By using purified TbCRK3/H blotted onto filters it should be possible to purify TbCRK3 specific antibodies from the TC3H/G and TC3H/S antisera. These purified antibodies could also be used for localisation studies, with particular regard to whether TbCRK3 is associated with the cytoskeleton, or even a particular part of it. Double label *in situ* localisation could be performed alongside either gamma-tubulin or Tyrosine-tubulin antibodies to look for an association with microtubule organising centres or growing microtubule ends.

Another method to further analyse the TbCRK proteins would be to use epitope tagged constructs integrated into the genomic loci. These proteins could then be immunoprecipitated for kinase assays, for assessment of complex components, or the antibodies could be used to localise the tagged proteins within the cells. This could allow appraisal of the kinase activity of specific TbCRK proteins in the various life cycle stages (assuming the mutant could be successful passaged through tsetse flies or that bloodstream form transformation could be performed). It may also be possible to follow the various kinase activities through the short stumpy to procyclic transformation. This differentiation is concurrent with the reinitiation of the cell cycle (Matthews and Gull, 1994a; Affranchino *et al.*, 1993) and provides a model for the initiation of a new round of cell division. Although the concomitant alteration of cell

type could pose problems for the analysis, the transformation step is, at present, the best synchronous model of the cell cycle available for *T. brucei.*

Further studies are available by using reverse genetics technology. Null mutants of *tbcrk2* are still to be assessed; will the gene be essential like *crk1* and *crk3* or does the probable lack of a leishmanial homologue imply redundancy (at least in culture)? The function/s, if any, of the unusual N-terminal extensions of both TbCRK2 and TbCRK3 have not begun to be elucidated. By using heterozygous strains of *T. brucei* haploid for *tbcrk2* or *tbcrk3* (generated by one round of targeted deletion) it would be relatively easy to create mutant cell lines containing one null gene and one gene lacking the region coding for the N-terminal extension of one of these proteins. Alternatively, especially if the double mutants are non-viable, these minus-N terminal mutants could also be epitope tagged and transfected into wild type cultures. Along with full length epitope tagged constructs it should be possible to analyse changes in kinase activity, complex subunit composition and cellular localisation caused by the loss of the N-terminus.

A way to side-step the inability to create null mutants of essential genes such as the *tbcrk*s in sufficient quantities for analysis would be to attempt to use the inducible expression systems becoming available for trypanosomes. One method is to create a gene under the control of an inducible/repressible promoter that is integrated into a silent region of the genome. Strains carrying this integration can then have the wild type chromosomal copies of the gene of interest removed by homologous recombination using the flanking regions of the gene locus. While the gene is being expressed, under the control of the *tet* repressor with tetracyclin added to the medium, there should be no phenotype, allowing large numbers of cells to be grown. When expression is shut off by the removal of the tetracyclin, it should be possible to analyse the result of a lack of the TbCRK protein i.e. do the cells stop growth at specific cell cycle points or at random. The draw back of this system is the need for multiple selectable markers (two for the knockout cassettes, one for the integrated gene and, because the inducible systems use components from heterologous systems, at least one

for the system supplying the regulatable expression components). There are enough selectable markers for trypanosomes (five) but the logistics and large amount of subcloning necessary can make the task time consuming. *cdc2* related genes in other organisms have been mutated to give dominant negative phenotypes (Fleig and Nurse, 1991; Fleig *et al.*, 1992). The altered proteins are apparently inactive but still capable of binding the cyclin regulatory subunits, and possibly other complex components as well. It is believed that these inactive kinase proteins titrate out the positive regulators of CDK function. The residues needing to be altered for this result are conserved between proteins and species, and are conserved in the TbCRK proteins. Therefore the same principle could be applied to the trypanosome *crk*s. If these alterations create dominant negative proteins in *T. brucei* then only two (or three) markers would be necessary to assess the mutant phenotype, an easier scenario.

7.3 Concluding remarks.

This study begins the analysis of the *tbcrk* family, and their roles in the control of the cell cycle in *Trypanosoma brucei*. The facts are at present unclear and although it seems likely that the TbCRK proteins are involved in regulation of trypanosome growth, it is still not known which, if any, of the three genes is the functional *cdc2* homologue.

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