Analysis of the CRK3 kinase of *Leishmania mexicana*

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This thesis is presented in submission for the degree of
Doctor of Philosophy in the Faculty of Veterinary Medicine

March 1999

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ABSTRACT

Control of cell cycle progression in eukaryotes is governed by members of a highly conserved family of serine threonine protein kinases, the cyclin-dependent kinases (CDKs), which associate with a regulatory cyclin partner protein to attain full activity. The *CRK3* gene product of *Leishmania mexicana* is one of two putative CDKs so far identified in this organism.

The possible function of CRK3 and the requirement of the *CRK3* gene for *Leishmania* survival was investigated using molecular genetic techniques. Attempts to create null mutants lacking an intact *CRK3* locus failed repeatedly and instead a series of transgenic cell lines were generated that had undergone changes in ploidy or genomic rearrangements. However, both alleles could be successfully disrupted when extra copies of *CRK3* were introduced on an episome into a heterozygote mutant, prior to disruption of the second chromosomal *CRK3* allele. Together these results provide evidence that *CRK3* is an essential gene in the promastigote form of *Leishmania mexicana*.

CDKs are regulated not only by cyclin binding, but also by inhibitory and activatory phosphorylation events. Inhibition of *Leishmania mexicana* phosphotyrosine phosphatase activity by bpV(phen) resulted in inhibition of cell cycle progression, and led to an accumulation of cells in the G1 and S-phases of the cell cycle. Cells treated with bpV(phen) had a low level of CRK3 activity and upon release from inhibition CRK3 activity increased. Inhibition of a phosphotyrosine phosphatase activity could be involved in regulation of CRK3 activity, either directly or indirectly.

The CDK inhibitor flavopiridol is a potent inhibitor of mammalian cdk1, cdk2 and cdk4, enzymes, which all have a role in cell cycle progression. Flavopiridol was found

to be a potent inhibitor of *L. mexicana* CRK3 activity and inhibits purified CRK3 with an IC_{50} value of 100 nM. Flavopiridol inhibited growth of *L. mexicana* promastigotes in liquid culture, with 50% inhibition of growth achieved with a concentration of 250 nM. Inhibition of growth is due to inhibition of cell cycle progression, as cells were found to accumulate in the G2 phase of the cell cycle, probably due to inhibition of CRK3 activity. Flavopiridol-induced growth inhibition is reversible up to 24 hours after addition of the drug. Release from flavopiridol inhibition resulted in a partially synchronous re-entry into the cell cycle. This method may be used to obtain cell samples enriched for cells in particular phases of the cell cycle.

In contrast to several plant and animal CDKs, CRK3 failed to complement for loss of function of CDC28 activity in the *Saccharomyces cerevisiae cdc28-1N*^{ts}, *cdc28-4*^{ts} and *cdc28-13*^{ts} mutants, suggesting that mechanisms of cell cycle control in *Leishmania* may be less conserved than those of other eukaryotes.

The findings that *CRK3* is an essential gene in *Leishmania*, that CRK3 is inhibited by specific inhibitors and that CRK3 has features that distinguish it from mammalian homologues, make CRK3 a novel drug target of some promise.

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LIST OF ABBREVIATIONS

cAMP	cyclic adenosine monophosphate
bpV(phen)	bis peroxovanadium-1,10-phenanthroline
CAK	Cdk activating kinase
CAKAK	CAK activating kinase
CDK	Cyclin dependent kinase
CIP	Calf intestinal alkaline phosphatase
CRK	Cdc2-related kinase
CTD	Carboxy terminal domain
DABCO	Diazobicyclo-octane
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetate
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
GIPL	Glycoinositol phospholipid
GPI	Glycosyl phosphatidylinositol
HRP	Horseradish peroxidase
LPG	Lipophosphoglycan
MHC	Major histocompatibility complex
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PV	Parasitophorous vacuole
RNA	Ribonucleic acid
RNase A	Ribonuclease A
SDS	Sodium dodecyl-sulphate
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
(S)-HPMPA	(S)-9-(3-hydroxy-2-phosphoenolmethoxypropyl)adenine
TFIIH	Transcription factor IIH
Tris	Tris(hydroxymethyl)amino methane
UV	Ultraviolet
VSG	Variant surface glycoprotein

Measurements

bp	base pairs
kb	kilobase pairs
kD	kilodaltons
μg	microgram
mg	milligram
ng	nanogram
M	molar
mM	millimolar
μM	micromolar
nM	nanomolar

NOTE ON GENETIC NOMENCLATURE

Gene and protein names for *Leishmania* and trypanosomes follow the guidelines outlined in Clayton, *et al.* 1998. The system for naming the genes of other organisms discussed in this thesis is unique for each particular organism. For clarification, examples of the nomenclature used to name a hypothetical gene of interest (GOI1) for each organism mentioned in this thesis are shown below.

Leishmania and trypanosomes:

Gene: *GOI1* Protein: GOI1 Disrupted allele: *GOI::NEO*

Saccharomyces cerevisiae:

Gene: *GOI1* Protein: GOI1 Temperature-sensitive mutant: $goi1^{ts}$

Schizosaccharomyces pombe:

Gene: *goi1* Protein: Goi1 Temperature-sensitive mutant: $goi1^{ts}$

Caenorhabditis elegans:

Gene: *goi1* Protein: GOI-1

DECLARATION

I declare that the work presented in this thesis is my own except where otherwise stated.

Paul Hassan

March 1999

ACKNOWLEDGEMENTS

I'm very grateful to the following people for all their help and advice during the course of this PhD. Jeremy Mottram, who showed the right balance between patience and impatience needed in a PhD supervisor; Karen Grant, for help and advice on western blots, CRK3 purification and kinase assays; Darren Brooks, for useful molecular biology hints; Phil Halford, who cloned the CRK3 knockout constructs; Jim Hilley, for being such a good-humoured bench neighbour; and Kathryn Brown, for help and advice with cell culture and FACS analysis.

I'd also like to thank past and present staff at WUMP for contributing to the unique WUMP atmosphere and for providing such good company on pub visits.

I must also acknowledge Dr. Martin Olivier, who shared unpublished data and provided a sample of bpV(phen); and also thank Prof. Steven Reed (Scripps Institute, California) for providing the *cdc28* mutant yeast strains; Dr. Paul Sternberg (Howard Hughes Medical Institute, Pasadena, California) who provided the C. elegans *ncc1* construct; and Dr. Swati Bal-Tembe (Hoechst Marion Roussel, Bombay) who provided many milligrams of flavopiridol.

In addition I must thank my family, particularly my parents who have supported and encouraged me throughout all my studies.

I'd like to dedicate this thesis to Tommy and Annie Murphy, Jean Hassan, and my nephew and niece; Jack and Molly, who were both born during the frustrating struggle to get this thing written.

CHAPTER 1

INTRODUCTION

1.1 The trypanosomatids

The trypanosomatid protozoa include various species that are parasites of mammals and have widespread medical and economic importance in many parts of the world, particularly in developing countries. The trypanosomatids are studied not solely because they are mammalian pathogens, but also because many trypanosomatid species exhibit a number of interesting biological phenomenon, some of which are rare, if not unique, in the eukaryotes. These features are of great biological interest in their own right and in some cases shed light on general questions of biological interest. This chapter will introduce some of the unique biological characteristics of these organisms, features which may be exploited to develop new, much needed, drugs or vaccines for the treatment and prevention of these diseases, and will introduce the aims and rationale behind the work described in this thesis.

The most important trypanosomatid pathogens are:

(I) The African trypanosome, *Trypanosoma brucei brucei*, the causative agent of Nagana in cattle, and the related subspecies *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*, the causes of acute and chronic African sleeping sickness respectively. The relationship between *T. brucei brucei* and *T. b rhodesiense* is unclear. The only noticeable difference between these parasites is that *T. b. brucei* is lysed by human serum, whereas *T. b rhodesiense* is not. Epidemics of African sleeping sickness may be due to the aquisition of human serum resistance by *T.*

brucei parasites, possibly through genetic exchange in the tsetse fly vector (Gibson and Mizen, 1997).

(II) The South American trypanosome, *Trypanosoma cruzi*, causative agent of Chagas' disease. This disease is chronic and involves infection and destruction of many tissue types, particularly cardiac muscle, eventually leading to heart failure.

(III) The leishmaniases, a group of diseases caused by various species of *Leishmania*. The leishmaniases can be divided into three major disease classes.

(A) Cutaneous leishmaniasis. Causes formation of skin ulcers which can be accompanied by secondary infections, and may spread to surrounding tissue or may be limited to the site of infection, dependent on the particular subspecies involved. Can be caused by *L. major*, *L. mexicana* and *L. tropica*.

(B) Mucocutaneous leishmaniasis. This is a more serious disease that can cause gross disfiguration and may also cause complications that are life threatening, such as sepsis and bronchopneumonia. Can be caused by infection with *L. braziliensis*

(C) Visceral leishmaniasis or kala-azar. The most serious form of disease, in which macrophages within the major organs are infected, causing inflammation and organ damage. Caused by *L. donovani* and *L. infantum*.

The diseases caused by these parasites vary in their geographical distribution. The African trypanosomes occur in sub-Saharan Africa, *T. b. rhodesiense* in East Africa, and *T. b. gambiense* in Central and West Africa. They are endemic in 36 countries and 55 million people are at risk of contracting the disease (Molyneux, 1997).

T. cruzi, the South American trypanosome, as the name suggests, occurs in South and Central America in 19 countries with 90 million people at risk and 16 million people infected by the parasite (Molyneux, 1997).

Leishmania is the most widespread of the trypanosomatid diseases. It is endemic in 88 countries in the tropics and sub-tropics, but not Southeast Asia and Oceania. 350 million people are at risk of contracting the disease and 119 million people are currently infected (Molyneux, 1997).

Like many other protozoan parasites the trypanosomes and *Leishmania* are transmitted from one infected host to the next via infection of an intermediate insect vector. The vector for African trypanosome transmission is the tsetse fly (*Glossina*). The infective metacyclic form develops in the salivary glands of the fly and is transmitted when the fly takes a blood meal. When parasites enter the blood they rapidly differentiate into the proliferative long slender bloodstream form. At the peak of infection the long slender form undergoes differentiation into the non-dividing short stumpy bloodstream form. The short stumpy form is preadapted for survival in the insect vector, and upon ingestion by a feeding fly, differentiates to the proliferative procyclic form in the fly mid-gut. Procyclics then migrate to the salivary glands where they complete their differentiation to the metacyclic form, thereby completing the life-cycle. The vector for *T. cruzi* transmission is the Triatomine bug. Infective forms develop in the hind gut and are excreted during feeding. They are then introduced into the site of the bite when infected faeces is inadvertently rubbed into the wound. Like the African trypanosome, *Leishmania* is introduced directly into the bloodstream when the insect vector feeds (Fig. 1.1). The vector in this case is either the *Lutzomyia* sandfly (New World) or the *Phlebotomus* sandfly (Old World). Like the trypanosomes, metacyclic parasites, the non dividing infective form, develop in the insect vector, in this case in the mouthparts. When introduced into the blood of the mammalian host, metacyclics are phagocytosed by host macrophages where they differentiate into proliferative amastigotes. Amastigotes multiply within the parasitophorous vacuole and eventually lead to lysis of

the macrophage, releasing free amastigotes into the blood and initiating another round of macrophage infection. The life cycle of *Leishmania* is represented in Fig. 1.1. The complex developmental changes these parasites undergo are a common feature of vector borne diseases, which encounter very different environments as they progress through their life-cycle and must adapt accordingly. Such unusual developmental programs are one feature of the biology of the trypanosomes and *Leishmania* that make them interesting organisms to study. Other peculiarites of their biology are discussed below.

1.1.2 The single organelles of trypanosomes and *Leishmania*

The trypanosomes and *Leishmania* belong to the Order *Kinetoplastida*, so called because of the presence of the kinetoplast, a DNA containing organelle associated with the single mitochondrion. The kinetoplast DNA (kDNA) is analogous to the mitochondrial genome of higher eukaryotes. kDNA however is very different in organisation from mitochondrial or nuclear DNA, being arranged as a network of interlocking circular DNA molecules which fall into two classes, maxicircles and minicircles (Shapiro and Englund, 1995). The maxicircles bear most resemblance to mitochondrial DNA in that they encode rRNA genes and proteins involved in mitochondrial energy production such as cytochrome oxidase subunits and NADH dehydrogenase(Simpson, 1987). The expression of maxicircle genes is unusually complex. The mRNAs for these genes undergo extensive processing via a procedure termed RNA editing, which involves the addition or deletion of uridine nucleotides at precise positions in the message to generate the final mRNA (Feagin *et al.,* 1988). The process of editing involves the use of small guide RNA templates involved in controlling the specificity of editing. These small guide RNA molecules are encoded by

the minicircles (Sturm and Simpson, 1990). Both the maxicircle and the minicircle DNAs must be replicated during each cell cycle to form two complete and identical new kinetoplasts, which are segregated into each daughter cell during cell division.

Another organelle unique to the kinetoplastids is the glycosome. A membrane bound organelle that contains the glycolytic enzymes (Visser *et al.,* 1981). In *T. brucei* compartmentalisation of the glycolytic enzymes greatly increases the efficiency of glycolysis, allowing the parasites to rely exclusively on glycolysis for their energy production in the glucose rich environment of the host (Opperdoes, 1987). As the parasite is transmitted to the vector however, where the environment is less rich in nutrients, the mitochondrion resumes its role in energy production via oxidative phosphorylation, and the levels of glycolytic enzymes decline (Vickerman *et al.,* 1988).

1.1.3 Avoidance of host immune responses

Leishmania and trypanosomes need to avoid detection and elimination by the mammalian host immune system in order to proliferate to a level that will increase their chances of uptake by feeding insect vectors. *T. brucei*, achieves this by the process of antigenic variation, in which the major constituent of the parasite surface, the variant surface glycoprotein (VSG), is sequentially replaced by a new variant, not yet encountered by the host immune system. In the course of an infection this gives rise to waves of parasitaemia as the immune system kills off parasites expressing one particular VSG, reducing the parasite load, until VSG switching occurs to generate antibody-resistant parasites that give rise to a new peak in parasitaemia (Barry, 1997a). The switch to expression of a new VSG gene reportedly occurs through three separate mechanisms: (1) Translocation of an inactive VSG gene from a silent locus to a

telomeric, transcriptionally active region known as an expression site, (2) an *in situ* switch, resulting in activation of a silent expression site or (3) recombination between the active VSG and an inactive gene, generating a hybrid gene (Barry, 1997b).

Unlike *T. brucei*, both *T. cruzi* and *Leishmania* are intracellular parasites and have evolved mechanisms to allow their survival inside the cells of the host (Mauel, 1996). The surface of *Leishmania* promastigotes consists mainly of the surface metalloprotease gp63 (Russell, 1994), a lipophosphoglycan, LPG (Turco and Descoteaux, 1992), and glycoinositol phospholipids, GIPLs, (McConville and Blackwell, 1991). Modification of the structure of LPG occurs as promastigotes differentiate into infective metacyclics (Sacks *et al.,* 1990). In amastigotes both LPG and gp63 are downregulated and replaced on the parasite surface by a structurally distinct LPG (McConville and Blackwell, 1991; Moody *et al.,* 1993; Turco and Sacks, 1991). It is thought that LPG and molecules on the surface of metacyclics bind to components of complement, avoiding complement mediated lysis and allowing uptake of the parasites by type I, and type III, complement receptors (CR1 and CR3) on the macrophage surface (Mauel, 1996). Metacyclics are then taken into the macrophage by phagocytosis. Phagocytotic vesicles, containing parasites, then fuse to lysosomes and/or late endosomes to form a modified organelle termed the parasitophorous vacuole (PV) (Chang, 1983; Alexander and Russell, 1992). During the formation of the PV metacyclics differentiate into amastigotes (Antoine *et al.,* 1998). Amastigotes proliferate in the PV and manage to avoid host defence systems. Densely packed glycoinositol phospholipid molecules GIPLs on the parasite surface may help to protect the amastigote from the degradative host enzymes present in the PV (Winter *et al.,* 1994). There is also evidence that *Leishmania* can internalise and degrade major histocompatibility complex (MHC) class II molecules, thereby avoiding presentation of *Leishmania* antigens by the infected macrophage (De Sousa Leao, 1995;

Russell *et al.,* 1992). Intracellular amastigotes must also obtain nutrients and do so by endocytosis through the flagellar pocket, a specialised, invaginated region of the plasma membrane located at the point where the flagellum emerges from the cell (Overath *et al.,* 1997). Amastigotes acquire host-derived molecules from the lumen of the PV (Russell *et al.,* 1992), and can also acquire molecules from the host macrophage cytosol via two separate routes; active transport of small anionic molecules into the PV lumen, and import via fusion of autophagous vesicles with the PV (Schaible *et al.,* 1999).

Because the surface of *Leishmania* and *T. brucei* are coated in densely packed protective molecules, secretion and uptake of nutrients, metabolites etc. is confined to a specialised area of the surface, the flagellar pocket, where the flagellum emerges from inside the cell (Webster and Russell, 1993).

1.1.4 Molecular genetic analysis of trypanosomatid gene function

There are a number of aspects of the biology of *Leishmania* that aid the molecular genetic analysis of the biology of these parasites (Beverley and Turco, 1998).

(1) The complete life cycle of many *Leishmania* species can be replicated *in vitro* (Doyle *et al.,* 1991; Bates, 1993), allowing the growth of large numbers of parasites for the purification or analysis of organelles, membranes, or enzyme activities.

(2) Clonal populations can easily be isolated on solid agar medium (Iovannisci and Ullman, 1983).

(3) Selected genes can be disrupted by gene targeting. A process which occurs with high efficiency via homologous recombination (Cruz and Beverley, 1990; Laban *et al.,* 1990; Tobin and Wirth, 1992; Tobin *et al.,* 1991)

(4) A large number of positive and negative selectable markers can be used to select for the integration of gene targeting constructs. These marker genes are discussed below.

(5) Plasmid expression vectors bearing a gene of particular interest can be introduced into the parasites where they are stably maintained as extrachromosomal episomes (LeBowitz *et al.,* 1990; Kelly *et al.,* 1992).

(6) Cosmids containing large genomic DNA fragments can be used to screen for genes that complement a mutant phenotype (Ryan *et al.,* 1993a; Ryan *et al.,* 1993b; Kelly *et al.,* 1994).

(7) *Leishmania* artificial chromosomes (LACs) have been developed, allowing the analysis of chromosomal structural elements (Beverley and Turco, 1998).

(8) The mariner transposable element from *Drosophila* may also be used to generate mutant parasites by insertional mutagenesis (Gueiros-Filho and Beverley, 1997). LAC, cosmid and plasmid libraries may then be used to screen for genes that restore the mutant phenotype.

(9) The genome of the Freidlin strain of *L. major* is currently being sequenced as part of a co-ordinated effort (http://www.ebi.ac.uk/parasites/leish.html). This resource, once complete, when combined with the use of the technologies described above, should help to significantly advance the understanding of the biology of *Leishmania.*

The development of the aforementioned technologies began with the modification of circular, amplified DNA fragments for use as vectors for the introduction and expression of genes of interest in *Leishmania.* Gene amplification upon drug selection is a known mechanism by which trypanosomatids become drug resistant (Callahan and Beverley, 1992). Many of these gene amplification events involve the generation of autonomously replicating extrachromosomal elements bearing the drug resistance gene.

An example of this phenomenon has been described in the case of spontaneous resistance to methotrexate, where two different regions of the genome, the R locus and the H region, can be amplified (Beverley, 1991). The amplified R locus, isolated from methotrexate resistant mutants of *L. major*, is a 30 kb extrachromosomal element which is stably maintained in the parasite. The R locus contains the open reading frame (ORF) for the dual activity dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) gene, plus a large region of flanking DNA (Mori *et al.,* 1994). The amplified R region was used as the basis for the generation of *pR NEO*, a 30 kb circular DNA construct in which the *DHFR-TS* gene has been replaced by the *NEO* gene, conferring resistance to the aminoglycoside antibiotics G418 and Geneticin (LeBowitz *et al.,* 1990). *pR NEO* also contains pUC derived sequence which allows the maintenance of this construct in *Escherichia coli* (Kapler *et al.,* 1990). The rather unwieldy *pR NEO* vector was further modified to reduce the total *Leishmania* derived DNA sequence to generate a smaller plasmid. This resulted in the pX vector which contains only 2.3 kb of *L. major DHFR-TS* flanking DNA. Reporter genes which were cloned into pX (eg. *E. coli* β-galactosidase or *L. amazonensis* GP46A) were transcribed and polyadenylated by processing events that occurred at the same sites used in processing of the *DHFR-TS* mRNA (LeBowitz *et al.,* 1990). Other investigators had also developed systems for expression of the *NEO* gene in other trypanosomatid species, such as the insect trypanosomatid *Leptomonas seymouri* (Bellofatto *et al.,* 1991). This system relied on the use of α-tubulin gene sequence, fused to a truncated *NEO* gene. The plasmid was maintained as a high copy extrachromosomal circular DNA containing several head to tail copies of the original plasmid. The α-tubulin-neomycin phosphotransferase fusion RNA transcribed from this episome, was polyadenylated and trans-spliced with the splice leader RNA (Bellofatto *et al.,* 1991). The pTEX vector, which contains flanking

and intergenic sequence from the *T. cruzi* glycosomal glyceraldehyde 3-phosphate dehydrogenase (g*GAPDH*) gene array, can be used to express genes in both *T. cruzi* and *Leishmania* species (Kelly *et al.,* 1992).

The presence of parasite derived sequence, such as *DHFR-TS*, g*GAPDH* or α-tubulin sequence, is required for the expression of the drug resistance gene or expression of other genes inserted into the plasmid. Such sequence is not however, necessarily needed for maintenance and replication of the episome. It has been demonstrated that bacterial plasmid DNA which does not contain any *Leishmania* derived sequence, is stably maintained, replicated and segregated in the absence of any kind of selection (Papadopoulou *et al.,* 1994). It may be therefore that *Leishmania* can use bacterial origins of replication or alternatively, *Leishmania* may have no strong requirement for specific sequence elements to act as replication origins. The situation in *T. brucei* is very different. In contrast to *Leishmania*, *Leptomonas*, and *T. cruzi*, it is much more difficult to obtain extrachromosomal, replicating episomes in *T. brucei*. There seems to be a distinct preference for integration of exogenous DNA rather than extrachromosomal maintenance (ten Asbroek *et al.,* 1993), and episomes will only be maintained if they contain transcriptional promoters (Patnaik *et al.,* 1993; Metzenberg and Agabian, 1994; Patnaik, 1997).

Other more sophisticated systems have recently been developed which allow the stage-specific expression of gene products in *Leishmania* (Ghedin *et al.,* 1998). The *Leishmania* vector (pGEMneo-TK) relies on the use of regulatory sequence derived from the amastigote specific A2 gene, to provide the splice addition and polyadenylation signals for amastigote-specific gene expression. The gene used in this case was the herpes simplex virus thymidine kinase gene. This gene has been used previously as a negative selectable marker in transfected *Leishmania* (LeBowitz *et al.,*

1992). Thymidine kinase phosphorylates gancyclovir, a nucleoside analogue, which is taken up by the parasites from the culture medium. In the unphosphorylated state, gancyclovir is non-toxic. However, in the phosphorylated form, gancyclovir is highly toxic to *Leishmania* as it is incorporated into replicating DNA and halts further replication. Expression of thymidine kinase therefore results in phosphorylation and activation of the cytotoxicity of gancyclovir. As the thymidine kinase gene was flanked by sequence derived from the amastigote specific A2 gene locus, then only amastigotes and not promastigotes were sensitive to gancyclovir, due to stage specific expression of the thymidine kinase gene (Ghedin *et al.,* 1998). This system represents a useful assay for testing the toxicity of expressed genes in *Leishmania* and may also lead to the development of attenuated live vaccines.

Another important development in the molecular genetic analysis of *Leishmania* was the development of techniques, in *L. major*, for targeted gene disruption (Cruz and Beverley, 1990). Heterozygous and null mutants of a particular gene can be obtained by sequential rounds of targeted gene disruption. Gene disruption or gene replacement constructs containing a drug resistance gene, flanked by sequences derived from the target locus can be introduced into the parasite by the same electroporation method used to introduce plasmid constructs (LeBowitz, 1994). Such disruption constructs integrate into the genome by homologous recombination mediated by matching sequence within the knockout construct and the target locus. Homologous recombination was demonstrated in *L. enrietti* by the introduction of constructs containing two separate, non-overlapping inactivating mutations in the chloramphenicol acetyltransferase (*CAT*) reporter gene. The subsequent detection of CAT activity indicated that homologous recombination had occurred between the two mutant *CAT* genes, resulting in reconstitution of a functional gene (Tobin *et al.,* 1991). Early attempts at gene

disruption focussed on the α-tubulin gene array of *L. enriettii*. The *pALT-Neo-Tub* construct, which contains the *NEO* gene flanked by α-tubulin flanking sequence was introduced into *L. enriettii* cells by electroporation and stably integrated at the α-tubulin locus (Tobin and Wirth, 1992). The same investigators also showed that it was possible to integrate a gene disruption construct to completely delete one of the two α-tubulin gene arrays (Curotto de Lafaille and Wirth, 1992).

The efficiency of homologous recombination in *Leishmania* has been reported to be dependent upon and proportional to, the extent of shared, homologous sequence (Papadopoulou and Dumas, 1997). This is therefore an important consideration when designing a construct for gene disruption or gene replacement. However as little as 400 bp of homologous sequence was sufficient to disrupt the 50 kb *CPB* gene array of *L. mexicana* (Mottram *et al.,* 1996b). Integration of knockout constructs in the related parasite, *Trypanosoma brucei* seems not to require such a large region of homology for recombination to occur, and it has been demonstrated that as little as 20 bp of homologous sequence can be used to achieve proper integration at the correct locus (Gaud *et al.,* 1997). This may reflect differences in the recombination machinery between these two trypanosomatid species, as homologous recombination is an integral and important aspect of the biology of antigenic variation in African trypanosomes (Borst et al., 1997). Inactive variant surface glycoprotein genes (VSG) can be activated by integration into a transcriptionally active expression site, thereby replacing one VSG type with a different variant which cannot yet be recognised by the host immune system (Borst et al., 1997). *Leishmania*, being intracellular parasites in the mammalian host, do not undergo antigenic variation

In order to generate null mutants by successive rounds of gene disruption it is necessary to use separate marker genes for each integration event. Fortunately in *Leishmania* a

number of separate marker genes exist for the selection and maintenance of stable genetic integration events. The first marker gene to be used in *Leishmania* was the neomycin phosphotransferase (*NEO*) gene which confers resistance to aminoglycoside antibiotic G418 or Geneticin (Laban *et al.,* 1990). The hygromycin (*HYG*) gene, which confers resistance to the antibiotic hygromycin B, works in a similar way to the *NEO* gene and has also been used as a stable genetic marker in *Leishmania* (Lee and Van der Ploeg, 1991). Other reporter genes which have been used include the *BLE* gene, conferring resistance to the DNA binding drug phleomycin (Souza *et al.,* 1994); the *PAC* gene which confers resistance to the glycopeptide antibiotic puromycin (Freedman and Beverley, 1993); the *SAT* gene encoding streptothricin acetyltransferase, and conferring resistance to nourseothricin (Joshi *et al.,* 1995), and the N-acetylglucosamine-1-phosphate transferase gene which confers resistance to tunicamycin (Liu and Chang, 1992). A mutant version of the *DHFR-TS* gene derived from a methotrexate resistant *L. major* strain has also been suggested for use as a selectable marker in *Leishmania* (Arrebola *et al.,* 1996). However, given that spontaneous resistance to methotrexate through gene amplification is a common phenomenon in *Leishmania* (Beverley, 1991), then it is likely that difficulties will be encountered in distinguishing true transfectants from false positive clones that have undergone such a gene amplification event. In addition to the positive selection methods described above, negative selection based on the use of the herpes simplex virus thymidine kinase gene (*HSV-TK*) has been used to demonstrate loss of heterozygosity of integrated knockout constructs (Gueiros-Filho and Beverley, 1996). An important development in the search for a method to remove drug resistance genes that have been integrated to knock out a particular target gene. As *HSV-TK* expression is cytotoxic to the parasites when grown in the presence of gancyclovir, then integration of an *HSV-TK*

construct to generate a heterozygous mutant, followed by selection with gancyclovir, leads to recombination at the target locus as a means of losing the integrated *HSV-TK* gene. Repeating the procedure on the remaining wild type locus should result in a cell line that has lost both copies of the targeted gene, but contains no drug resistance gene (Gueiros-Filho and Beverley, 1996). Other markers, which do not rely on conferring drug resistance phenotypes, may also be used to select transfected *Leishmania*. The green fluorescent protein (GFP) of *Aequorea victoria* was expressed in *Leishmania* and transfected parasites, expressing the protein were selected and sorted from non-transfected parasites by fluorescence activated cell sorting (FACS) (Ha *et al.*, 1996). In this instance the parasites were fixed, prior to sorting. However the technique may be modified to separate live parasites expressing GFP. This technique may be useful in the generation of live attenuated *Leishmania* vaccines as it has the advantage that it does not rely on the use of drug resistance genes as markers for gene deletion. It would not be desirable to use attenuated parasites containing drug resistance genes as live vaccines due to the possibility that these drug resistance genes may be acquired by other pathogenic micro-organisms (Gueiros-Filho and Beverley, 1994).

As an alternative to using a gene disruption approach to study gene function it has been reported that transfection of *T. brucei* with double-stranded RNA can be used to abolish the function of a particular gene. The mechanism by which this occurs is not yet known, but probably involves disruption of mRNA processing and translation. This effect is not general, and can be used to target a specific gene (Ngo *et al.*, 1998). This procedure has yet to be tested in other trypanosomatid species.

The techniques described in this section allow the detailed analysis of gene function in *Leishmania*. Transgenic mutant clones can be selected on solid media containing the appropriate antibiotic and can be transferred and cultured in liquid medium. Such

mutants can then be analysed for phenotypic changes arising from loss of gene function. For example, a decrease in the ability to form lesions in mice has been observed for null mutants of the *CPB* genes, an array of 19 copies encoding isoforms of a cathepsin-L like cysteine proteinase (Mottram *et al.,* 1996b). The *CPB* null mutants were then used in complementation assays using different individual members of the array. This work showed that not all of the members of the array were identical and there were significant differences in the stage specificity of the enzyme activities encoded by members of the array (Mottram *et al.,* 1997). It is not always possible to obtain a distinct, observable phenotype for particular null mutants (Souza *et al.,* 1994; Bart *et al.,* 1997; Webb and McMaster, 1994). Many *Leishmania* genes do not seem to be essential and their loss may be compensated for by the action of other proteins. Alternatively the phenotype of null mutants may not be observable *in vitro* or the wrong phenotypic tests may be used. No phenotype was observed for null mutants of the *HEXBP* gene of *L. major* (Webb and McMaster, 1994). This gene encodes a single strand DNA binding protein that specifically binds a region of sequence on the antisense strand of the *GP63* 5' flanking region *in vitro* (Webb and McMaster, 1993). This 5' flanking region is highly conserved in *Leishmania* species and it was proposed that the HEXBP protein may be involved in regulating the expression of *GP63* (Webb and McMaster, 1993). Null mutants of *HEXBP* showed no difference in transcription of the *GP63* mRNA and were able to form lesions in BALB/c mice (Webb and McMaster, 1994).

To date null mutants of a particular gene have only been obtained if that gene is non essential in promastigotes, as no transfection protocol has yet been developed for amastigotes. If a gene is essential for growth and division of the parasite then it is clearly not possible to generate null mutants. In some cases mutant lines that have undergone sequential rounds of gene disruption undergo changes in ploidy, or genomic

rearrangements, that are believed to result from attempts to knock out essential genes (Cruz *et al.,* 1993). These phenomena are discussed in more detail in section 3.3.1.

In some cases it is still possible to analyse the function of essential genes using a gene disruption strategy. Though it may not be possible to obtain null mutants in which both copies of the gene have been disrupted, it is usually possible to disrupt one allele to generate heterozygote mutants. In the case of the trypanothione reductase (*TR*) gene of *L. donovani*, a phenotype was observed for heterozygote mutant parasites (Dumas *et al.,* 1997). Trypanothione reductase is an enzyme involved in the cellular defence mechanism of trypanosomatids against oxidative stress and is analogous to glutathione reductase of mammalian cells. Heterozygote *TR* mutants of *L. donovani* had reduced levels of TR activity and showed a decreased ability to survive in an *in vitro* macrophage infection (Dumas *et al.,* 1997).

Gene function may also be analysed by expressing epitope tagged or mutated genes in a wild type or null background. Mutant versions of the *L. mexicana CPB* genes have been re-expressed in a *CPB* null background in order to verify which residues are required for enzymatic activity (Brooks *et al*, unpublished data). A hexahistidine tagged version of the *CRK1* gene, encoding a cdc2 related kinase, has been expressed in a null background allowing purification of the kinase by affinity chromatography on a Nickel NTA agarose matrix, and assessment of the stage regulated nature of this kinase activity (Mottram *et al.,* 1996a). A trans-dominant mutant version of the trypanothione reductase gene has been used to analyse the function of this enzyme (Tovar *et al.,* 1998). The active enzyme is a homodimeric complex. Expression of a trans-dominant mutant form of the *T. cruzi* enzyme in *L. donovani* resulted in a formation of heterodimers between the native enzyme and the exogenous enzyme (Tovar *et al.,* 1998). This resulted in an inactive complex. The effect of expression of the trans-dominant form was to titrate out

the activity of the native enzyme, causing a decrease in the ability of these parasites to survive in a murine model of a *Leishmania* infection (Tovar *et al.,* 1998).

Another approach to identifying genes involved in a particular process is to isolate mutants that display a characteristic, selectable phenotype. A cosmid library can then be introduced into the mutant, allowing the selection of transfected cell lines exhibiting repair of the mutant phenotype (Vasudevan *et al.,* 1998). Cosmids can be recovered from these cell lines and can be sequenced to determine which gene or genes may be involved in the repair of the mutant phenotype. This approach was used to isolate two genes involved in the biosynthesis of lipophosphoglycan (LPG) a major surface glycoprotein of *Leishmania* implicated in virulence and in host parasite interactions (Beverley and Turco, 1998). LPG is present on the surface of promastigotes of all *Leishmania* species. The molecule consists of repeating disaccharide units, a glycan core, a phospholipid anchor and an oligosaccharide-phosphate cap (Turco and Descoteaux, 1992; McConville and Ferguson, 1993). In *L. major* LPG is capped with a β-linked terminal galactose residue. The cytotoxic lectin ricin, binds to terminal β-linked galactose residues and, because it is bivalent, will agglutinate *Leishmania* promastigotes. This property was exploited to select for *L.* major mutants that were defective in LPG biosynthesis and were therefore resistant to ricin agglutination (King and Turco, 1988). A cosmid library was then screened to isolate genes that restored the phenotype. This led to the identification of genes involved in the biosynthesis of LPG (Ryan *et al.,* 1993b; Garraway *et al.,* 1993). The ability to clone genes by complementation in this way is a very powerful and useful technique. However it relies on the availability of a powerful method of screening for a mutant phenotype. For some aspects of the biology of *Leishmania* this is difficult if not impossible, and reverse genetic methods must be used instead. The development of the genetic manipulation

techniques described above is therefore extremely valuable for the analysis of gene function in *Leishmania*.

1.2 Control of the Eukaryotic Cell Cycle

The ordered progression between alternating phases of cell growth, DNA replication and cell division is termed the cell cycle. The cell cycle consists of four distinct phases; a newly formed daughter cell progresses through a gap phase called G1 before entering S-phase, the DNA synthesis phase, when the total DNA content of the cell is replicated. After DNA replication there is a second gap phase, G2, before cells enter into mitosis or M-phase and divide to form two separate daughter cells. The lengths of each phase differ between different cell types and under different growth conditions. The *Saccharomyces cerevisiae* and mammalian cell cycles are represented in Figs. 1.3 and 1.4 respectively.

Transition from one phase of the cell cycle to the next is a highly ordered process. Cells must complete each cell-cycle phase in turn. For example; cells must properly replicate their genome during S-phase, before entering into M-phase to ensure that each daughter cell has a complete set of chromosomes, otherwise cell division would be lethal. The major transition points in the cell cycle are the transition from G1 to S-phase and G2 to M-phase and a point during G1 termed START, when cells commit to progression through the cell cycle or arrest and become quiescent. These transitions are controlled primarily by the activity of homologues of the *S. pombe* Cdc2 serine threonine protein kinase.

1.3 Cell cycle control in fission yeast

Studies of the mechanisms governing cell cycle progression have relied greatly upon the analysis of various temperature sensitive cell division cycle (cdc) mutants of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* (Forsburg and Nurse, 1991). The fission yeast *S. pombe* is a rod-shaped organism that grows by apical extension whilst retaining a constant diameter. The cell then divides by medial fission to produce two identical daughter cells. At the permissive temperature of 25°C, the cdc mutants of *S. pombe* grow and divide as normal. At the restrictive temperature of 37°C however, cdc mutants are unable to undergo normal division and continue to grow by apical extension, resulting in abnormally long cells. It is clear therefore that cell division but not cell growth has been blocked in these mutants. The cell cycle stage at which these cells are arrested can be determined by DNA content analysis. By these methods 26 separate cdc mutants were characterised (Hartwell, 1978). The gene responsible for a given cdc mutant phenotype can be isolated by screening genomic or cDNA libraries for plasmids which will rescue the mutant phenotype. This approach led to the identification of the *cdc2* gene as a key regulator of the entry into mitosis (Beach *et al.*, 1982).

A separate class of cell cycle mutants has also been identified which divide prematurely to form abnormally small daughter cells, the wee phenotype. At least one of these mutants is due to the expression of a dominant mutant form of Cdc2 (Nurse and Thuriaux, 1980; Fantes, 1981). These results made it clear that the *cdc2* gene had an important role in the regulation of mitosis as loss of Cdc2 activity resulted in a failure to undergo mitosis, whilst overexpression of a dominant mutant form promoted premature entry into mitosis (Nurse and Thuriaux, 1980; Fantes, 1981).

Analysis of the Cdc2 coding sequence revealed that it was a protein kinase, but it did not fall into any of the previously described protein kinase families. It is now known that Cdc2 is a member of a distinct group of protein kinases termed the cyclin dependent kinases (CDKs). These enzymes rely on the binding of a partner protein belonging to the cyclin family, to regulate their activity (see Fig. 1.2 for a representation of regulation of cdk activity). Cyclin was first identified in biochemical analyses of cell cycle regulated proteins in sea urchin embryos (Evans *et al.,* 1983). One particular protein showed an interesting pattern of expression. It was present in very low levels early in the cell cycle, and increased gradually, reaching a peak at mitosis. After entry into mitosis the protein was destroyed and had to be re-synthesised during the next cell cycle (Evans *et al.,* 1983). In *S. pombe* Cdc2 binds to a cyclin encoded by the *cdc13* gene to achieve full activation at the entry into mitosis. Deletion of the *cdc13* gene leads to cell cycle arrest at mitosis with an inactive Cdc2 kinase (Booher and Beach, 1988; Hagan *et al.,* 1988). Binding of Cdc13 to Cdc2 and degradation of Cdc13 at anaphase are key regulatory events in the cell cycle, as cyclin binding is required for activity of the complex (Moreno *et al.,* 1989; Booher *et al.,* 1989). Cyclin binding is not only dependent on the ratio of Cdc13 subunits to Cdc2 subunits, but is also regulated by phosphorylation of a conserved Thr 167 residue (Gould *et al.,* 1991). This residue must be phosphorylated to allow cyclin binding to occur (Fig. 1.4). Replacement of Thr 167 with a non-phosphorylatable alanine residue abolishes *in vitro* function, and dominant mutations cause a cell cycle arrest (Nigg *et al.,* 1992). The kinase involved in Cdc2 regulation by phosphorylation at Thr 167 is termed CAK (Cdc2 activating kinase). The CAK enzyme has been purified from *Xenopus laevis* and has been shown to be the previously identified $p40^{MO15}$ protein (Fesquet *et al.,* 1993; Poon *et al.,* 1993; Gautier *et al.,* 1990; Murray and Kirschner, 1989). Active Cdc2 kinase complex also contains a

third protein, $p13^{suc1}$, which is encoded by an essential gene and, when overproduced, causes accelerated entry into mitosis (Hayles *et al.,* 1986a; Hindley *et al.,* 1987). The *suc1* gene was cloned as a suppressor of various *cdc2* alleles, indicating an interaction with Cdc2 (Hayles *et al.,* 1986b). The function of $p13^{suc1}$ is not yet known, though it has been suggested that it may act as a 'hub' for localisation of multiple Cdc2/Cdc13 complexes (Vogel and Baratte, 1996). Cdc2 is also regulated by phosphorylation at another conserved residue, Tyr 15 (Lee *et al.,* 1988). Phosphorylation at Tyr 15 inhibits Cdc2 kinase activity (Gould and Nurse, 1989). Dephosphorylation of Tyr 15 by the Cdc25 phosphatase occurs just prior to mitosis and results in activation of the kinase complex, triggering entry into mitosis (Draetta and Beach, 1988; Morla *et al.,* 1989; Millar *et al.,* 1991b). Temperature sensitive mutants of Cdc25 arrest at mitosis when shifted to the restrictive temperature and have the phosphorylated Tyr 15 form of Cdc2. When shifted back to the permissive temperature these mutants promptly enter mitosis (Moreno *et al.,* 1989; Booher *et al.,* 1989). Overproduction of Cdc25 leads to advanced mitosis (Russell and Nurse, 1986) and substitution of Cdc2 Tyr 15 with a phenylalanine residue, which cannot be phosphorylated, renders cells independent of Cdc25 activity (Gould and Nurse, 1989). Both *cdc25* mRNA and protein levels increase throughout the cell cycle, reaching a peak in G2 phase (Moreno *et al.,* 1990; Ducommun *et al.,* 1990). It has been demonstrated that the human Cdc2 kinase is able to phosphorylate Cdc25C, one of three isoforms of the Cdc25 phosphatase in human cells. This phosphorylation leads to activation of the phosphatase and raises the possibility that a positive feedback loop operates to trigger mitosis. An accumulation of active Cdc2 complex reaches a threshold level where the activity is great enough to stimulate Cdc25 phosphatase activity, which in turn activates more Cdc2 kinase complex (Hoffmann *et al.,* 1993;

Izumi and Maller, 1993). Such a mechanism would result in an extremely rapid increase in Cdc2 kinase activity, providing an irreversible trigger for entry into mitosis.

Cdc2 Tyr 15 is phosphorylated by the Wee1 kinase. This kinase was cloned by complementation of mutant yeast strain that exhibited a wee phenotype (Russell and Nurse, 1987), ie. cells prematurely entered mitosis and divided at a smaller than normal cell size (Nurse, 1975). Overproduction of Wee1 results in formation of abnormally long cells that have grown, but failed to undergo mitosis (Russell and Nurse, 1987). It was demonstrated that Wee1 kinase inhibits Cdc2 kinase complex by phosphorylating Tyr 15 (McGowan and Russell, 1993).

1.4 Cell cycle control in budding yeast

Important insights into the control of cell cycle progression have also been gained from the analysis of cdc mutants of the budding yeast *Saccharomyces cerevisiae* (Nasmyth and Reed, 1980) (Fig. 1.3 shows arepresentation of the *S. cerevisiae* cell cycle). Cell division in *S. cerevisiae* occurs by budding of the daughter cell from the mother cell (Hartwell, 1971). The newly formed daughter cell is smaller than the mother cell and must grow before forming it's own bud and undergoing cell division. Bud formation occurs in G1 phase of the cell cycle after a completion of a regulatory event known as START. Passage through START is required for completion of the remainder of the current cell cycle (Reed, 1980). Under conditions of nutrient limitation cells fail to progress through START and enter a quiescent state. Upon restoration of appropriate nutrient conditions quiescent cells will re-enter the cell cycle, passing through START and progress to completion of DNA synthesis and cell division. Under more severe nutrient limitation conditions cells will undergo sporulation to form haploid spores of

either the MATa or MATα mating type. Haploid spores are capable of mitotic growth under appropriate growth conditions but will undergo cell cycle arrest in response to mating pheromones secreted by haploid spores of the opposite mating type. Such a cell cycle arrest may act to synchronise spores prior to conjugation and re-entry into the cell cycle. Phenotypic analysis of the *cdc28* temperature sensitive mutant strain indicated that at the restrictive temperature cells resembled those that had arrested due to nutrient limitation, mating pheromone action or sporulation (Wittenberg and Reed, 1988). It seemed therefore that the *cdc28* mutant was defective in the completion of START (Reed, 1980). The *CDC28* gene was cloned by complementation and was shown to be a homologue of the fission yeast *cdc2* gene (Lorincz and Reed, 1984; Reed *et al.,* 1985). CDC28 had kinase activity (Reed *et al.,* 1985) that increased at START, and it was shown that monomeric CDC28 was inactive (Wittenberg and Reed, 1988). This suggests that, like Cdc2, cyclin binding is required for kinase activity. G1 specific cyclins were cloned as high dosage suppressers of the $cdc28^{ts}$ mutation, indicating a functional interaction with CDC28 (Hadwiger *et al.,* 1989). Three G1 cyclin genes were identified, *CLN1*, *CLN2* and *CLN3* (Hadwiger *et al.,* 1989; Sudbery *et al.,* 1980). Mutational elimination of CLNs 1, 2, and 3 confers a G1 arrest phenotype (Richardson *et al.,* 1989) and CLN proteins co-precipitate with CDC28 (Wittenberg *et al.,* 1990). Overexpression of G1 cyclins advances progression through START (Richardson *et al.,* 1989). Truncated forms of the G1 cyclins are hyperstable and also cause advanced completion of START by prematurely activating CDC28 kinase activity (Hadwiger *et al.,* 1989; Wittenberg *et al.,* 1990). *CLN1* and *CLN2* mRNA levels fluctuate during the cell cycle, accumulating to a peak level immediately before the G1/S transition and almost completely disappearing in the subsequent G2 interval (Wittenberg *et al.,* 1990). CLN1 and CLN2 protein levels and the associated kinase activity mirror the fluctuations

in mRNA levels (Wittenberg *et al.,* 1990). *CLN3* mRNA levels are constant throughout the cell cycle, but protein levels are controlled by a translational control element in the 5' region of the *CLN3* mRNA (Polymenis and Schmidt, 1997) that is regulated by the Ras-cyclic AMP pathway (Hall *et al.,* 1998). This control element is required for repression of CLN3 synthesis under conditions of slow growth or diminished protein synthesis (Gallego *et al.,* 1997), thereby coupling cell growth with cell cycle progression. All three CLNs have short half-lives (Wittenberg *et al.,* 1990; Tyers *et al.,* 1992; Cross, 1990) and may be specifically degraded by the same mechanism. All three proteins contain PEST sequences which, when removed by truncation mutations, confer increased protein stability (Hadwiger *et al.,* 1989; Wittenberg *et al.,* 1990; Cross, 1990). When *S. cerevisiae* cells undergo sporulation the haploid spores generated by meiosis can undergo two rounds of mitotic division to form one cell of each mating type, MATa and MATα. At the first division, a MATa cell will divide to form two MATa cells. The cell corresponding to the daughter cell will divide again to form two MATa cells, whilst the original mother cell will switch mating type and divide to form two MATα cells. Cells of opposite mating type can then mate to from a diploid MATa/MATα cell. The mating type switch of the mother cell involves the action of the *HO* gene product, an endonuclease that introduces a site-specific double strand break in the *MAT* gene. The *HO* gene product is regulated during the cell cycle with an expression pattern identical to that of the *CLN1* and *CLN2* genes. The element controlling the periodic expression of the *HO* gene is termed the SCB (SWI4 cell cycle box) (Nasmyth, 1985a; Nasmyth, 1985b; Breeden and Nasmyth, 1987). SCB consensus sequences are also present upstream of the *CLN1* and *CLN2* genes (Ogas *et al.,* 1991). Transcription of the *HO* gene is controlled by two transcription factors, SWI4 and SWI6 (Andrews and Herskowitz, 1989). DNA binding analysis indicates that only SWI4 binds to DNA

(Primig *et al.,* 1992; Sidorova and Breeden, 1993) but both factors are bound as a complex to SCB DNA (Nasmyth and Dirick, 1991). The *SWI4* gene itself is transcribed specifically in G1 (Breeden and Mikesell, 1991). It has been demonstrated that SCB transcription is dependent on CDC28 activity. The expression of *CLN1* and *CLN2* therefore forms part of a positive feedback loop. Newly born cells in G1 have no significant levels of *CLN1* and *CLN2* transcripts. In a $cdc28^{ts}$ mutant line maintained at the restrictive temperature *CLN1* and *CLN2* transcripts appear when the cell has achieved sufficient growth. These levels do not increase further (Dirick and Nasmyth, 1991; Cross and Tinkelenberg, 1991). Wild type cells however, increase their levels of *CLN1* and *CLN2* transcripts from the same starting point (Dirick and Nasmyth, 1991). The mechanism of CDC28 dependent activation of *CLN1* and *CLN2* transcription may act through activation of the SWI6 transcription factor, which contains consensus sites for CDC28 phosphorylation (Sidorova and Breeden, 1993).

During the mating pheromone response, extracellular signals impinge on the cell cycle control machinery to regulate progression through START. Mating pheromone causes a repression of *CLN1*, and *CLN2* transcription (Wittenberg *et al.,* 1990; Dirick and Nasmyth, 1991). *CLN3* transcription however, is induced (Wittenberg *et al.,* 1990), but CLN3-associated kinase activity is repressed (Jeoung *et al.,* 1998). The *FAR1* gene was identified as a mutation that uncouples cell cycle arrest from mating pheromone response. FAR1 is regulated by phosphorylation and binds to CDC28/CLN complexes (Chang and Herskowitz, 1992). Phosphorylation of FAR1 is due to the action of FUS3, a MAP kinase homologue (Peter *et al.,* 1993). FUS3 may also be involved in inactivation of CLN3 in a FAR1 independent manner, possibly by direct phosphorylation (Elion *et al.,* 1991).

Passage through START does not necessarily commit cells to DNA synthesis. A number of cdc mutants arrest after completion of START, but before entry into S-phase (Reed, 1980). The initiation of S-phase is therefore controlled separately from START control. There are six B-type cyclins in *S. cerevisiae*; CLB1-6. CLB1 and CLB2 have a role in initiation of mitosis (Surana *et al.,* 1991; Ghiara *et al.,* 1991; Fitch *et al.,* 1992) but also appear to have a mid-S-phase role in conjunction with CLBs 3 and 4. Deletion of all four genes simultaneously arrests cells in S-phase with a half-replicated DNA content (Richardson *et al.,* 1992). CLB5 and CLB6 have a direct role in controlling entry into S-phase, as deletion of both genes impairs S-phase initiation, though START progression is unaffected (Epstein and Cross, 1992; Schwob and Nasmyth, 1993; Kuhne and Linden, 1993). The pattern of expression of the budding yeast B-type cyclins mirrors their activities, with CLB5 and CLB6 being synthesised in G1 (Schwob and Nasmyth, 1993; Kuhne and Linden, 1993), CLB3 and CLB4 being produced during S-phase and CLB1 and CLB2 are synthesised primarily in G2 (Fitch *et al.,* 1992; Epstein and Cross, 1992; Grandin and Reed, 1993). These data suggest that, 1: S-phase initiation involves the action of CDC28 with CLB5/CLB6, 2: S-phase progression requires interaction of CDC28 with CLB3/CLB4, and, 3: Entry into mitosis is controlled by CDC28 bound to CLB1/CLB2.

The regulation of START progression by CDC28 is very similar to the regulation of G2/M phase progression by Cdc2 in fission yeast. Both events require the binding of a regulatory cyclin partner protein which is itself regulated by proteolysis or degradation.

Not all temperature sensitive mutant alleles of *CDC28* result in G1 arrest. The *cdc28-1N* mutant arrests in G2 (Piggott *et al.,* 1982), suggesting that CDC28 plays a role not only in G1 progression, but is also involved in signalling the entry into mitosis in the same way as Cdc2 governs mitotic entry in the fission yeast. The identification of the mitotic

(B-type) cyclins, CLBs 1-4, which show a relatively high degree of homology to fission yeast Cdc13, and the demonstration that these cyclins interact with CDC28, suggested that CDC28 did indeed have a dual role in cell cycle progression; governing both G1 progression and entry into mitosis (Surana *et al.,* 1991; Ghiara *et al.,* 1991; Richardson *et al.,* 1992).

A number of other homologues of Cdc2/CDC28 exist in *S. cerevisiae*, not all of which have a direct role in cell cycle progression (see Table 1.1). The product of the *SRB10/SSN3* gene forms a kinase complex with the SRB11 cyclin. This complex forms part of the RNA polymerase II holoenzyme (Kuchin *et al.,* 1995; Liao *et al.,* 1995) and has kinase activity towards the carboxy terminal repeat domain (CTD) of the polymerase *in vitro* (Hengartner *et al.,* 1995; Liao *et al.,* 1995). The SRB10/SRB11 complex probably has a role in repression of transcription (Kuchin and Carlson, 1998). A second kinase/cyclin complex, KIN28/CCL1, also associates with the RNA polymerase II holoenzyme and has CTD kinase activity (Valay *et al.,* 1995).

The CAK1/CIV1 kinase is distantly related to the cdk family but is active as a monomer and has not been found to bind to any of the yeast cyclins (Kaldis *et al.,* 1996; Thuret *et al.,* 1996; Espinoza *et al.,* 1996). CAK1 phosphorylates the Thr 161 residue of CDC28 that is necessary for full activity of the kinase (Kaldis *et al.,* 1996; Thuret *et al.,* 1996; Espinoza *et al.,* 1996). CAK1 activity is also required for phosphorylation and activation of KIN28 (Espinoza *et al.,* 1998).

The last member of the cdk family in *S. cerevisiae* is encoded by the *PHO85* gene. PHO85 interacts with an extensive number of cyclins to carry out a wide variety of cellular functions (Measday *et al.,* 1997; Kaldis *et al.,* 1998). For example PHO85 acts in combination with the PHO80 cyclin to regulate cell cycle progression in response to phosphate levels. In low phosphate conditions Pho4 binds to DNA and activates the

transcription of genes involved in phosphate metabolism such as the acid phosphatase, *PHO5* (Jayaraman *et al.,* 1994). Under high phosphate conditions however, Pho4 is phosphorylated by PHO85/PHO80 and cannot bind to DNA (Kaffman *et al.,* 1994). The PHO85/PHO80 complex can be inhibited by PHO81, a protein that is bound to the complex in high and low phosphate conditions. The inhibitory activity of PHO81 must therefore be post-translationally modified (Schneider *et al.,* 1994; Hirst *et al.,* 1994). PHO85 also has a role in glycogen metabolism (Timblin *et al.,* 1996) in conjunction with the PCL8 and PCL10 cyclins (Huang *et al.,* 1998). These complexes control glycogen biosynthesis by phosphorylation of the glycogen synthase GSY2 (Huang *et al.,* 1998). PHO85 also has a role in cell cycle control in combination with the cyclins, PCL1 and PCL2 (Espinoza *et al.,* 1994; Measday *et al.,* 1994). The activity of these complexes may be required for progression through G1. Expression of both genes, like that of *CLN1* and *CLN2*, is controlled by SBF, and reaches a peak at START (Espinoza *et al.,* 1994; Measday *et al.,* 1994). PHO85/PCL1 is able to phosphorylate the SIC1 protein, the *S. cerevisiae* homologue of *Schizosaccharomyces pombe* Rum1 (Sanchez-Diaz *et al.,* 1998), *in vitro* (Nishizawa *et al.,* 1998). Phosphorylation of SIC1 results in its rapid degradation, a process that is required for DNA synthesis to occur (Nishizawa *et al.,* 1998). Both PHO85/PCL1 and PHO85/PCL2 are able to phosphorylate the RVS167 protein (Lee *et al.,* 1998). Deletion of RVS167 results in defects in endocytosis and organisation of the actin cytoskeleton, phenotypes that are also observed when PCL1 and PCL2 are deleted (Lee *et al.,* 1998). PHO85 may also have a role in late M-phase as part of a complex with the PCL9 cyclin, expression of which is controlled by the SWI5 transcription factor, and reaches a peak level at M/G1 (Tennyson *et al.,* 1998). Deletion of PCL9 causes defects in bud formation (Tennyson *et al.,* 1998).

1.5 Vertebrate cell cycle control

Homologues of the yeast *cdc2* and *CDC28* genes have been found in a wide range of vertebrate species and are known as cyclin-dependent kinases (cdks) (Pines, 1995) (the vertebrate cell cycle is represented in Fig. 1.4). In humans there are nine cdks, cdk1 to cdk9 (see Table 1.2), which interact with members of the human cyclin family, which consists of fourteen members: cyclin A (Henglein *et al.,* 1991), cyclin B1, B2, B3 (Gallant and Nigg, 1994), cyclin C (Lew *et al.,* 1991), cyclin D1, D2, D3 (Xiong *et al.,* 1991; Motokura *et al.,* 1992), cyclin E (Koff *et al.,* 1991), cyclin F (Bai *et al.,* 1994), cyclin G (Tamura *et al.,* 1993), cyclin H (Mäkelä *et al.,* 1994; Fisher and Morgan, 1994), cyclin I (Nakamura *et al.,* 1995), and cyclin T (Wei *et al.,* 1998). Homologues of other cell cycle control genes such as *wee1*, *cdc25* and *suc1*, have also been found (McGowan and Russell, 1993; Millar *et al.,* 1991a; Draetta *et al.,* 1987). Although there are many similarities between the mechanisms of cell cycle control in yeast and vertebrates there are also a number of important differences that are discussed below.

Experiments involving the fusion of mitotic and inter phase cells identified the existence of a factor dubbed MPF (M-phase promoting factor), which was capable of inducing chromosome condensation when interphase cells were fused to cells blocked in mitosis (Johnson and Rao, 1970). Another factor termed SPF (S-phase promoting factor), induced DNA replication in the nuclei of G1 cells fused to cells synchronised in S-phase (Rao and Johnson, 1970). Biochemical purification of MPF from *Xenopus laevis* eggs, showed that it contained two proteins of 32 and 45 kDa. The 32 kDa polypeptide cross-reacted with antibodies raised against a conserved region of the fission yeast Cdc2 protein (Gautier *et al.,* 1988). MPF was also shown to bind to *Schizosaccharomyces pombe* $p13^{suc1}$, as MPF activity is depleted from a crude preparation of MPF passed

through a $p13^{suc1}$ column (Dunphy *et al.,* 1988). These results suggested that cell cycle control mechanisms in vertebrates might resemble those found in yeast. A human homologue of the fission yeast *cdc2* gene had previously been cloned by complementation of $cdc2^{ts}$ mutant strains (Lee and Nurse, 1987), demonstrating a remarkable degree of conservation of function throughout eukaryotic evolution. The human cdc2 gene is also known as cdk1 and will be referred to as such in this thesis. Microinjection of anti-cdc2 antibodies into mouse cells indicated that mammalian cdk1 functioned at G2/M but not at G1/S (Riabowol *et al.,* 1989). After microinjection, the mouse cells underwent DNA replication but failed to initiate cell division, indicating a G2/M specific role for cdk1 and suggesting that it plays no role in G1/S. This hypothesis is supported by the finding that the mouse FT210 cell line, which contains a temperature sensitive cdk1 gene, completes DNA replication but fails to enter mitosis at the restrictive temperature (Hamaguchi *et al.,* 1992). This situation is unlike that observed in *S. pombe* and *Saccharomyces cerevisiae*, where Cdc2/CDC28 acts to initiate both transitions. G1/S phase progression in mammalian cells involves the action of other members of the mammalian cdk family, cdk2 (Pagano *et al.,* 1993) and cdk3 (Meyerson *et al.,* 1992). The kinase encoded by the cdk2 gene shares the greatest degree of homology with cdk1 of all the cdks: 65% of all amino acids are identical in both kinases. Expression of dominant negative mutant versions of cdk2 and cdk3 block cells at the G1/S-phase transition (Van den Heuvel and Harlow, 1993). The cyclin partner to which cdk2 binds during S-phase is cyclin A, which is synthesised at the beginning of S-phase (Tsai *et al.,* 1991; Pines and Hunter, 1990). It is believed that cdk2/cyclin A is required for DNA synthesis, but not for transition into S-phase. Microinjection of cells with anti-cyclin A antibodies abolishes DNA replication as judged by BrdU incorporation (Girard *et al.,* 1991; Zindy *et al.,* 1992), and overexpression of cyclin A

causes premature initiation of DNA synthesis (Rosenberg *et al.,* 1995). There is also a role for cdk2 in the G1/S-phase transition in conjunction with cyclin E. The mRNA and protein levels of cyclin E and the activity of the cdk2/cyclin E complex all peak at the G1/S transition and decline as cells progress through S-phase (Dulic *et al.,* 1992). Functional evidence to support a role for cdk2/cyclin E in G1/S progression comes from studies on *Drosophila* embryos with a mutant cyclin E. In these developing flies, once the maternal store of cyclin E mRNA is degraded in cycle 15, cells arrest in G1 (Knoblich *et al.,* 1994). Furthermore, overexpression of cyclin E in mammalian cells causes accelerated progression through G1 and into S-phase (Ohtsubo and Roberts, 1993). The cdk2/cyclin E and cdk2/cyclin A complexes associate with the E2F transcription factor (Mudryj *et al.,* 1991; Devoto *et al.,* 1992; Pagano *et al.,* 1992a). The E2F family of transcription factors control the transcription of a number of genes required for DNA synthesis, such as ribonucleotide reductase, dihydrofolate reductase, thymidylate synthase, PCNA and DNA polymerase α (Nevins, 1992). The E2F transcription factors therefore play a role similar to that of the SBF and MBF transcription factors of *S. cerevisiae.* It is not yet clear how the binding of cdk2/cyclin E complex to E2F regulates expression of E2F controlled genes. One suggested mechanism is that binding of E2F/cdk2/cyclin E complex to DNA allows the phosphorylation of proteins forming part of the transcription machinery. One such candidate is the CCG1 protein, which associates with the TFIID complex and forms part of the RNA polymerase II initiation complex (Hisatake *et al.,* 1993; Ruppert *et al.,* 1993). Of all the human cdks identified, cdk1-9, only cdks 1, 2, and 3 can complement a budding yeast *cdc28*^{ts} mutant, suggesting that cdks 5 to 9 have roles not directly associated with cell cycle progression (Meyerson *et al.,* 1992). In the G2 phase of the cell cycle cdk1 binds to both cyclin A and cyclin B. However, it is the cdk1/cyclin B

complex that is responsible for signalling entry into mitosis (Draetta and Beach, 1988). During S-phase and G2 the cdk1/cyclinB complex is located in the cytoplasm, becoming associated with the centrosomes. At mitosis, the complex rapidly enters the nucleus (Pines and Hunter, 1991). The cdk1/cyclin A complex on the other hand, is located in the nucleus during S-phase and G2 (Pagano *et al.,* 1992b), and localises to DNA replication sites in S-phase (Cardoso *et al.,* 1993). The nuclear located cdk1/cyclin B complex is responsible for initiating nuclear lamina breakdown, one of the earliest events of mitosis (Peter *et al.,* 1990). Other possible substrates of the cdk1/cyclin B kinase include cytoskeletal elements such as microtubules (Verde *et al.,* 1992) and Golgi membrane proteins (Lowe *et al.,* 1998).

The other members of the mammalian cdk family have diverse functions, some of which play an indirect role in cell cycle progression. The cdk4 kinase for example associates with cyclin D1 just before the initiation of S-phase and the complex reaches a peak level in early S-phase and declines in late S-phase and G2 (Matsushime *et al.,* 1992). Accumulation of cyclin D1 is stimulated by growth factors. Mouse macrophages stimulated with colony stimulating factor (CSF) begin to accumulate cyclin D1 protein 4 to 6 hours after stimulation, at a time which corresponds to the beginning of DNA replication (Matsushime *et al.,* 1991). Withdrawal of growth factor stimulation leads to cessation of cyclin D1 transcription and disappearance of the mRNA and the protein itself, as the D-type cyclins are short lived proteins (Won *et al.,* 1992). Experiments involving microinjection of anti-cyclin D1 antibodies into serum starved cells indicate that it's activity is required in mid to late G1 and not in S-phase, because antibody microinjection up to 8 hours after serum starvation (when cells are in mid G1) prevented DNA replication, whereas microinjection after 16 hours (when cells were already beginning to enter S-phase) did not affect DNA replication (Baldin *et al.,* 1993). Further

evidence that the cdk4/cyclin D1 kinase couples growth factor signalling pathways to cell cycle control comes from experiments in which cyclin D1 overexpression resulted in a diminished dependence on growth factor stimulation and caused a more rapid progression through the cell cycle (Quelle *et al.,* 1993). D-type cyclins have also been identified as oncogenes (Motokura *et al.,* 1991; Withers *et al.,* 1991; Lammie *et al.,* 1992) and experiments in which cells overexpressing cyclin D1 were injected into nude mice showed that they were capable of tumour formation (Jiang *et al.,* 1993). The mechanism by which the cdk4/cyclin D1 complex promotes DNA replication was initially thought to involve modulation of the inhibitory properties of the retinoblastoma protein pRb (Fig. 1.5). The Rb protein can be phosphorylated by cdk4/cyclin D1 *in vitro* at the same sites that are phosphorylated *in vitro* at the G1/S transition (Kato *et al.,* 1993). Activity of the cdk4/cyclin D2 kinase is capable of reversing the Rb mediated inhibition of SAOS-2 cell growth. This effect is concomitant with hyperphosphorylation of Rb (Dowdy *et al.,* 1993; Ewen *et al.,* 1993). The Rb protein is known to bind to, and inhibit, the E2F transcription factor, which controls the expression of a number of S-phase genes. Phosphorylation of pRb by the cdk4/cyclin D1 complex is thought to cause the dissociation of Rb from E2F, allowing S-phase specific gene expression (Sherr and Roberts, 1995). Expression of both cyclin E and cyclin A is dependent on E2F mediated transcription (Devoto *et al.,* 1992; Ohtani *et al.,* 1995; DeGregori *et al.,* 1995), as is expression of the Cdc6 protein that is involved in DNA replication (Yan *et al.,* 1998). This led to the proposal of a model in which growth factor induced activity of cdk4/cyclin D1 kinase resulted in regulation of cdk2/cyclin E and cdk2/cyclin A activity via phosphorylation of Rb, resulting in release of E2F and subsequent expression and accumulation of cyclin E and cyclin A. The cdk inhibitor (CKI), $p16^{INK4a}$, was also shown to be expressed in an E2F dependent manner (Hartwell, 1978). $p16^{INK4a}$ is an

inhibitor of cdk4/cyclin D (Serrano *et al.,* 1993) and would therefore seem to form part of a feedback loop, negatively regulating the activity of cdk4/cyclin D. The proposed model therefore links extracellular signalling events to passage through START, and progression into S-phase. Other evidence, however, contradicts this presumed role of cdk4/6 complexes, and suggests instead that they are involved not in inhibitory hyperphosphorylation of Rb, but in hypophosphorylation, that leads to activation of Rb (Ezhevsky *et al.,* 1997). It is argued that the reported ability of cdk4/cyclin D to hyperphosphorylate Rb occurred under non-physiological conditions, such as in *in vitro* kinase assays and in experiments where cdk4/cyclin D was overexpressed. It is suggested that inhibition of Rb by hyperphosphorylation is carried out by the cdk2/cyclin E complex (Ezhevsky *et al.,* 1997). The precise role of cdk4/cyclin D mediated hypophosphorylation of Rb is still unclear. However it has been observed that prior hypophosphorylation of Rb is required for subsequent hyperphosphorylation by cdk2/cyclin E (Hatakeyama *et al.,* 1994). Unphosphorylated Rb is therefore not a substrate for inactivating hyperphosphorylation and maintains cells in a G0 state by inhibiting E2F mediated transcription . The cdk4/cyclin D complex may therefore regulate cell cycle re-entry from a G0 state (Fig 1.6).

The cdk5 protein is present in various mammalian tissues, but cdk5-associated kinase activity has been found only in brain tissue. This is due to the restricted expression of the activators of cdk5, p35, and two proteins derived from p35 by proteolysis, p25 and p23 (Lew and Wang, 1995). Although cdk5 is highly homologous to other members of the cdk family, p35 shows no resemblance to any of the known cyclins. The cdk5/p35 kinase phosphorylates the microtubule associated protein, tau, and the neuron specific intermediate filament proteins, NF-M and NF-H (Lew and Wang, 1995). This suggests that cdk5/p35 is involved in the regulation of neurite outgrowth and cytoskeletal

assembly. Evidence that this is the case comes from a study in which neurons were co-transfected with cdk5 and p35 and produced longer neurites. In contrast, neurons transfected with a dominant negative cdk5 or with antisense p35, produced fewer and shorter neurites (Nikolic *et al.,* 1996).

The function of cdk6 may be similar to that of cdk4. It is known that cdk6 associates with D-type cyclins in G1, but the precise role is not yet understood (Meyerson and Harlow, 1994; Bates *et al.,* 1994). It may be that cdk4 and cdk6 have overlapping functions or may be controlled by different growth factor signalling pathways. Another possibility is that cdk4 and cdk6 perform similar functions, but are expressed in different tissues.

The CAK activity of vertebrates may be formed by another member of the cdk family, cdk7, in associatiation with cyclin H. Purified CAK activity from *Xenopus laevis* oocytes was identified as being encoded by the MO15 gene. This was later renamed cdk7, when it was demonstrated that it's activity was dependent on binding to cyclin H (Solomon *et al.,* 1993; Fesquet *et al.,* 1993; Poon *et al.,* 1993). The cdk7/cyclin H complex co-purifies with a 36 kDa protein known as MAT1 (menage-à-trois 1). The precise function of MAT1 is not yet known but it may have a role in assembly of the complex. A heterotrimeric complex of cdk7/cyclin H/MAT1 is more stable than a heterodimer of cdk7/cyclin H, when assembled *in vitro* (Tassan *et al.,* 1995a). Whether cdk7/cyclin H functions as the physiological CAK has not yet been fully determined (Harper and Elledge, 1998). The budding yeast homologue of cdk7 is KIN28, which does not have CAK activity but associates with the TFIIH transcription complex and has CTD kinase activity (Valay *et al.,* 1995). The CAK activity of *S. cerevisiae* is due to the activity of CAK1 (CIV1), a kinase that is related to the cdk family but does not bind to a cyclin partner, and is active as a monomer (Kaldis *et al.,* 1996; Thuret *et al.,* 1996;

Espinoza *et al.,* 1996). There is also a great deal of difference between the mechanism of action of CAK1 and cdk7 (Kaldis *et al.,* 1996). This raised the possibility that the true *in vitro* function of cdk7 was as a CTD kinase, and that cdk7 associated CAK activity was an *in vitro* artifact (Harper and Elledge, 1998). Studies in *Drosophila* however, have provided genetic evidence that cdk7 does indeed function as the physiological CAK (Larochelle *et al.,* 1998). It is possible, therefore, that cdk7 in metazoans fulfils the functions of CAK1 and KIN28 in yeast. As, like KIN28, the cdk7/cyclin H complex also associates with the TFIIH transcription factor and functions has CTD kinase activity (Serizawa *et al.,* 1995). Phosphorylation of the CTD of RNA pol II may be important in controlling transcription and the role of cdk7/cyclin H in this process may provide a link between cell cycle control and general transcriptional control. Transcription is repressed when cells enter mitosis. Experiments utilising cell extracts from mitotic cells or using recombinant cdk1/cyclin B in an *in vitro* transcription assay, have indicated that cdk1/cyclin B activity is responsible for this repression. Repression of transcription is concomitant with inhibition of CTD kinase activity of TFIIH associated cdk7/cyclin H suggesting that cdk1/cyclin B, activated by cdk7/cyclin H, negatively regulates TFIIH-associated cdk7/cyclin H (Long *et al.,* 1998). CAK is responsible for phosphorylating the Thr 161 residue of cdk1 and the corresponding Thr 160 of cdk2, which is required for full activation of the kinases. The cdk7 kinase itself contains a similar residue, Thr 170, suggesting that it is also regulated by an upstream activator. A mutant form of cdk7, in which Thr 170 has been mutated to an alanine residue, which cannot be phosphorylated, has reduced CAK activity (Poon *et al.,* 1994). Phosphorylation of Thr 170 is not required when the cdk7/cyclin H complex is bound to MAT1. When the mutant cdk7 binds to both cyclin H and MAT1, the complex has full CAK activity (Fischer *et al.,* 1995). This implies that two separate pathways of CAK

regulation exist; binding of MAT1, or phosphorylation at Thr 170. The kinase that is responsible for phosphorylating the cdk7 Thr 170 residue is known as CAK activating kinase (CAKAK) and has yet to be identified. It has been demonstrated however, that cdk2/cyclin A is capable of functioning as a CAKAK *in vitro*. This raises the possibility that CAK is regulated by a positive feedback loop involving cdk2 (Fischer *et al.*, 1995). Studies in *Drosophila* have shown that cdk2/cyclin A requires CAK activity for activation *in vitro* (Larochelle *et al.*, 1998).

The cdk8 kinase is the mammalian homologue of *S. cerevisiae* SRB10, a kinase that forms part of the RNA polymerase II holoenzyme. Immunoprecipitation of cdk8, co-precipitates cyclin C (Tassan *et al.*, 1995b), a cyclin that is able to complement an *S. cerevisiae* G1 cyclin mutant (Lew *et al.*, 1991; Leopold and O'Farrell, 1991). The cdk8/cyclin C complex has been shown to interact with the large subunit of RNA pol II (Liao *et al.*, 1995; Leclerc *et al.*, 1996) and has also been shown to act as a CTD kinase *in vitro* (Rickert *et al.*, 1996). Affinity chromatography using antibodies directed against cdk8 has been used to purify a human RNA pol II complex which contains the histone acetyltransferases CBP and PCAF which are chromatin remodelling factors (Cho *et al.*, 1998).

The human cdk9 gene was initially isolated using a PCR based approach (Grana *et al.*, 1994). Similarly to cdk5, cdk9 appears to have a role in differentiation. The protein is detected in higher levels in differentiated tissues than in non-differentiated tissue and the kinase activity is highest in terminally differentiated tissues such as brain and muscle (Bagella *et al.*, 1998). Cdk9 forms part of a multimeric protein complex (Garriga *et al.*, 1996), and associates with cyclin T (Wei *et al.*, 1998) and is capable of phosphorylating the Rb protein *in vitro* (De Luca *et al.*, 1997). Cdk9 also forms part of the *Drosophila* P-TEFb elongation complex and can bind to the HIV Tat protein, resulting in increased

transcription elongation of the HIV genome. Both the P-TEFb and Tat complexes are able to phosphorylate the CTD of RNA pol II (Mancebo *et al.,* 1997). These findings point to role for cdk9 activity in transcriptional control of differentiation. Further studies which utilised the yeast two hybrid system have shown that cdk9 interacts with TRAF2, a tumour necrosis factor signal transducer. This interaction was also shown to occur by immunoprecipitation (Maclachlan *et al.,* 1998). It seems therefore that cdk9 plays a role in integrating extracellular signals, transcription and differentiation.

1.6 Cyclin dependent kinase inhibitors (CKIs)

Another class of proteins which regulate the function of cdks are the cdk inhibitors (CKIs). Currently, seven CKIs have been identified in human cells, which fall into two major families; INK4 and CIP/KIP. The INK4 family includes p16, p15, p18, and p19 (Sherr and Roberts, 1995). The p16 inhibitor was identified in a yeast two-hybrid screen for proteins that interacted with cdk4 (Serrano *et al.,* 1993). The interaction between cdk4 and p16 prevents association with cyclin D and therefore inhibits cdk4 associated kinase activity (Serrano *et al.,* 1993). Inhibition of cdk4/cyclin D was thought to lead to cell cycle arrest because pRb remains hypophosphorylated and bound to the E2F transcription factor (Kato *et al.,* 1993). However, given that cdk4/cyclin D may actually be responsible for hypophosphorylation of Rb (Ezhevsky *et al.,* 1997), inhibition of cdk4/cyclin D may prevent exit from a G0 state. Whatever the mechanism, inhibition of cdk4/cycin D results in inhibition of transcription of E2F-associated genes required for S-phase progression. Given the role of p16 in inhibiting the activity of cdk4/cyclin D, maintaining cells in a G0 state, it is no surprise to find that it is frequently mutated, or deleted in many tumours, as are other members of the INK4 family (Orlow *et al.,* 1995;

Okamoto *et al.,* 1995; Okuda *et al.,* 1995). A role in tumour formation has been confirmed in p16 null mutant mice. 69% of these animals develop tumours and they are more susceptible to carcinogenic treatments than wild type littermates (Serrano *et al.,* 1996).

The CIP/KIP family of CKIs function in a different way to the INK4 inhibitors. They do not bind to monomeric cdks, but bind to cdk/cyclin complexes. The first of the CIP/KIP family of CKIs to be discovered was p21. It was identified in four separate screens; (1) through interaction with cdk1 in a yeast two hybrid assay (Harper *et al.,* 1993), (2) as an inhibitor derived from senescent cells (Noda *et al.,* 1994), (3) as a protein induced by the action of p53 (El-Deiry *et al.,* 1993) and (4) as a protein that binds strongly to cdk2/cyclin complexes (Gu *et al.,* 1993). p21 can form complexes with cdk2 bound to cyclin A, cyclin D1, and cyclin E. In addition it can also bind to complexes containing cdk1 and cdk3 (Harper *et al.,* 1993; Xiong *et al.,* 1993). The promoter of the p21 gene contains a p53 binding site that can confer p53 dependent expression on a reporter gene, and p21 can be induced by wild type, but not mutant p53 (El-Deiry *et al.,* 1993). p21 is induced when cells are irradiated, but not in p53 mutant cells (Dulic *et al.,* 1994). Analysis of the levels of p21 in quiescent human fibroblasts, stimulated to grow, has shown that levels of monomeric, unbound p21 fall as cells approach S-phase. This occurs at the same time as cyclin A levels are increasing, lending weight to the hypothesis that G1 to S-phase progression occurs when the concentration of cdk2/cyclin A exceeds that of p21 (Cai and Dynlacht, 1998). The function of p21 however, is not limited to controlling progression into S-phase, a recent report has suggested that it may also have a role in correctly co-ordinating mitosis (Dulic *et al.,* 1998).

Mammalian cells arrest in G1 in response to treatment with the antimitogen transforming growth factor β (TGF-β). This arrest correlates with inhibition of

cdk2/cyclin E activity (Koff *et al.,* 1993), and involves the action of p27, another member of the CIP/KIP family of CKIs (Polyak *et al.,* 1994b; Toyoshima and Hunter, 1994). p27 is also involved in cell cycle arrest due to contact inhibition (Polyak *et al.,* 1994a). Although p21 and p27 are related, they differ in their substrate specificities. p21 binds strongly to cdk2, whereas p27 binds more to cdk4. Though p27 can inhibit cdk2/cyclin E, it has a much greater affinity for cdk4/cyclin D (Polyak *et al.,* 1994b). Immunoprecipitates of p27 from Swiss 3T3 cells, co-precipitate cdk4/cyclin D1, but no other cdk/cyclin complexes (Toyoshima and Hunter, 1994). The activity of p27 is masked by binding of a heat-labile 'masking' factor, and becomes unmasked when cells are treated with TGF-β, or become contact inhibited (Polyak *et al.,* 1994a). Because p27 can inhibit cdk2/cyclin E and can be sequestered by cdk4/cyclin D2 complex, it has been suggested that cdk2/cyclin E can only be activated when there is enough cdk4/cyclin D to sequester p27 (Polyak *et al.,* 1994a).

Another member of the CIP/KIP family of inhibitors, $p24^{Cdi1}$, identified in a yeast two-hybrid assay, has significant homology to VH1 type protein tyrosine phosphatases, and has phosphotyrosine phosphatase activity *in vitro* (Gyruis *et al.,* 1993). Overexpressing p24 causes a delay in S-phase. However, overexpression of a mutated version of the protein, which has no phosphatase activity, suppresses this phenotype (Gyruis *et al.,* 1993).

1.7 Chemical inhibitors of cyclin-dependent kinases

Because cdks play such a pivotal role in cell cycle progression, and because cell cycle regulatory mechanisms are frequently disturbed in tumour cells, a great effort has been

invested in identifying compounds that have inhibitory effects on cdks. At least nine such inhibitors now exist (Meijer, 1996).

The mechanism of inhibition by a number of these inhibitors is known; staurosporine, butyrolactone I (Kitagawa *et al.,* 1993), flavopiridol (Losiewiecz *et al.,* 1994; De Azevedo *et al.,* 1996), olomoucine (Abraham *et al.,* 1994), roscovitine (De Azevedo *et al.,* 1997). Although the chemical inhibitors of the cdks are structurally diverse, they appear to inhibit kinase activity by the same mechanism; ie. competitive binding to the ATP binding pocket. They differ however, in their substrate specificities. Some of the inhibitors are not specific to cdks, and can inhibit a wide range of kinases. This is the case for staurosporine , which inhibits cdk1/cyclin B and protein kinase C (PKC) with an IC_{50} value of 3-6 nM (Rialet and Meijer, 1991; Gadbois *et al.,* 1992), and suramin, which inhibits a wide range of nuclear enzymes such as DNA primase, DNA polymerase α, and DNA topoisomerase II, as well as cdk1 (Mahoney *et al.,* 1990). Butyrolactone I on the other hand displays good specificity against cdk1 and cdk2. It competes for ATP binding and inhibits the phosphorylation of cdk1 consensus sites of histone H1 and pRb (Kitagawa *et al.,* 1993). Another specific inhibitor of cdk1 and cdk2, namely toyocamycin, has been identified from cultures of *Streptomyces* sp. Toyocamycin shows good specificity towards both cdk1 and cdk2 but does not inhibit in the nanomolar range, as do some other cdk inhibitors (Park *et al.,* 1998).

Studies on the cleavage of sea urchin eggs identified the puromycin derivative 6-dimethylaminopurine (6-DMAP), as a potent inhibitor of cell division (Rebhun *et al.,* 1973). Further studies showed that, though not specific, 6-DMAP inhibited cdk1/cyclin B. Inhibitor screening of derivatives of 6-DMAP identified olomoucine as a specific, and potent inhibitor of cdk1/cyclin B (Vesely *et al.,* 1994). Olomoucine inhibits cdk1, cdk2 and cdk5, but does not inhibit cdk4 and cdk6, suggesting that it may be possible to

develop inhibitors that are specific for a particular cdk (Meijer, 1995). Analysis of the crystal structure of cdk2 bound to olomoucine, showed that it binds to the ATP binding pocket, though the purine ring of olomoucine binds in a completely different orientation to the purine ring of the ATP molecule (Schulze-Gahmen, 1995).

Another highly specific inhibitor of cdks is the semi-synthetic flavone, flavopiridol. Flavopiridol was initially identified as a compound that was a potent inhibitor of the growth of several breast and lung cancer cell lines (Kaur *et al.,* 1992) and was later shown to specifically inhibit the cdk1 kinase by competition for ATP binding (Losiewiecz *et al.,* 1994). It is now known that cdk1, cdk2 and cdk4 are equally sensitive to flavopiridol (Meijer, 1995). Flavopiridol is highly specific as an inhibitor of cdks and can inhibit the growth of human tumour xenografts at concentrations that apparently do not affect the growth of normal cells (Sedlacek *et al.,* 1996), and is currently undergoing phase II clinical trials as an anti-cancer drug (Stadler *et al.,* 1998).

Artificial peptides have also been shown to function as inhibitors of cdk2. Expression of a library of random peptides in a yeast two hybrid-based assay allowed the identification of peptides that specifically interacted with cdk2 and could inhibit its kinase activity *in vitro* (Colas *et al.,* 1996). More detailed analysis of one of these peptides, pep8, indicated that inhibition of cdk2 activity was not absolute, pep8 inhibited activity towards histone H1 but not pRb, raising the possibility that different peptide inhibitors may be developed to inhibit cdk2 activity towards an individual substrate (Cohen *et al.,* 1998).

1.8 Cell division and cell cycle control in trypanosomatids

Control of cell division in trypanosomes and *Leishmania* is different from that of yeast or mammalian cells in that there has to be co-ordination of replication of the single organelles; the kinetoplast, flagellum and the basal body. These organelles must then be evenly distributed between both daughter cells. The control of cell division in trypanosomatids must therefore include mechanisms to initiate and monitor the proper replication and segregation of these organelles. There must also be strict controls linking cell cycle progression with differentiation, as the parasite alternates between proliferative and non-proliferative life cycle stages. An understanding of the basic mechanism of cell cycle control in trypanosomatids is therefore necessary to gain a proper insight into control of the parasite life cycle (Matthews and Gull, 1994b).

The cell division cycle of the procyclic form of *Trypanosoma brucei* has been studied in great detail and the timing of a number of characteristic events is known (Sherwin and Gull, 1989). The first event to occur is the initiation of kinetoplast and nuclear DNA synthesis (Gull *et al.,* 1990; Woodward and Gull, 1990). These events occur almost simultaneously and are quickly followed by elongation of the pro-basal body adjacent to the mature basal body subtending the flagellum. The elongated pro-basal body will initiate the growth of a new daughter flagellum. Flagellum growth occurs for a large proportion of the cell cycle. During this time kinetoplast replication and division occurs (Sherwin and Gull, 1989). The two kinetoplasts, together with the two new flagellar basal bodies separate together as there is a physical link between the basal body and the kinetoplast (Robinson and Gull, 1991). The next event to occur is mitosis, which involves the segregation of both the large and minichromosomes. Segregation of the large chromosomes occurs by the action of kinetochore microtubules. The partitioning of the minichromosomes, however, occurs by a another method that may involve the action of microtubule motor proteins (Ersfeld and Gull, 1997). Organelle repositioning

occurs so that longitudinal cell division segregates a single nucleus, kinetoplast and flagellum between each daughter cell (Sherwin and Gull, 1989). Inhibition of microtubule dynamics disrupts cell division, resulting in mis-segregation of organelles and the formation of anucleate 'zoids' (Robinson *et al.,* 1995). This suggests that the checkpoint mechanisms observed in other eukaryotes do not exist in trypanosomes.

In yeast and mammalian cells, signalling pathways impinge on the mechanism of cell division to co-ordinate the expression of genes required for further progression through the cell cycle or for cell cycle arrest and differentiation (Pines, 1994; Zetterberg *et al.,* 1995). Control of cell cycle progression and control of differentiation of *T. brucei* bloodstream form cells to procyclics are linked (Matthews and Gull, 1994a). This differentiation requires the perception of an unidentified signal in a particular stage of the cell cycle, G1 or G0 (Matthews and Gull, 1994a). Studies of the differentiation from the proliferative long slender bloodstream form of *T. brucei*, to the non-dividing short stumpy form is due to the accumulation of an as yet unidentified stumpy induction factor (SIF) that promotes differentiation via the cAMP signalling pathway (Vassella *et al.,* 1997). Adenylate cyclase activity is also known to be stimulated during the transition from the bloodstream to the procyclic form (Rolin *et al.,* 1993). The pp44/46 proteins of *T. brucei* are tyrosine phosphorylated proteins with RNA binding characteristics that are developmentally regulated (Parsons *et al.,* 1994). Proteins such as these may provide a link between cell signalling pathways controlling differentiation and mRNA stability, degradation or translation. This is a possible way that trypanosomatids may control developmental gene expression. Current evidence suggests that the expression of the majority of protein coding genes in trypanosomatids is not regulated at the level of transcription initiation, but that gene expression is controlled post-transcriptionally, by modulation of RNA stability and RNA processing events such

as trans-splicing and polyadenylation (Agabian, 1990; Vanhamme and Pays, 1995). Many homologues of mammalian cell signalling molecules exist in trypanosomatids, such as MAP kinases (Wiese, 1998), adenylate cyclase (Paindavoine *et al.,* 1992), protein kinase A and protein kinase C homologues (Boshart and Mottram, 1997), and trypanosomatids are thought to utilise host-derived molecules to regulate growth and differentiation (Barcinski and Costa-Moreira, 1994). *T. brucei* growth can be stimulated by epidermal growth factor (EGF) and a protein that cross-reacts with an antibody against the human EGF receptor can be detected in *T. brucei* cell extracts (Hide *et al.,* 1989). It is likely therefore that trypanosomatids use similar signalling pathways to control growth and division. However, although there will be similarities in the mechanisms linking extracellular signals with cell cycle progression between trypanosomatids, yeast, and mammalian cells, there are also likely to be important differences that may be targets for the development of parasite specific drugs.

Homologues of components of the cell cycle machinery have been identified in trypanosomes and *Leishmania*. The *CRK1* (cdc2-related kinase) gene of *Leishmania mexicana* was isolated from a cDNA library during an experiment unrelated to the study of CDK homologues in *Leishmania* (Mottram *et al.,* 1993). The protein encoded by *CRK1* contained many of the residues known to be important for the regulation of the activity of cdks, such as homologues of the Tyr 15 and Thr 161 residues involved in regulation of kinase activity. CRK1 also contained a modified PSTAIR box, with a single amino acid substitution. CRK1 was found to have stage regulated kinase activity against histone H1, with activity being detected in log phase and stationary phase promastigotes but not in amastigotes. This was despite the fact that the protein was detected at equal levels in all three life cycle stages, and suggested that the kinase activity was post-translationally regulated in the same way as cdks from other

organisms; by the binding of regulatory proteins such as cyclins, CKIs or by inhibitory and activatory phosphorylations. Because the activity of CRK1 is not ubiquitous in all three life cycle stages it is likely that it plays a role in differentiation. A direct role in cell cycle progression is unlikely, as CRK1 activity is present in cell cycle arrested, stationary phase promastigotes and is absent in proliferative amastigotes (Mottram *et al.,* 1993). A separate kinase activity that binds to $p13^{suc1}$ was detected in *Leishmania.* This activity, originally termed SBCRK (suc1-binding cdc2-related kinase), was found to be present only in the proliferative promastigote and amastigote stages of the life cycle, and was not detected in the cell cycle arrested metacyclic stage (Mottram *et al.,* 1993). Further investigation suggested that the CRK3 kinase is responsible for this activity (Grant *et al.,* 1998). The *CRK3* gene of *Leishmania mexicana* is homologous to a previously identified gene of *Trypanosoma brucei* that had been identified by heterologous screening using the *L. mexicana CRK1* gene as a probe. This approach identified a family of CRKs in *Trypanosoma brucei* (*CRK1-CRK3*) (Mottram and Smith, 1995). A fourth *CRK* gene has been identified in *Trypanosoma brucei* (Ford and Mottram, unpublished). This gene, *CRK4*, is homologous to a previously identified CRK from the insect trypanosomatid, *Crithidia fasciculata* (Brown *et al.,* 1992). A fifth member of the *T. brucei* CRK family, *CRK5*, was identified from the EST sequencing project. The kinase encoded by *CRK5* is most homologous to the mammalian cdk5 and may therefore play an important role in differentiation (Van Hellemond and Mottram, unpublished). As well as catalytic kinase subunits, a number of regulatory cyclins have been cloned from *Trypanosoma brucei* and *Trypanosoma cruzi.* The *CYC1* gene of *T. brucei* was able to complement an *S. pombe* $cdc13^{ts}$ mutant and co-purified, by immunoprecipitation, with a 34kDa protein that cross-reacted with a monoclonal antibody raised against the PSTAIR epitope of $p34^{cdc2}$ (Affranchino *et al.,* 1993). Two

more cyclins were identified from *T. brucei* through complementation screening of *T. brucei* cDNA library, expressed in an *S. cerevisiae* G1 cyclin mutant (Neuville and Mottram, unpublished). CRKs have also been found in *Trypanosoma cruzi*. Co-expression of *T. cruzi* CRK1 with mammalian cyclins in COS-7 cells, demonstrated that CRK1 was capable of binding mammalian cyclins E, D3 and A (Gomez *et al.*, 1998).

The work described in this study involves the use of molecular genetics, chemical inhibition and yeast complementation analysis to gain more knowledge of the function of the *CRK3* kinase of *Leishmania mexicana* and it's possible role in cell cycle control.

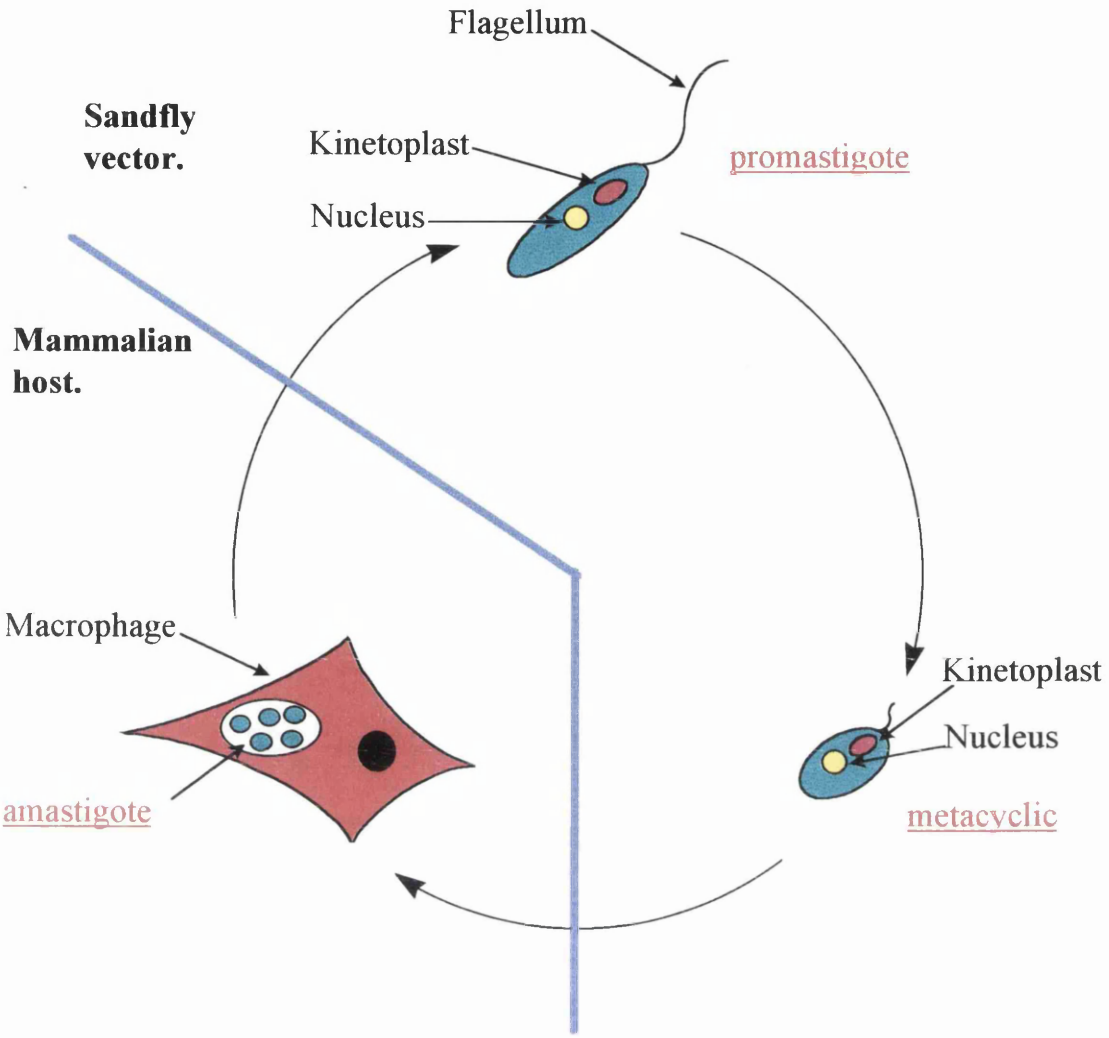

Fig. 1.1 The life cycle of *Leishmania mexicana*

The life cycle of *Leishmania mexicana* is represented. The proliferative promastigote and the cell cycle arrested metacyclic exist in the sandfly vector. Upon transmission to the mammalian host metacyclics differentiate into proliferative amastigotes that are phagocytosed by host macrophages where they survive in the parasitophorous vacuole.

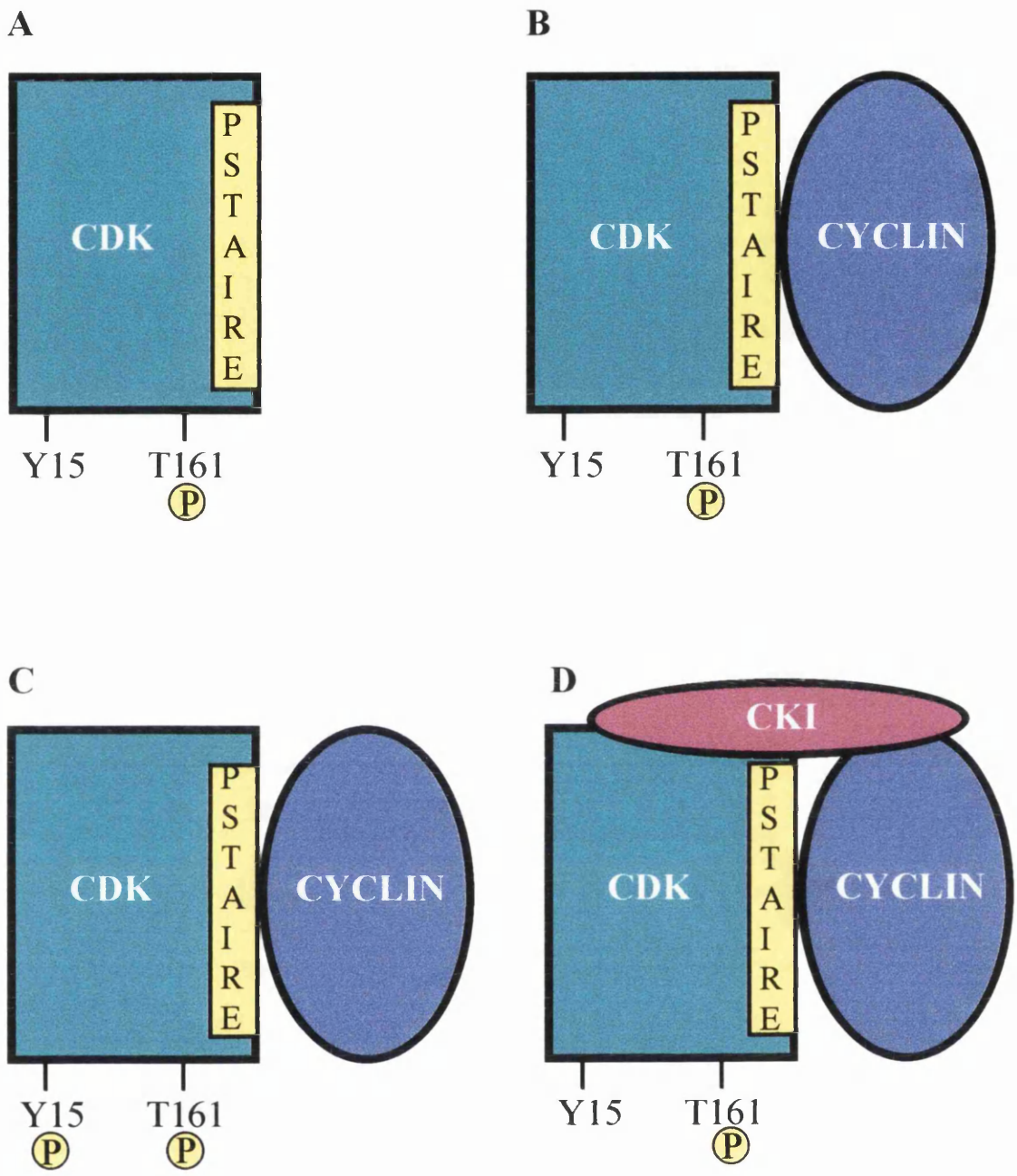

Fig. 1.2. Activation and inhibition of cyclin-dependent kinases

Phosphorylation of Thr161 (Panel A) allows the binding of a cyclin partner via interaction with the PSTAIR box (Panel B). This results in a fully active kinase complex. The complex can be inhibited by phosphorylation of Tyr15 (Panel C) or by the binding of a CDK inhibitor protein, CKI, (Panel D).

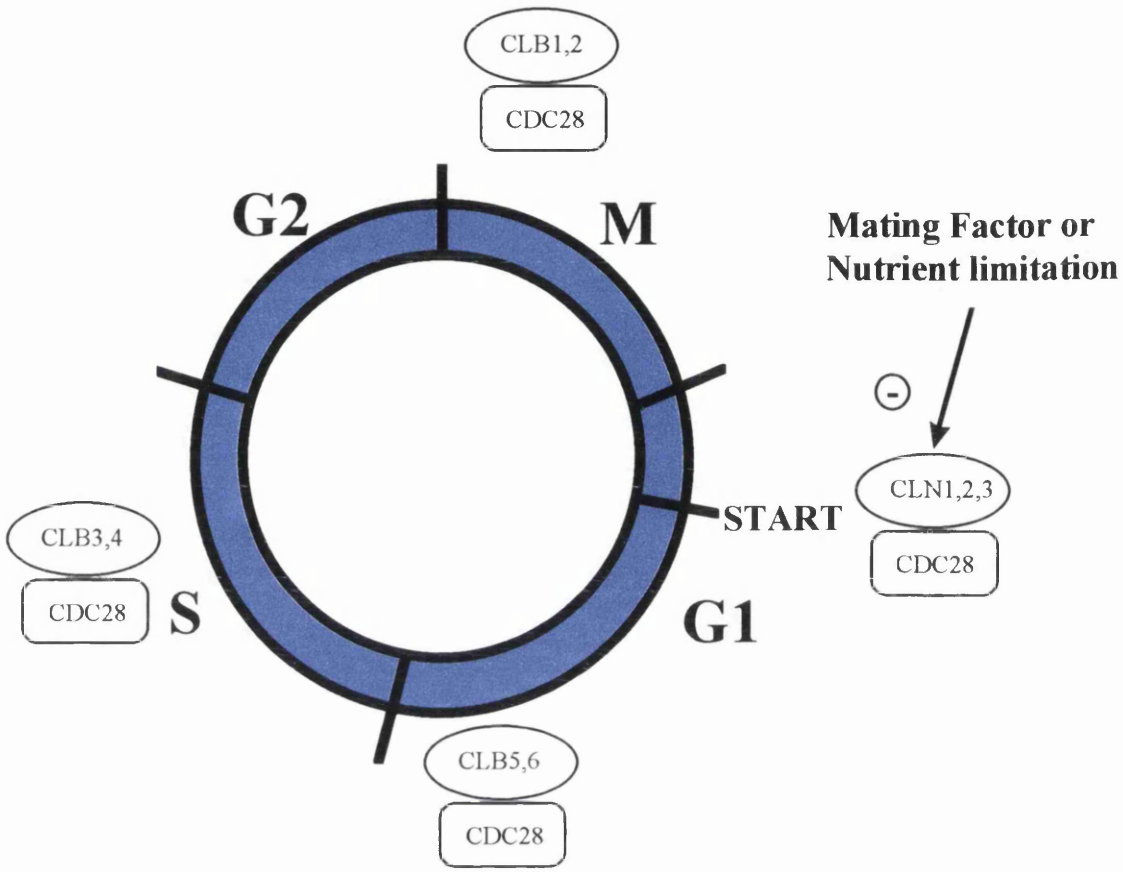

Fig 1.3 The *Saccharomyces cerevisiae* cell cycle

The *S. cerevisiae* cell cycle is depicted with the position of each cdk/cyclin complex representing the point in the cycle at which it functions. Inhibition of cell cycle progression due to nutrient limitation, or mating factor signalling, occurs at START.

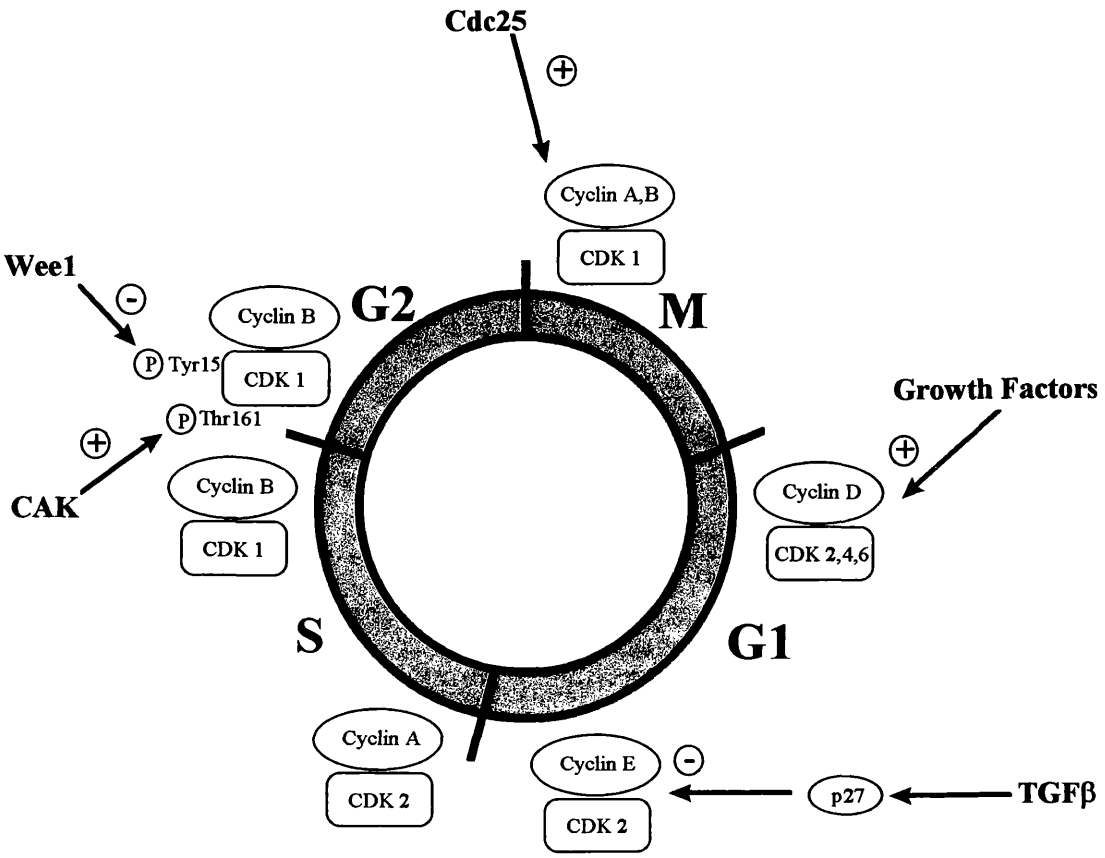

Fig 1.4 The mammalian cell cycle

The mammalian cell cycle is depicted with the position of each cdk/cyclin complex representing the point in the cycle at which it functions. The point at which inhibitory (-) and activatory (+) signals act on cdk/cyclin complexes is indicated by the arrows.

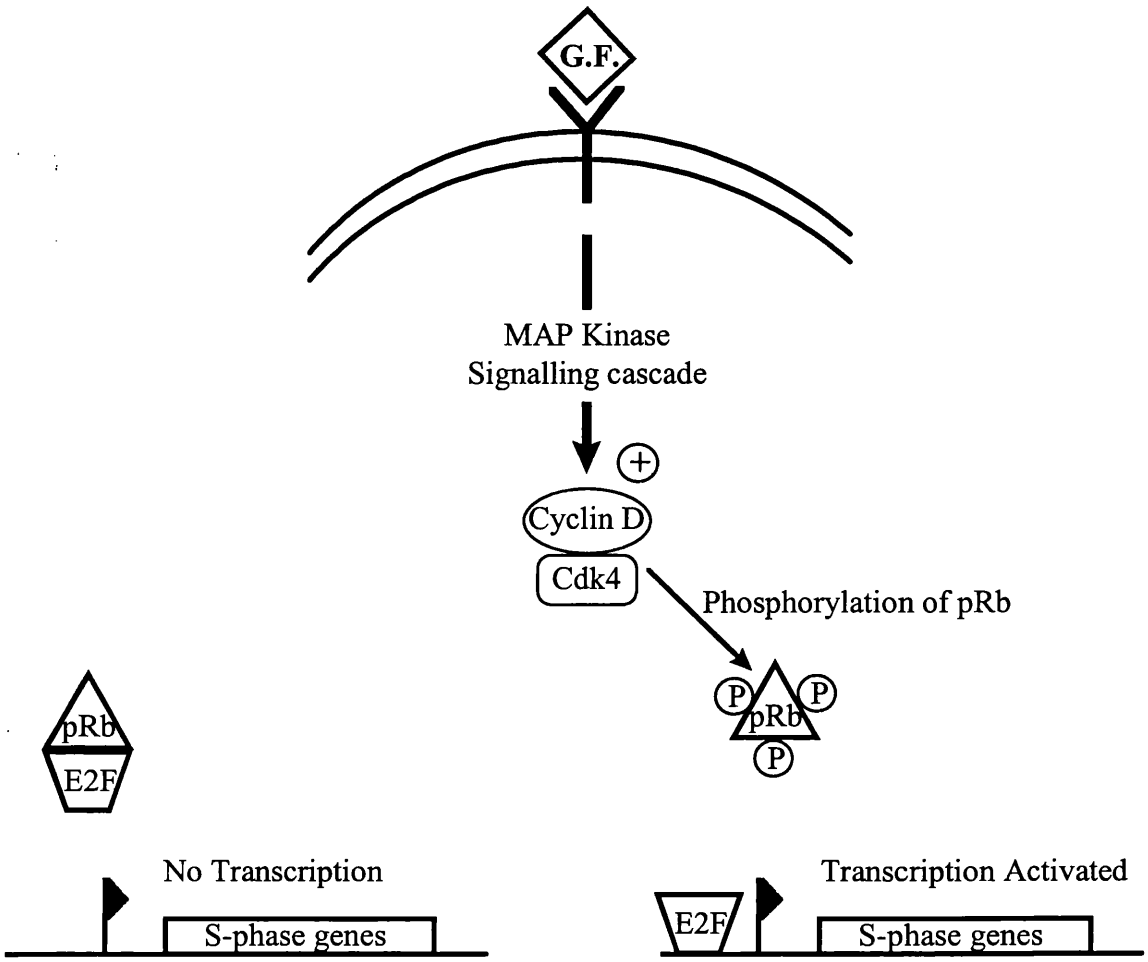

Fig. 1.5 Model for regulation of S-phase progression by growth factors, via modulation of Cdk4/cyclin D activity

Transduction of extracellular growth factor signals, via the MAP kinase cascade, leads to activation of Cdk4/cyclin D. Phosphorylation of the retinoblastoma protein (pRb) by Cdk4/cyclin D causes it's release from binding to the E2F transcription factor. This allows E2F to participate in activation of transcription of genes required for S-phase progression.

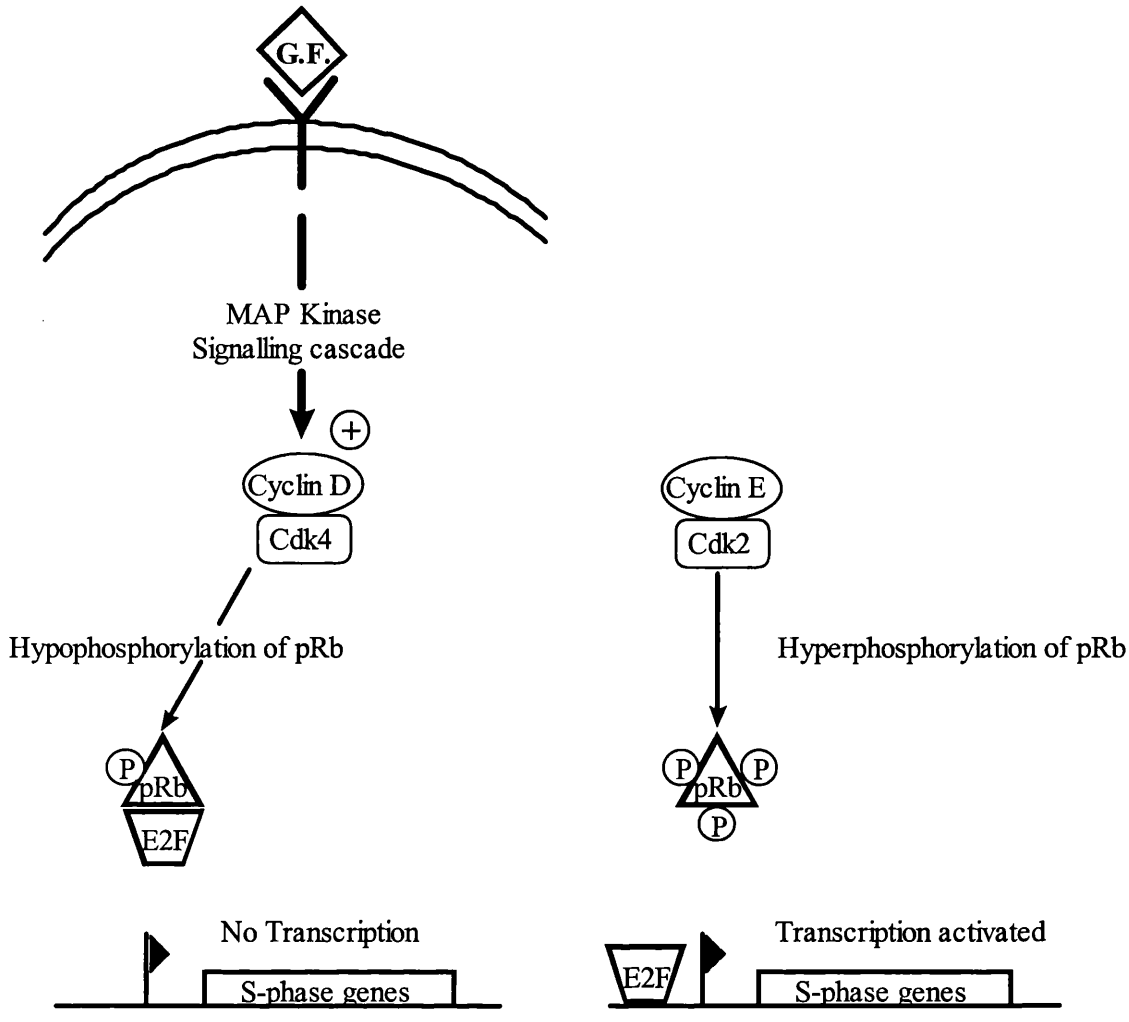

Fig. 1.6 Revised model for regulation of S-phase progression by growth factors, via modulation of Cdk4/cyclin D activity

Transduction of extracellular growth factor signals, via the MAP kinase cascade, leads to activation of Cdk4/cyclin D. Hypohosphorylation of the retinoblastoma protein (pRb) by Cdk4/cyclin D primes the Rb protein for activity. Subsequent hyperphosphorylation by Cdk2/cyclin E activates causes release ofthe E2F transcription factor allowing transcription of genes required for S-phase progression.

CDK	CYCLIN	FUNCTION
CDC28	CLN1, CLN2, CLN3 CLB5, CLB6 CLB1, CLB2, CLB3, CLB4	Passage through START DNA replication Mitosis
CAK1	CLN2	Activation of Cdc28 by T167 phosphorylation
KIN28	PCL1	Part of RNA pol II holoenzyme
SRB10	SRB11	Part of RNA pol II holoenzyme
PHO85	PHO80 PCL1, PCL5, PCL9	Transcriptional control of phosphate metabolism

Table 1.1 The cyclin-dependent kinases of *Saccharomyces cerevisiae*

CDK	CYCLIN	FUNCTION
Cdk1	Cyclin A, B, B2, B3	G2/M phase transition
Cdk2	Cyclin A, E, D1, D2, D3	G1/S transition and S-phase progression
Cdk3	Unknown	G1/S transition
Cdk4	Cyclin D1, D2, D3	G1/S phase progression in response to growth factors
Cdk5	p35 and Cyclin D1, D3	Neuronal differentiation
Cdk6	Cyclin D1, D2, D3	G1/S phase progression in response to growth factors
Cdk7	Cyclin H	Cdk activating kinase and RNA polII CTD kinase
Cdk8	Cyclin C	Component of RNA pol II holoenzyme
Cdk9	Cyclin T	Probable role in differentiation

Table 1.2. The mammalian cyclin-dependent kinases

CHAPTER 2

Materials and methods

2.1 Molecular methods

2.1.1 Bacterial strains

The *Escherichia coli* strain used throughout this study was XL1-Blue MRF' (Stratagene). Cultures were grown overnight at 37°C with rotation at 220 rpm.

2.1.2 Glycerol stocks

A single colony was selected from an LB-agar plate and inoculated into 1 ml LB broth. The culture was grown overnight at 37°C and 0.5 ml was mixed with an equal volume of 2% peptone/40% glycerol solution. Samples were stored at -70°C.

2.1.3 Bacterial culture

2 ml LB broth in a sterile glass test tube was inoculated with an individual colony selected from an LB-agar plate using a sterile tooth pick. 100 μg ml^{-1} ampicillin was added and caps were placed on the tubes. This culture was incubated in a rotary incubator at 220 rpm, 37°C, overnight and was either used to perform a plasmid mini-prep, or was used to initiate a larger culture. Briefly, 1 ml of the pre-culture was used to inoculate 20 ml (for midi prep) or 400 ml (maxi-prep) LB-broth which was grown overnight at 220 rpm, 37°C.

2.1.4 Preparation of competent cells

1 ml LB-broth containing 20 μg ml^{-1} tetracycline was inoculated with a single bacterial colony selected from an LB-agar plate. This culture was grown overnight at 37°C, 220 rpm, in a rotary incubator. This pre-culture was inoculated into 200 ml LB-broth containing 20 μg ml^{-1} tetracycline and was incubated at 37°C, 220rpm till the $O.D._{600}$ value was between 0.6 and 0.7. (The $O.D._{600}$ value was determined by transferring a 0.5 ml sample to a disposable plastic cuvette (Sarstedt) at various time points. The optical density at 600 nM was then determined using a DU 650 spectrophotometer (Beckman). When the culture had reached the appropriate density, cells were harvested by centrifugation at 2000g for 10 min. From this point onwards cells were kept at 4°C. The cell pellet was washed once in ice cold distilled H_2O. Cells were then resuspended in 20 ml TFG I buffer and incubated on ice for 15 min. Cells were harvested, resuspended in buffer TFG II and incubated on ice for 15min. The cell suspension was then divided into 250 μl aliquots in eppendorf tubes. Tubes were then snap-frozen by submersion in a dry-ice/ethanol bath. Competent cells were stored at -70°C.

2.1.5 Plasmid preparation

Plasmids were prepared from bacterial strains using the mini and midi-prep plasmid preparation kits (Qiagen) according to the manufacturers protocol. Briefly, cells were pelleted, resuspended, lysed and neutralised. The samples were then centrifuged and the clear supernatant, containing the plasmid DNA, was loaded onto a column containing a plasmid DNA-binding resin. After washing, plasmid DNA was eluted from the column, precipitated, and pelleted by centrifugation. DNA was then resuspended in 100 μl of

sterile water or TE buffer. This typically resulted in a concentration of approximately 1 mg ml^{-1} as measured by U.V. absorption at 260nm.

2.1.6 Large scale plasmid preparation

A single bacterial colony was inoculated into 3 ml LB medium and grown overnight with shaking at 37°C. 1 ml of this overnight culture was inoculated into 400 ml LB medium and grown overnight at 37°C at 220 rpm. Cultures were then harvested by centrifugation at 4000g for 15 min. Pelleted cells were resuspended in 10 ml sucrose resuspension buffer (SRB). 2 ml lysozyme stock solution was added and cells were incubated at 4°C for 15 min. 4 ml 0.25M EDTA (pH 8.0) was added and samples incubated at 4°C for a further 5 min before the addition of 16 ml Triton mix and incubation on ice with gentle shaking. Samples were then centrifuged at 30,000g, 4°C for 30 min. The supernatant was then transferred to a fresh container and 0.5x volumes of ice cold PEG 8000 solution was added. NaCl was added to a final concentration of 1.5M and samples were shaken vigorously on ice for 15 min. Precipitated DNA was pelleted by centrifugation at 30,000g, 4°C for 30 min. The DNA pellet was then resuspended in 6.188 ml TE buffer to which was added 6.63 g CsCl and 442 µl Ethidium Bromide (10 mg ml^{-1} stock). Samples were spun at 1000g for five minutes to separate precipitated material. The samples were then loaded into quick-seal tubes (Beckman) which were heat-sealed, loaded into a TLN-100 rotor (Beckman) and then centrifuged at 60,000 rpm for 24 hours in an Optima™ ultracentrifuge (Beckman). The top of the tube was punctured with a syringe needle to form an air hole and the plasmid band (which was clearly visible) was recovered by aspirating with a syringe directed through the wall of the tube into the relevant band. Ethidium bromide was extracted

against butan-1-ol. The plasmid solution was mixed with an equal volume of H_2O-equilibrated butan-1-ol, and the phases were allowed to separate. The organic phase, the upper phase, was removed and the extraction process was repeated until the aqueous phase became colourless. Plasmid DNA was recovered by diluting the clear aqueous phase with TE buffer, and precipitating the DNA by the standard ethanol precipitation procedure.

2.1.7 Polymerase chain reaction (PCR) amplification of DNA fragments

PCR was used to amplify DNA fragments required for cloning purposes or for identification purposes by PCR screening of transformed *E. coli* or transfected *L. mexicana*. All reactions were carried out in a 10µl volume consisting of 0.9 µl 11.1x PCR mix, 20 ng of sense and antisense oligonucleotide primer, approx 50 ng genomic DNA or a partial bacterial colony as template, and 2U Taq polymerase (Applied Biosystems). Vent polymerase (NEB) was used for cloning experiments as it has 5' to 3' exonuclease activity. The sequence of PCR-amplified products used in cloning experiments was determined by automated DNA sequencing of both strands using appropriate primers (eg. T7 and SP6 for PCR products cloned into pGEM-T). The amplification protocol was as follows: An initial denaturing step of 4 min at 95°C, followed by 30 cycles at 94°C for 1 min (denaturing), 60°C for 1 min (annealing), and 72°C for 1 min (primer extension). A final step of primer extension for 5 minutes at 72°C was followed by incubation of the samples at 4°C.

For screening of transformed bacteria denaturing, annealing and extension times were reduced to 30 seconds. For amplification of *L. mexicana* genomic DNA 20 µl volumes were used and DMSO was added to a final concentration of 5%.

2.1.8 Restriction digestion

Restriction enzyme digests were carried out according to instructions supplied with the enzymes (NEB). Typically 1 μg of DNA was digested in a 20 μl reaction with 10 units of restriction enzyme at 37°C for 1 hour. The reaction was halted by the addition of 1/6th volume DNA loading buffer. For double digests the reaction was carried out in a buffer that was compatible with both enzymes. If this was not possible the DNA was digested with one enzyme, this was then heat inactivated by incubation at 65°C for 20 min (where appropriate)., then the sample was precipitated, resuspended in restriction enzyme buffer and digested with the second enzyme. 5 μg of *L. mexicana* genomic DNA was digested with 40 units of enzyme at 37°C for 4 hours.

2.1.9 Gel electrophoresis

DNA fragments were separated and analysed by gel electrophoresis. For analysis gels were typically 0.8 to 1.2 % agarose/TBE gels. Samples were mixed with 6x DNA loading dye to allow visualisation of the progress of electrophoresis. Samples were run at 40 to 100V until the dye front migrated to two thirds of the distance of the gel. Gels were stained in a bath of 300 ng ml^{-1} ethidium bromide solution and images were obtained using The Imager™ gel documentation system (Appligene) under UV illumination.

2.1.10 DNA extraction from agarose gel

DNA fragments to be recovered were separated by electrophoresis through a 0.8% agarose/TAE gel. For small scale recovery, < 1 μg, the Qiaquick gel extraction kit (Qiagen) was used according to the manufacturers instructions. For large scale recovery the appropriate fragment was excised from the gel using a scalpel blade. The gel fragment was diced into small cubes approximately 1 mm^3. Gel fragments were placed into Spin-X tubes (Costar), and incubated at -20 and 37°C respectively for 15 min. Samples were then centrifuged at 13,000 rpm in a microcentrifuge for 15 min. Excess agarose was discarded and the resulting solution, containing the DNA, was extracted twice against equal volumes of dH_2O equilibrated chloroform. DNA was then precipitated by ethanol precipitation and was resuspended in TE buffer at a concentration of 1 mg ml^{-1}.

2.1.11 Phosphatase treatment

To prevent religation of plasmids digested with only one restriction enzyme, the 5' terminal phosphate was removed by treatment with calf intestinal alkaline phosphatase (CIP). 5U of CIP (NEB) was added to the reaction mix and incubated at 37°C for 20 min. The reaction mix was then extracted once against an equal volume of H_2O-equilibrated phenol and twice against an equal volume of H_20-equilibrated chloroform. The aqueous phase was removed and DNA was precipitated by the addition of 2.5 volumes of a 9:1 mixture of ethanol and 3M sodium acetate.

2.1.12 DNA Ligation

Vector and insert DNAs were combined in a 10 µl reaction in a range of ratios, from 3:1 to 1:3. 200U (equivalent to 3 Weiss units) T4 DNA ligase and T4 DNA ligase buffer (NEB) were added and the reaction was incubated at 16°C overnight.

PCR products were ligated into the pTag (Ingenius) or the pGEM-T (Promega) PCR cloning vectors according to the manufacturers instructions.

2.1.13 Bacterial transformation

1.0 µl of a ligation reaction was added to 50 µl of competent *E. coli* in a 15 ml falcon tube (Greiner). Cells were incubated on ice for 30 min. then subjected to heat shock at 42°C for 45 seconds. After heat shock cells were placed on ice for 3 min and then 950 µl SOC medium was added. The samples were then incubated for 1 hour at 37°C, 220 rpm in a shaking incubator. 100µl of cells was plated onto LB-agar plates containing 100 µg ml^{-1} ampicillin. Plates were incubated overnight at 37°C.

2.1.14 DNA sequencing

DNA sequencing reactions utilised the ABI-Prism™ cycle sequencing kit (Perkin-Elmer) according to the manufacturers instructions using template DNA and the appropriate oligonucleotide primer. Sequence was obtained for both strands. Samples were then run on an ABI 373 automated DNA sequencer. The University of Wisconsin Genetics Computer Group (GCG) software package (Version 7.0) was used to analyse nucleic acid and amino acid sequence.

2.2 Generation of constructs described in this thesis

2.2.1 pGL89

The pGL89 construct containing the 2.0Kb *Hin*dIII fragment from the *CRK3* locus was generated as described previously (Grant *et al.,* 1998).

2.2.2 pGL97 (*CRK3::BLE* knockout construct)

The pGL97 (*CRK3::BLE*) construct was generated by P. Halford. The *LmCPA::BLE* construct (Souza *et al.,* 1994) was used as the basis for generation of pGL97. *LmCPA::BLE* consists of the *BLE* gene, flanked by 5' and 3' *L. major DHFR-TS* sequence that is in turn flanked by *LmCPA* 5' and 3' sequence. The 1.1 kb fragment of pGL89 was released by digestion with *Hin*dIII and *Sal*I. The fregment was gel-purified and ligated into the *LmCPA::BLE* construct digested with *Hin*dIII and *Sal*I. This formed an *LmCPA/CRK3::BLE* fusion construct. The *CRK3* 3' flank was generated by PCR using the OL322 and OL323 oligonucleotide primers (See Table 2.2). These primers contain engineered *Sma*I and *Bgl*II restriction sites. The PCR product was cloned into the pTAg vector (Ingenius) and the insert was sequenced. The 339 bp insert was released by digestion with *Sma*I and *Bgl*II and cloned into the *LmCPA/CRK3::BLE* fusion plasmid digested with *Sma*I and *Bgl*II.

2.2.3 pGL105 (*CRK3::HYG* knockout construct)

The pGL105 (*CRK3::HYG*) construct was generated by digesting pGL97 with *Spe*I and *Bam*HI to release the *BLE* drug resistance gene. This was replaced by the 1.0 kb

*Spe*I/*Bam*HI fragment of *LmCPA::HYG* (Souza *et al.,* 1994) that contains the *HYG* drug resistance gene

2.2.4 pGL96 (pX*CRK3*His)

The pGL96 construct was generated by Dr. Karen M. Grant as described previously (Grant *et al.,* 1998).

2.2.5 pGL100 (pTEX*CRK3*)

The pGL89 construct was digested with *Eco*RI and *Hin*dIII. The 1.0 kb fragment containing the *CRK3* gene was gel-purified and ligated into the pTEX vector (Kelly *et al.,* 1992).

2.2.6 pGL310 (pTEX*CRK3*His)

The pGL91 plasmid containing the *CRK3* gene fused to a 6-histidine tag (Grant *et al.,* 1998) was digested with *Eco*RI and *Hin*dIII. The 800 bp fragment was gel-purified and ligated into the pTEX vector (Kelly *et al.,* 1992). Subsequent sequence analysis indicated that there was a serine to alanine substitution at the second amino acid position of the *CRK3* gene in the pGL91 plasmid. This results in the production of an inactive kinase (Grant *et al.,* 1998).

2.2.7 pGL120 (pRS416MET*CRK3*)

The pGL89 construct was digested with *Eco*RI and *Xho*I. The 1.1 kb fragment containing the *CRK3* gene was gel-purified and ligated into the pRS416MET yeast expression vector (Mumberg *et al.,* 1994).

2.2.8 pGL332 (pRS314MET*CYC1*)

The pGL106 plasmid containing the *T. brucei CYC1* gene (generated by R. Ford), was digested with *Eco*RI and *Xho*I. The 1.3 kb fragment was gel-purified and ligated into the pRS416MET vector (Mumberg *et al.,* 1994). This was then digested with *Kpn*I and *Sac*I. The 2.1 kb fragment containing the *T. brucei CYC1* gene flanked by the *S. cerevisiae MET25* promoter and *CYC1* terminator was gel-purified and ligated into pRS314 (Sikorski and Hieter, 1989). pRS314 was a kind gift from Dr. J.V. Gray, Department of Molecular Genetics, University of Glasgow.

2.2.9 pGL292 (pRS314MET*CYC2*)

The pGL123 plasmid containing the *T. brucei CYC2* gene flanked by the *S. cerevisiae MET25* promoter and *CYC1* terminator (Neuville and Mottram, unpublished), was digested with *Kpn*I and *Sac*I. The 1.6 kb fragment containing the *CYC2* gene, *MET25* promoter and *CYC1* terminator, was gel-purified and ligated into the pRS314 vector (Sikorski and Hieter, 1989).

2.2.10 pGL293 (pRS314MET*CYC3*)

The pGL124 plasmid containing the *T. brucei CYC3* gene flanked by the *S. cerevisiae MET25* promoter and *CYC1* terminator (Neuville and Mottram, unpublished), was digested with *Kpn*I and *Sac*I. The 2.4 kb fragment containing the *CYC3* gene, *MET25* promoter and *CYC1* terminator, was gel-purified and ligated into the pRS314 vector (Sikorski and Hieter, 1989).

2.3 *Leishmania mexicana* methods

2.3.1 *Leishmania mexicana* cell line

The original *L. mexicana* cell line used in this study, and from which all transgenic cell lines were derived, was *Leishmania mexicana mexicana* (MNYC/BZ/62/M379).

2.2.2 *Leishmania mexicana* cell culture

L. mexicana promastigotes were cultured at 25°C in HOMEM medium (Berens *et al.*, 1976), supplemented with 10% heat inactivated foetal calf serum (FCS) (Labtech). Cells were seeded at a density of approximately $1x10^6$ cells ml^{-1} and cultures were maintained by serial passage when the cell density reached approximately $1.5x10^7$ cells ml^{-1}. Transgenic cell lines were grown in the presence of appropriate antibiotics at the following concentrations: hygromycin B (Boehringer Mannheim) 50 μg ml^{-1}, phleomycin 10 μg ml^{-1} (Cayla, France) and geneticin (Gibco BRL) 50 μg ml^{-1}.

2.3.3 Determination of cell density

Cell numbers were determined by counting cells in an improved Neubauer haemocytometer (Weber scientific). 20 µl of cell culture was mixed with 20 µl of 4% formaldehyde in PBS in a 1.5 ml eppendorf tube to kill the parasites. The sample was then loaded onto the haemocytometer and cells were counted at x20 objective magnification.

2.3.4 Preparation of *L. mexicana* promastigote cell pellets

Cells were centrifuged at 2000g for 5 min. Cells were then washed twice by resuspending in 10 ml PBS and centrifuging again at 2000g for 5 min. Cell pellets were then stored at -70°C until required.

2.3.5 Preparation of stabilates

Stabilate stocks of all cell lines described in this thesis were prepared by addition of 10% (v/v) sterile dimethylsulphoxide (DMSO) to 0.5 ml of log-phase cell culture in a Cryotube™ vial (Nunc). Samples were frozen overnight at -70°C and transferred to a liquid nitrogen storage tank.

2.3.6 Electroporation procedure

Transfection of *Leishmania mexicana* followed the procedure of C.M. Coburn *et al* (1991). Cells were harvested in mid log phase of growth by centrifugation at 2,000g, 4°C for 5 min. Cells were washed once in electroporation buffer (EPB). Cells were then resuspended to a density of $1x10^8$ cells ml^{-1} in EPB. Samples were kept on ice

throughout this procedure. 0.4 ml (ie. $4x10^7$ cells) were resuspended in a 0.2 cm electroporation cuvette (Biorad). 20 μg of plasmid or gel-purified targeting construct DNA was added and cells were immediately transfected by electroporation at 2.25 kV cm^{-1} with the Genepulser II apparatus (Biorad). After electroporation cells were placed on ice for 10 min before being transferred to liquid medium (HOMEM). Cells were incubated overnight to allow expression of the drug selectable marker, before plating on solid HOMEM agar plates containing the appropriate antibiotics. Antibiotics were used at the following concentrations: hygromycin B 50 μg ml^{-1} , geneticin 50 μg ml^{-1}, and phleomycin 10 μg ml^{-1}. Plates were wrapped in parafilm and incubated at 25°C for 10 to 15 days to allow colonies to form. Colonies were picked from plates using a sterile pasteur pipette and transferred into 10 ml liquid medium (HOMEM, 10% FCS plus appropriate antibiotics). Cultures were incubated at 25°C.

2.3.7 Preparation of *Leishmania mexicana* genomic DNA. Large scale prep.

30 ml of stationary phase cultures of *L. mexicana* were harvested by centrifugation at 1000g for 10 min at 4°C. The pellet was washed twice in 10 ml PBS (phosphate buffered saline) before being resuspended in 5 ml SE buffer. 0.25 ml of a 20% SDS solution was added and the sample incubated at 65°C for 30 min. Proteinase K was then added to a final concentration of 50 μg ml^{-1} and samples were incubated at 37°C for 30 min. The samples were then extracted twice against phenol (equilibrated to pH >7.0) and twice against H_2O-equilibrated chloroform. DNA was precipitated from the aqueous layer by addition of 2.5 volumes of a 9:1 mixture of ethanol and 3M sodium acetate before centrifugation at 30,000g for 30 min at 4°C. The pellet was then washed with 3ml 70% cold ethanol and centrifuged as before. DNA was resuspended in 200 μl TE buffer,

and RNAse A was added to a final concentration of 20 μg ml^{-1}. Samples were incubated at 37°C for 30 min, and were then extracted twice against phenol and chloroform, and precipitated as before. DNA was then resuspended in TE at a concentration of approximately 1 mg ml^{-1}.

2.3.8 Preparation of genomic DNA. Mini-prep method

0.5 ml of late log-phase *L. mexicana* culture was harvested and washed twice in PBS. The cell pellet was then resuspended in 200 μl TELT buffer. Samples were incubated at room temperature for 10 min before extraction with H_2O equilibrated phenol. The samples were then extracted twice with H_2O equilibrated chloroform. The aqueous layer was removed and DNA was precipitated by the addition of 2 volumes of cold 100% ethanol and centrifugation in a microcentrifuge at 13,000 rpm, 4°C for 30 min. The DNA was washed with 70% cold ethanol, centrifuged as before, dried briefly at room temperature, and then resuspended in 50 μl TE buffer. RNAse A was added to a final concentration of 20 μg ml^{-1} and samples were incubated at 37°C for 30 min.

2.3.9 Southern blot transfer

L. mexicana DNA was digested overnight with *Hin*dIII at 37°C. Fragments were separated by electrophoresis through a 0.8% TBE agarose gel. The gel was stained with a 300 ng ml^{-1} solution of ethidium bromide. The gel was analysed under low power UV illumination to ascertain whether digestion had gone to completion and to obtain a photograph of the gel placed next to a ruler to allow a comparison of the position of hybridisin bands with the molecular weight markers. The gel was washed for 10 min in

distilled water, then incubated for 30 min with two changes of depurination solution. After brief rinsing with dH_2O the gel was incubated in two changes of denaturing solution for 30 min. The gel was again rinsed briefly before transfer into neutralisation solution with two changes over a 30 min period. Finally the gel was incubated in 10x SSC for 30 min. DNA fragments were transferred to Hybond-N, positively charged nylon membrane (Amersham) by capillary transfer (Sambrook et al., 1989).

2.3.10 Nucleic acid hybridisation

Nucleic acids were covalently cross-linked to Hybond -N membrane by exposure to 1200 J UV radiation in a UV crosslinker (Stratagene). The membrane was then incubated with Southern blot hybridisation solution at 65°C for 2 hours. Radiolabelled probe DNA was prepared by random primer labelling with the Prime-It II kit (Stratagene) according to the manufacturers instructions, using 30 ng of template DNA. Radiolabelled probe was purified on NucTrap® columns (Stratagene) and added to the hybridisation solution and the membrane was incubated overnight at 65°C. Excess radiolabel and non-specifically bound probe was removed by washing the membrane with three washes of 2x SSC/0.1% SDS at 65°C for 10 min each wash, followed by the same washing protocol using 0.2x SSC/0.1% SDS. The membrane was then sealed in polythene and exposed to Reflection™ autoradiography film (NEN™ Life Sciences). Film was developed using a Kodak X-omat automated developer.

2.3.11 bpV(phen) block and release

L. mexicana promastigotes were seeded at a density of $1x10^7$ cell ml^{-1} and incubated in the presence of 10 μM bpV(phen) (a kind gift of Dr. Martin Olivier, Laval university, Quebec, Canada) for 24 hours to block cell cycle progression. Cells were released from the block by centrifuging the cells at 2000g for 5 min in a Beckman S4180 swing bucket rotor. Cells were then resuspended in cold (4°C) PBS and the process was repeated. Cells were then resuspended in a volume of fresh medium equal to the original volume.

2.3.12 Flavopiridol block and release

L. mexicana promastigotes were seeded at a density of $1x10^6$ cell ml^{-1} and incubated in the presence of 0, 1.0, 2.5 or 5.0 μM flavopiridol (a kind gift of Dr. Swati Bal-Tembe, Hoechst Marion Roussel Ltd, Bombay, India) to block cells at the G2/M transition. Cells were released from the block by centrifuging the cells at 2000g for 5 min in a Beckman S4180 swing bucket rotor. Cells were then resuspended in either serum-free medium, complete medium, or cold (4°C) PBS and the process was repeated. Cells were then resuspended in a volume of fresh medium equal to the original volume.

2.3.13 DNA content analysis by flow cytometry

Mid-log phase *L. mexicana* promastigotes were harvested by centrifugation at 2000g, 4°C for 5 min. Cells were washed once in 10ml PBS. Samples were then fixed for at least one hour in 70% methanol/30% PBS. Prior to analysis, fixed cells were harvested by centrifugation at 1000g, 4°C for 10 min. Cells were washed in 10 ml PBS then resuspended in a further 1 ml PBS. RNAse A and propidium iodide were both added to final concentration of 10 μg ml^{-1} and cells were incubated at 37°C for 45 min. After

staining cells were analysed on a Coulter Epics/XL or a Becton Dickinson FACScalibur flow cytometer. 10,000 cells were analysed for each sample. Cell cycle distribution was determined using the ModFit LT software package.

2.3.14 SDS-PAGE

Proteins were separated by the discontinuous SDS-PAGE method (Laemmli, 1971). 12% acrylamide mini-gels (0.75 mm thickness) were cast, loaded and run using a Bio-Rad Mini-Protean II dual slab cell according to the manufacturers instructions. Rainbow markers (Amersham) were also run to allow the estimation of protein molecular weights.

2.3.15 Preparation of $p13^{suc1}$ beads

$p13^{suc1}$ protein, purified as described previously (Mottram and Grant, 1996; Grant *et al.*, 1998), was provided by Dr. Karen M. Grant. 5 mg ml^{-1} protein was cross-linked to AminoLink beads (Pierce) following the manufacturers protocol. Prepared beads were stored as a 50% slurry in 0.05% sodium azide at 4°C.

2.3.16 $p13^{suc1}$ Binding Kinase Assay

Cell pellets were resuspended at a density of $1x10^8$ cells ml^{-1} in LSGI. Samples were incubated on ice for 20 minutes, then subjected to centrifugation at 100,000g for 45 mins in a Beckman ultracentrifuge. After centrifugation the supernatant was diluted 1 in 3 with LSGI and 200 µl of the sample was incubated with 40 µl of p13 bead slurry for 1

Hr at 4°C. Beads were then washed with 5 ml LSG, 5 ml HSLS then 5 ml KAB. The beads were then resuspended in 1 ml KAB transferred to a 1.5 ml screw capped eppendorf tube and centrifuged at 13,000 rpm in a microcentrifuge. Samples were resuspended in 20 µl KAM and incubated at 30°C for 30 mins. Reactions were stopped by addition of 20µl SDS sample buffer and samples were boiled for 5 min before electrophoresis on a 12 % SDS-PAGE gel. Gels were dried under a vacuum, wrapped in cellophane and exposed to X-ray film or a phosphorimaging plate for quantification of relative radioctivity on a Fuji phosphorimager using MacBas v2.2 software.

2.3.17 Nickel NTA agarose selection and flavopiridol IC_{50} determination

Cell pellets were resuspended at a density of $1x10^8$ cells ml^{-1} in LSGI and incubated on ice for 30 min. Samples were then centrifuged at 100,000g for 45 min. at 4°C. Ni-NTA agarose beads were transferred to a 2 ml disposable plastic column (Pierce) and washed with 0.5 ml LSGI. The supernatant from the S100 lysate was diluted 1:1 with 100 mM imidazole to give a final concentration of 50 mM. Diluted supernatant was added to the column containing the Ni-NTA agarose beads and was incubated at 4°C on a tube roller for 1 hour. The column was then drained and washed with the following regime; 5ml LSG plus 50 mM imidazole, 5 ml HSLS, and 5 ml LS-T. Bound kinase was then eluted with 100 mM EDTA. The kinase was then bound to $p13^{suc1}$ beads as described previously. Beads were washed as described, aliquoted into screw-capped eppendorf tubes and assayed for kinase activity in the presence of a range of concentrations of flavopiridol.

2.3.18 DAPI staining and microscopy

Slides were prepared by spreading a 50 µl volume of cell suspension on a glass microscope slide. Slides were air-dried then fixed by incubation in 75% methanol/25% PBS at 4°C for 1 Hr. Slides were stained with DAPI by a 20 min incubation in a 20 µg ml^{-1} solution in PBS. Slides were mounted in Mowiol containing DABCO (anti-fading agent) (Sigma) and visualised under phase contrast and UV illumination using a Zeiss Axioplan microscope fitted with a CCD camera (Hamamatsu photonics). Images were captured on a Power-macintosh computer (Apple) using Openlab 2.0.2 software (Improvision).

2.4 Yeast methods

2.4.1 *Saccharomyces cerevisiae* strains

The genotypes of the *S. cerevisiae* strains used in this study are as follows:-

(1) *cdc28-1N, ade1, ura3, trp1, his2*

(2) *cdc28-4, ade1, ura3, leu2, trp1, his2*

(3) *cdc28-13, ade1, ura3, leu2, trp1, his2*

All of the above strains were the kind gift of Dr. S.I. Reed, Scripps Research Institute, La Jolla, California, USA.

2.4.2 *Saccharomyces cerevisiae* transformation

S. cerevisiae cells were transformed using the lithium acetate method (Ito *et al.*, 1983). 10 ml of YPD broth was inoculated with a single yeast colony isolated from a YPD agar plate. The culture was incubated at 25°C overnight. 1 ml of overnight culture was used

to inoculate 10 ml YPD and the culture was grown for three hours or until the OD_{600} value reached 0.6. Cells were pelleted by centrifugation at 2000g for 5 min. The pellet was resuspended in 10 ml dH_2O and cells were again centrifuged at 2000g for 5 min. The pellet was then resuspended in 1 ml TE/LiAc and transferred to an eppendorf tube. Cells were pelleted by centrifugation at 13,000rpm for 5 min. The pellet was then resuspended in 30 µl TE/LiAc and the following components were added; 5 µg sheared Herring sperm DNA, 5 µg plasmid DNA, and 250 µl 40% PEG 6000 in TE/LiAc. Samples were incubated at 25°C for 30 min. then heat-shocked at 37°C for 15 min. Cells were then pelleted by centrifugation at 13,000rpm for 5 min. Pellets were washed in dH_2O to remove excess PEG, then resuspended in 200 µl dH_2O and plated onto YNB-agar plates containing the appropriate amino acids for plasmid selection. Plates were sealed with parafilm and incubated at 25°C to allow colony formation (approximately 4 days).

2.4.3 *S. cerevisiae* cell lysates

10 ml YPD broth was inoculated with a colony selected from an agar plate and grown overnight at 25°C. Cells were pelleted by centrifugation at 200g for 5 min. Pellets were washed once with dH_2O and cells were transferred to an eppendorf tube. Pellets were resuspended in 100 µl breaking buffer and an equal volume of acid washed glass beads (0.5 mm diameter) was added (Sigma). Samples were vortexed for 30 seconds, then incubated on ice for 30 seconds. This process was repeated eight times. Samples were centrifuged at 13,000rpm for 10 min. The supernatant was removed, mixed with 4x SDS-PAGE sample loading buffer, and boiled for 10 min.

2.4.4 Western blot transfer

Proteins, separated by SDS-PAGE, were transferred to polyscreen PVDF membrane (NEN™ Life Sciences) by electroblotting (Towbin *et al.,* 1979) using a Bio-Rad mini transblot cell according to the manufacturers instructions.

After transfer the membrane was soaked in methanol for 5 min. then washed in TBST (Tris buffered saline with 0.1% Tween 20) for 5 min. The membrane was then incubated in blocking reagent (BLOTTO) overnight at room temperature in a 50 ml falcon tube, on a tube roller. This causes protein to bind to sites on the membrane that are not yet protein bound. This prevents the non-specific binding of proteins during the subsequent antibody incubation steps.

2.4.5 Antibody hybridisation

Anti-CRK3 antibody (Grant *et al.,* 1998) was added at a titre of 1 in 500 in BLOTTO and the membrane was incubated at room temperature for 2 hours in the presence or absence of 2 $\mu g\ ml^{-1}$ competing peptide. The blot was washed for 30 min. with three changes of TBST on a shaking platform and probed with a mouse anti-rabbit IgG HRP conjugate (Promega) secondary antibody at a titre of 1 in 10,000 for 45 min. The blot was washed for 30 min. in three changes of TBST. Hybridising antibody was detected using the Supersignal™ CL-HRP substrate chemiluminescent detection system (Pierce). Signal was detected by autoradiography with Reflection™ autoradiography film (NEN™ Life Sciences). Film was developed using a Kodak X-omat automated developer.

2.5 Buffers and reagents

Ampicillin: 100 mg ml^{-1} stock in 50% ethanol. Stored at -20°C.

BLOTTO: 5% non-fat dried milk, 5% heat inactivated horse serum (Life technologies) in TBS. Stored at 4°C.

bpV(phen): 10 mM stock in PBS. Stored at -20°C.

Breaking buffer: 50 mM sodium phosphate pH7.4, 1 mM EDTA, 5% glycerol, protease inhibitors; 1 mM 1,10 phenanthroline, 5 μg ml^{-1} pepstatin A, 100 μg ml^{-1} leupeptin, 50 μg ml^{-1} pefabloc SC (Boerhinger Mannheim). Stored at 4°C.

Depurination solution: 0.125M HCl. Stored at room temp.

Denaturing solution: 1.5M NaCl, 0.5M NaOH. Stored at room temp.

Denhardt's solution (50x): 1% (w/v) BSA, 1% (w/v) ficoll, 1% (w/v) polyvinyl pyrolidine. Stored at -20°C.

DNA loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose in dH_2O. Store at 4°C.

Electroporation buffer: 21 mM HEPES pH7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM phosphate buffer, 5 mM glucose. Stored at 4°C.

Ethidium Bromide: 10 mg ml^{-1} stock in distilled water. Stored at room temp.

Flavopiridol: 10 mg ml^{-1} stock in 100% ethanol. Stored at -20°C.

HSLS (high salt lysis solution): 50 mM MOPS pH7.2, 500 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100. Stored at 4°C.

KAB (kinase assay buffer): 50 mM MOPS pH7.2, 20 mM $MgCl_2$, 2 mM DTT, 10mM EGTA. Stored at 4°C.

KAM (kinase assay mix): 5 µl histone H1 (10 mg ml^{-1} stock) (BRL), 8 µl ATP (100 µM stock), 186 µl KAB, 1 µl γ-^{32}P-ATP (50 µCi). Stored at 4°C.

LELSG (low EDTA lysis solution plus glycerol): 50 mM MOPS pH7.2, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% Triton X-100, 10% glycerol, 10 mM NaF, 1 mM Na orthovanadate. Stored at 4°C.

LELSGI (LELSG plus inhibitors): As LELSG plus protease inhibitors; 1 mM 1,10 phenanthroline, 5 µg ml^{-1} pepstatin A, 100 µg ml^{-1} leupeptin, 50 µg ml^{-1} pefabloc SC (Boerhinger Mannheim). Stored at 4°C.

Luria-Bertani (LB) agar: As LB broth with 0.8% (w/v) agar (Difco). Autoclaved and stored at room temp.

Luria-Bertani (LB) broth: 1% bactotryptone (Difco), 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl in distilled water. Sterilised by autoclaving and stored at room temp.

LS (lysis solution): 50 mM MOPS pH7.2, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 1 mM Na orthovanadate. Stored at 4°C.

LSG (lysis solution plus glycerol): 50 mM MOPS pH7.2, 100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 10% glycerol, 10 mM NaF, 1 mM Na orthovanadate. Stored at 4°C.

LSGI (LSG plus inhibitors): As LSG plus protease inhibitors; 1 mM 1,10 phenanthroline, 5 $\mu g\ ml^{-1}$ pepstatin A, 100 $\mu g\ ml^{-1}$ leupeptin, 50 $\mu g\ ml^{-1}$ pefabloc SC (Boerhinger Mannheim). Stored at 4°C.

Lysozyme: 5 $mg\ ml^{-1}$ stock in 50 mM Tris-HCl pH 7.4. Prepared fresh prior to use.

Mowiol: 6 g glycerol, 2.4 g Mowiol 4-88 (Calbiochem) in 6 ml dH_2O. 12 ml 0.2M Tris-HCl (pH8.5) added and mixture incubated on shaking platform for half a day. The mixture is then left for 2 hours, incubated at 50°C for 10 min then centrifuged at 5000g for 15 min. DABCO (Sigma) was added to a final concentration of 0.1% (w/v) and the supernatant was aliquoted and stored at -20°C.

Neutralisation solution: 1.5M NaCl, 0.5M Tris-HCl pH7.0. Stored at room temp.

PBS: 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH7.4. Autoclaved and stored at room temp.

PCR mix (11.1x): 45 mM Tris-HCl (pH8.8), 11 mM ammonium sulphate, 4.5 mM $MgCl_2$, 6.7 mM β-mercaptoethanol, 4.4 mM EDTA (pH8.0), 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP and 113 μg ml^{-1} BSA. Stored at -20°C.

40%PEG/TE/LiAc: 40% PEG 6000 in 10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0, 100 mM Lithium acetate. Store at room temp.

RNase A: 10 mg ml^{-1} stock in sterile water. Stored at -20°C.

SDS-PAGE sample loading buffer: 200 mM Tris-HCl pH6.8, 400 μM β-mercaptoethanol, 8% SDS, 40% glycerol, a few crystals of bromophenol blue. Stored at room temp.

SOC medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 0.4% (w/v) glucose. Glucose added after autoclaving. Stored at room temp.

Southern blot hybridisation solution: 6X SSC, 0.1 % SDS, 0.1 % Na pyrophosphate, 10X Denhardts, 100 μg ml^{-1} sheared herring sperm DNA. Stored at room temp.

SRB: 50 mM Tris-HCl pH8.0, 50 mM EDTA pH 8.0, 25% (w/v) sucrose. Stored at room temp.

SSC: 3M NaCl, 0.3M tri-sodium citrate pH 7.0. Stored at room temp.

TAE: 40 mM Tris-acetate, 1 mM EDTA. Stored at room temp.

TBE: 0.9M Tris-HCl, 0.9M Boric acid, 25 mM EDTA. Stored at room temp.

TBS: 20 mM Tris, 137 mM NaCl pH7.6. Stored at room temp.

TBST: 20 mM Tris, 137 mM NaCl pH7.6, 0.1% Tween 20. Stored at room temp.

TE buffer: 10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0. Stored at room temp.

TE/LiAC: 10 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0, 100 mM Lithium acetate. Store at room temp.

TELT: 50 mM Tris-HCl pH 8.0, 62.5 mM EDTA pH 9.0, 2.5M LiCl, 4% Triton X-100. Stored at room temp.

Tetracycline: 10 mg ml^{-1} stock in 100% ethanol. Stored at -20°C.

TFG I: 30 mM Potassium acetate, 100 mM rubidium chloride, 10 mM $CaCl_2$, 50 mM manganese chloride, 15% (v/v) glycerol, pH 5.8 with dilute acetic acid. Filter-sterilised and stored at 4°C.

TFG II: 10 mM MOPS, 75 mM CaCl2, 10 mM Rubidium chloride, 15% (v/v) glycerol, pH 6.5 with dilute NaOH. Filter-sterilised and stored at 4°C.

Triton mix: 50 mM Tris-HCl pH8.0, 62.5 mM EDTA pH8.0, 1% Triton X-100. Stored at room temp.

Western blot transfer buffer: 5 mM Tris, 2 mM glycine, 20% methanol in distilled water. Stored at 4°C.

YNB agar: 0.67% (w/v) yeast nitrogen base (without amino acids), 2.0% (w/v) glucose, 2.0% (w/v) bacto-agar (Difco). Supplemented with appropriate amino acids, autoclaved, and stored at room temp.

YNB broth: 0.67% (w/v) yeast nitrogen base (without amino acids), 2.0% glucose (w/v). Supplemented with appropriate amino acids, autoclaved, and stored at room temp.

YPD agar: 1.0% (w/v) yeast extract, 1.0% (w/v) bacto-peptone, 2.0% (w/v) glucose, 2.0% (w/v) bacto-agar (Difco). Supplemented with appropriate amino acids, autoclaved, and stored at room temp.

YPD broth: 1.0% (w/v) yeast extract, 1.0% (w/v) bacto-peptone, 2.0% (w/v) glucose. Supplemented with appropriate amino acids, autoclaved, and stored at room temp.

Oligonucleotide	Sequence
OL322	GGCCATGGCTTCGTTTGGCCGTGTG
OL323	GCGGATCCCTACCAACGAAGGTCGCTG
OL324	CCTGCACGAATGCAGAAAGTGATA
OL326	GCAGATCTCCCGGGCAGTTGTTTGAGAT
OL327	GCAGATCTCCCGGGCAGTTGTTTGAGAT
OL382	GCTCAGGGCAATGTTCAG
OL383	TTGTAGCACGGAGAAAGG

Table 2.1 List of oligonucleotides

CHAPTER 3

Targeted gene disruption of *Leishmania mexicana CRK3*.

3.1 Introduction

A number of molecular genetic tools exist for the analysis of gene function in *Leishmania* (Beverley and Turco, 1998). Genes can be targeted for disruption to generate null cell lines for phenotypic analysis (Mottram *et al.*, 1996b). Expression of modified or mutated genes can also prove valuable in determining the function of a given protein (Mottram *et al.*, 1997). Transient transfection with a reporter gene can be used to screen for sequences with promoter activity, (Uliana *et al.*, 1996; Gay *et al.*, 1996) or for sequences involved in mRNA processing (Curotto de Lafaille *et al.*, 1992; Ramamoorthy *et al.*, 1996). Gene disruption experiments can also be used to determine whether a given gene is essential (Cruz *et al.*, 1993) and can therefore prove important in identifying and validating potential drug targets (Barrett *et al.*, 1999).

A family of cdc2-related kinases (CRKs) exist in the trypanosomatids (Mottram and Smith, 1995), two of which have been found in *Leishmania* (CRK1 and CRK3). These kinases are highly related to the cyclin dependent kinase family of serine threonine protein kinases that appear to be ubiquitous throughout the eukaryotes. The primary role of the CDKs is to regulate and co-ordinate progression through the cell cycle with growth signals, transcription, and differentiation (Nigg, 1995; Gao and Zelenka, 1997). Activity of the CDKs is regulated by reversible phosphorylation of a number of highly conserved residues (Lew and Kornbluth, 1996), and binding to regulatory proteins such as cyclins, CDK inhibitors or homologues of the fission yeast $p13^{suc1}$ protein (Andrews and Measday, 1998; Vogel and Baratte, 1996).

Studies of the *CRK1* gene of *L. mexicana* have indicated that the gene is essential in promastigotes (Mottram *et al.,* 1996a), and encodes a stage-regulated kinase that is present in all three life cycle stages, but is active only in log-phase and stationary phase promastigotes (Mottram *et al.,* 1993).

The *CRK3* gene also encodes a stage regulated kinase which is active only in the proliferative promastigote and amastigote stages of the life cycle, and is absent from the cell-cycle arrested metacyclic stage (Grant *et al.,* 1998). CRK3 also binds to $p13^{suc1}$, suggesting that it may be the functional homologue of the fission yeast cdc2 gene, which plays a key role in signalling entry into mitosis (Nurse, 1990).

To gain a better understanding of the possible role of CRK3, attempts were made to use the available molecular genetic techniques to do the following: (1) to test whether the *CRK3* gene was essential and therefore a potential drug target and, (2) to express a hexa-histidine tagged version of the kinase in a null background to allow the purification and analysis of active or inactive kinase complex from each life-cycle stage.

3.2 RESULTS

3.2.1 Gene disruption of first and second alleles of *CRK3*

Targeted gene disruption experiments are informative in that they can elucidate the function of a particular gene or confirm whether or not a gene is essential. If the gene is essential it is not possible to obtain viable, null mutant parasites lacking a copy of the targeted gene. If, on the other hand, the gene is not essential then it is possible to obtain null mutants which can then be analysed for phenotypic changes from wild type. Such phenotypic analysis can provide valuable functional data on the gene of interest. In

order to gain more information on the possible function of the *CRK3* gene of *Leishmania mexicana* we designed gene targeting constructs based on those previously used for gene disruption of the *LmCPA*, *LmCPB* and *LmCPC* genes (Souza *et al.,* 1994; Mottram *et al.,* 1996b; Bart *et al.,* 1997). Two targeting constructs were created; these confer resistance to hygromycin and phleomycin respectively (see Fig 3.2). The extreme 5' end of the construct consists of the 1.2 kb *Hin*dIII/*Sal*I fragment of plasmid pGL89, which contains the 5' flank and 130 bp of the *CRK3* ORF. The 3' end of the targeting construct consists of the 339 bp *SmaI/Bgl*II fragment of plasmid pGL89. These flanking regions allow integration, and hence disruption, of the *CRK3* locus.

The original wild type cells used in this study were harvested as amastigotes from a mouse lesion. Amastigotes were then transformed in vitro into promastigotes by incubation at 25°C in HOMEM medium, supplemented with heat inactivated foetal calf serum (FCS). These were designated sub-passage 1 (sp1). Cells for transfection were used no later than sp7 and were derived from low sub-passage stabilate stocks. The *Hin*dIII/*Bgl*II insert from pGL105 (*CRK3::HYG*) was separated by agarose gel electrophoresis and recovered from the gel by purification using Spin-X columns (Costar). $4x10^7$ mid log-phase promastigotes of a wild type *Leishmania mexicana* culture were transfected using standard conditions, with 10 μg of the 4.8 kb *CRK3::HYG* targeting construct. Transfected clones were selected on solid HOMEM plates containing 50 μg ml^{-1} hygromycin. At this concentration no colonies were obtained for cells which had been electroporated in the absence of any DNA construct. Several colonies were selected from the plates and cultured in liquid medium in the presence of 50 μg ml^{-1} hygromycin. One of these clones, W583 (See Table 3.1) was cultured and prepared for introduction of the second targeting fragment conferring resistance to phleomycin. The *Hin*dIII/*Bgl*II insert from the *CRK3::BLE* construct,

pGL97, was digested and gel-purified in an identical way to the *CRK3::HYG* construct, and was introduced into both the W583 clone and wild type *L. mexicana* by electroporation. Drug resistant *L. mexicana* clones were selected by growth on solid medium plates containing 10 μg ml^{-1} phleomycin and 50 μg ml^{-1} hygromycin; or 10 μg ml^{-1} phleomycin alone. Two events could be envisaged as a result of this experiment; integration of the second knockout construct could occur at the remaining *CRK3* allele, resulting in a null mutant that is resistant to both hygromycin and phleomycin. Alternatively, the second construct may integrate at the previously disrupted locus, replacing the *HYG* gene with the *BLE* gene.

Only two clones were obtained for the transfection of the hygromycin resistant W583 clone; these were named W625 and W626 respectively. A number of clones were obtained on the plates containing phleomycin alone. One of these clones, W585, was selected for further analysis.

DNA was prepared from 10 ml of stationary phase promastigote cultures of wild type, W585, W583, W625 and W626 clones. 50 ng of DNA was used as the template in a PCR reaction with the primers OL322 and OL323 which anneal to the 5' and 3' end of the *CRK3* gene and anneal to 5' and 3' positions of the *CRK3::HYG* and *CRK3::BLE* constructs (see Fig.3.2). These primers amplify the complete *CRK3* ORF of 800 bp when the wild type gene is present and amplify a 3.8 kb PCR product from the locus in which the *CRK3::HYG* construct has integrated, and a 3.1 kb product from the locus in which the *CRK3::BLE* construct has integrated (see Fig. 3.2). Amplified PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and the resulting gel was photographed (Fig. 3.3). For wild type DNA an 800bp amplified fragment corresponding to the *CRK3* gene was detected (lane 1). The integrated *CRK3::HYG* and *CRK3::BLE* constructs were detected in the W583 and W585

heterozygote mutants (3.1 kb and 3.8kb fragments, lanes 2 and 3 respectively), as was the 800 bp wild type *CRK3* PCR product. This indicates that both constructs are present. Whether they have integrated at the correct locus cannot be determined from these results alone. Both of the clones derived from two rounds of transfection, which were found to be resistant to both hygromycin B and phleomycin, tested positive for the presence of *CRK3::HYG* and *CRK3::BLE* as predicted (lanes 4 and 5). However, it is also clear that a fragment corresponding to the wild type *CRK3* allele is still present in both of these clones. Several possibilities could account for this finding: (1) The introduced targeting constructs may not have integrated at the correct locus. (2) The original cell line used may have been triploid or aneuploid. (3) Cross contamination of PCR samples may have occurred. (4) Changes in ploidy may have occurred as has been described previously in *Leishmania* transfectants (Cruz *et al.*, 1993).

To further investigate these possibilities Southern blot analysis of genomic DNA was performed. Five μg of genomic DNA from wild type or mutant parasites was digested with *Hin*dIII, separated on an agarose gel, transferred to Hybond-N nylon membrane (Amersham) and probed with a radiolabelled 2.0 kb *Hin*dIII insert from plasmid pGL89. The results confirm those of the PCR analysis and indicate that the *CRK3::HYG* and *CRK3::BLE* constructs have integrated into the correct locus. A 2.0 kb *Hin*dIII fragment containing the wild type *CRK3* gene was detected in DNA prepared from wild type parasites (Fig. 3.4 lane 1). In the DNA prepared from the W583 or W585 heterozygotes a 4.8 kb and 4.1 kb fragment corresponding to the integrated *CRK3::HYG* or *CRK3::BLE* constructs were detected, as well as the 2.0 kb fragment containing the *CRK3* gene (lanes 2 and 3). This verified that these clones were indeed heterozygous at the *CRK3* locus. Clone W625 was found to contain both the 4.8 kb and 4.1 kb fragments indicating that both the *CRK3::HYG* and *CRK3::BLE* constructs have been targeted to

the correct locus, however the wild type gene was found to be still present (lane 4). Clone W626 showed an identical pattern of hybridising fragments to W625 (data not shown).

To analyse the DNA content of the mutant cell lines, fluorescence activated cell sorting (FACS) analysis was performed. Mid log phase promastigotes were fixed in methanol, washed and resuspended in phosphate buffered saline (PBS) then stained with propidium iodide, and incubated with 10 μg ml^{-1} RNAse A for 30 minutes. These samples were then analysed on an EPICS/XL flow cytometer (Coulter) in order to determine the overall DNA content. As the DNA of the cells was stained with the DNA intercalating dye propidium iodide, which fluoresces under excitation from a light source, then the level of fluorescence emitted from an individual cell after excitation by the laser source gives a direct indication of the DNA content of that cell. A higher level of fluorescence indicates a greater number of intercalated propidium iodide molecules and hence an increased DNA content. Another useful feature of the flow cytometer is that it allows the quantification of the DNA content of a large sample population, in this case 10,000 individual cells. The flow cytometry results were plotted as histograms of cell numbers versus fluorescence intensity (proportional to DNA content), see Fig. 3.5. Panel A shows the results for wild type parasites. There are two major peaks. These correspond to cells with either a 2N or 4N DNA content (a DNA content of 1N is equivalent to a haploid genome). The 2N peak corresponds to cells in the G1 phase of the cell cycle and the 4N peak is due to cells which are in the G2 phase of the cell cycle and have replicated their DNA but have not yet undergone cell division. The DNA content of the heterozygote mutant clones W583 and W585, panels B and C respectively, was found to be the same as for wild type parasites, with the two peaks corresponding to a 2N or 4N DNA content. The DNA content of the hygromycin and

phleomycin double -resistant clones obtained from two rounds of transfection, W625 and W626, was found to be double the normal DNA content, with a G1 content of 4N and a G2 content of 8N, indicating that these cells are aneuploid (Panels D and E). This change in ploidy has clearly occurred during the introduction of, and selection for, the second knockout construct, as happened when the *CRK1* gene was targeted for disruption (Mottram *et al.,* 1996a). The uneven nature of the DNA content histograms for the aneuploid clones W625, and W626, with a less distinct separation into two clear peaks, suggests that there may be some variability in the DNA content of these cells and they may not be completely tetraploid. The mechanism of such ploidy changes upon gene targeting is unknown at present but some possible explanations are provided in section 3.3.1.

3.2.2 Introduction of an episomal copy of *CRK3* followed by gene disruption

To test the hypothesis that the *CRK3* is an essential gene and that the ploidy changes result from the selection for maintenance of at least one gene copy, an attempt was made to express the gene from an episome prior to disrupting the remaining *CRK3* allele. In this instance it should be possible to disrupt both alleles without causing any changes in ploidy as the cells will still retain copies of the essential gene on an episome.

The *CRK3* gene was excised from plasmid pGL89 by digestion with *EcoRI/Hin*dIII and ligated into *EcoRI/Hin*dIII digested pTEX vector to give plasmid pGL100 (see Fig 3.6). The pTEX vector was designed for stable episomal expression in *Trypanosoma cruzi*; it contains upstream and intergenic regions of the glycosomal glyceraldehyde-3-phosphate dehydrogenase (g*GAPDH*) gene array which provide splice leader addition, and polyadenylation sites for production of mature RNAs. Although these sequences are

derived from *T. cruzi* they have been shown to function in various species of *Leishmania* and the pTEX vector has been used to express a number of genes including, trypanothione reductase (Tovar *et al.,* 1998), cysteine proteinases (Mottram *et al.,* 1997) and the *CRK1* gene in *L. mexicana* (Mottram *et al.,* 1993).

20 μg of the pGL100 plasmid was introduced by electroporation into the W583 cell line, and cells were plated onto solid HOMEM plates containing 50 μg ml^{-1} Geneticin (G418) and 50 μg ml^{-1} hygromycin B. Only a small number of clones were recovered from several attempts. One of these clones W638 was selected for further analysis. It is not clear why so few resistant clones were obtained from several experiments; but problems have been encountered in other laboratories where Geneticin and hygromycin were used to select at the same time (see below). The W638 clone was grown in liquid culture and tested for resistance to higher concentrations of Geneticin. Cells were resistant up to 500 μg ml^{-1}, the highest concentration used, with no noticeable adverse effects. It is not clear, however, whether the increase in episome copy number that occurred upon increasing the drug selection, resulted in a concomitant increase in expression of the *CRK3* gene.

The W638 clone was shown to contain an episomal copy of the *CRK3* gene and one remaining wild type allele. In theory, therefore, it should be possible to disrupt the second wild type allele, to create a null mutant background, without a resultant change in ploidy, as the essential *CRK3* gene would be expressed from the episome. This would confirm that both wild type alleles could be disrupted by the construct developed for this study. The standard electroporation procedure was used to introduce 10 μg of gel purified *CRK3::BLE* targeting fragment into the W638 cell line. These cells were then plated in triplicate on solid media containing 10 μg ml^{-1} phleomycin, 50 μg ml^{-1}

hygromycin and 50 μg ml^{-1} geneticin. The experiment was repeated a number of times without any success. One possibility is that the level of expression of *CRK3* from the episome is too high and cannot therefore complement for loss of the two *CRK3* alleles. Another possibility is that the triple drug selection used places cells under too much stress, or it may be that the three drugs used are not compatible. It should be noted however that this combination of drugs was successfully used to generate *CRK1* nulls expressing the *CRK1* gene from a pTEX episome (Mottram *et al.,* 1996a). Subsequently it was found that several investigators have encountered problems using the *NEO* and *HYG* genes in combination for drug selection. These problems may arise from the fact that both genes encode phosphotransferase enzymes, and the drugs themselves target and disrupt the machinery of protein synthesis. Possible 'crosstalk' or synergistic effects between the two selection mechanisms may make the selection procedure used in this study difficult.

3.2.3 Co-transfection of the *CRK3* episome with the second targeting construct

Because the *CRK3* gene was thought to be essential, it was hypothesised that there should be no need to initially select for the episome with Geneticin, as it should be possible to perform a co-transfection experiment, in which the episomal *CRK3* gene would be introduced simultaneously with the second targeting fragment. If *CRK3* is indeed essential, then this will provide a selection pressure for the maintenance of the episome. W583 cells were transfected with 20 μg plasmid pGL100 and 10 μg gel purified *CRK3::BLE* targeting fragment. Transfected cells were plated onto solid media containing 50 μg ml^{-1} hygromycin and 10 μg ml^{-1} phleomycin. Assuming *CRK3* was an essential gene, under this selection protocol one would expect to obtain two classes of

mutants; aneuploid cells that have integrated the *CRK3::BLE* cassette only, as described previously, and diploid cells which have integrated the *CRK3::BLE* cassette into the second *CRK3* wild type allele and have also taken up the episome, thereby retaining a functional *CRK3* gene. This combination of events would be expected to occur at a low frequency.

Clones derived from plates were transferred to liquid medium and grown for further analysis. Each clone was tested for resistance to Geneticin as before, by transferring approximately 200 µl of mid-log cell culture into 10 ml liquid medium containing 50 µg ml^{-1} Hygromycin, 10 µg ml^{-1} Phleomycin and 50 µg ml^{-1} Geneticin. Clones were also analysed for DNA content by FACS. Fig 3.7 shows the combined results of the analysis by FACS and geneticin resistance. Panels A corresponds to the Geneticin-sensitive wild type strain. Only one clone resistant to Geneticin was recovered, clone W635. This was found to be diploid (panel B). The majority of the geneticin sensitive clones were aneuploid, a representative FACS histogram is shown in panel D, clone W634. These aneuploid clones are most likely due to integration of the *CRK3::BLE* fragment without concomitant uptake of the *CRK3* episome, resulting in the characteristic ploidy changes seen previously upon disruption of the second *CRK3* allele (See section 3.2.1). One of the Geneticin sensitive clones W633 was diploid (panel C). To test the possibility that the W633 clone may contain the *CRK3* episome but was not resistant to geneticin due to some toxic side effect of overexpression, PCR analysis was performed on 50 ng of purified DNA using primers OL324 and OL323 (Table 2.1). This primer combination should specifically detect the presence of the pGL100 episome containing *CRK3* and not the wild type *CRK3* gene, as the OL324 primer is homologous to vector sequence which should only be present in the pTEX episome and is unlikely to be present in the *L. mexicana* genome. The results obtained from this analysis are shown in Fig. 3.8.

When wild type DNA was used as the template in the reaction there was, as predicted, no amplified DNA fragment produced (lane 1). When DNA from the Geneticin resistant clone W635 is analysed, an amplified fragment was produced (lane 2). This fragment corresponds in size to the fragment amplified from the plasmid alone (lane 5). The absence of an amplified fragment in the DNA from the W633 clone which is Geneticin sensitive, but is still diploid, suggests that the *CRK3* episome is not present in this cell line (lane 4). To further investigate whether the W633 clone still retained a copy of *CRK3* Southern blot analysis was performed as described previously in section 3.2.1. The results of this analysis are shown in Fig. 3.9. Lane 1 shows hybridisation to the 2.0 kb fragment containing the wild type *CRK3* allele in DNA from wild type parasites. Lanes 2, 3 and 4 correspond to DNA from heterozygote mutant clones W583, W585 and the aneuploid clone W625 described in section 3.2.1. The 2.0 kb fragment which indicates the presence of an intact wild type *CRK3* allele is still present in the W633 clone (lane 6), even although this clone is diploid and resistant to both Hygromycin and Phleomycin. It is clear however that the *CRK3::BLE* targeting fragment has not integrated at the correct locus as the characteristic 4.1 kb band is not present. It is possible that the introduced *CRK3::BLE* fragment has formed an episome as occurred during a similar study on *L. mexicana CRK1* (Mottram *et al.,* 1996a). Alternatively, the *CRK3::BLE* targeting fragment may have integrated at another locus, hence the 6.5 kb band seen in lane 6.

The Geneticin resistant clone W635 does not seem to contain a wild type copy of *CRK3* as judged by the absence of the 2.0kb DNA fragment containing the *CRK3* gene (lane 5). However, of the expected 4.8 and 4.1 kb fragments corresponding to the integrated *CRK3::HYG* and *CRK3::BLE* fragments, only the 4.1 kb *CRK3::BLE* fragment is present. The absence of the 4.8 kb *CRK3::HYG* fragment is puzzling as the original cell

line used in the transfection, W583, was a heterozygote mutant containing an integrated *CRK3::HYG* construct (4.8 kb fragment, Lane 2). Furthermore the W633 clone is still resistant to Hygromycin. The absence of the 4.8 kb *CRK3::HYG* fragment suggests that a recombination event has occurred, resulting in translocation of the integrated *CRK3::HYG* construct to another part of the genome. This possibility would need further investigation which is outwith the scope of this study. It is clear from this experiment that the approach taken still does not allow the disruption of the second genomic copy of the *CRK3* gene in the manner expected. Co-transfection with an episome and with a targeting construct, followed by triple drug selection results in unforeseen effects that complicate the analysis of clones obtained.

The problems encountered in this approach to gene disruption may be due to the vector being used. It is possible that the processing signals present in the pTEX vector are not sufficient for the proper processing of the *CRK3* gene, due to some unknown gene specific phenomenon. It has become clear from work performed in a number of laboratories that not all genes can be disrupted or re-expressed in the same way and careful consideration must be given to the design of experiments aimed at disrupting a given gene. It is feasible that flanking regions of a given gene may be as important as the ORF itself in some critical aspect of the gene function, indeed it seems certain that this must be the case in *Leishmania* where flanking DNA has a critical effect on mRNA stability. For example, for control of expression at particular points in the cell cycle or control of mRNA levels (Hines and Ray, 1997; Brown and Ray, 1997). The use of heterologous flanking DNA, controlling expression of any gene expressed from an episome may result in an unusually stable or unstable mRNA, this may not affect the expression of some genes but could conceivably affect the expression of genes whose mRNA levels are critically important.

3.2.4 Episomal expression of *CRK3* followed by second round gene disruption in the absence of Geneticin selection

As a final attempt to disrupt both alleles of *CRK3* an attempt was made to integrate the *CRK3::BLE* targeting fragment into the W638 heterozygote mutant, which has only one intact wild type *CRK3* allele and which also contains the pGL100 episome (Fig. 3.5). This experiment had been attempted three times previously with no success (section 3.2.2). In this instance however, the drug selection protocol was altered. Cells were electroporated with 10 μg of gel purified *CRK3::BLE* targeting fragment. Transfectants were plated and selected in the presence of 50 μg ml^{-1} hygromycin and 10 μg ml^{-1} phleomycin in the absence of Geneticin. This selection protocol should avoid any problems associated with the triple drug selection, whilst allowing the retention of the *CRK3* episome, as this is the only source of a functional *CRK3* gene if the *CRK3::BLE* targeting fragment integrates correctly, disrupting the single remaining wild type allele. Five clones were selected for analysis and all were found to be resistant to Geneticin. These clones (W1187 to W1191) were analysed by FACS to determine their DNA content (Fig. 3.10). All five clones were shown to have a normal diploid DNA content (Panels C to G). Panel A corresponds to the diploid, wild type control cells and panel B to the W638 cell line. DNA was prepared from each of the mutant clones for Southern blot analysis which was performed as described (section 3.2.1). The results are shown in Fig. 3.11. Lane 1 is wild type parasite DNA, with a single 2.0 kb fragment containing the wild type *CRK3* locus. Lanes 2 and 3 are the W585 and W583 heterozygote mutants with two fragments corresponding to one intact allele (the 2.0 kb band) and one disrupted allele (the 4.1 kb *CRK3::BLE*, or 4.8 kb *CRK3::HYG* band). The heterozygote

mutant W638 used in this experiment, which contains the pGL100 episome is represented in lane 4. This clone contains both the 2.0 kb wild type allele and the 4.8 kb *CRK3::HYG* disrupted allele. The *CRK3::HYG* allele is partially obscured by the hybridisation to the pGL100 plasmid bands which range from 5.0 to 10.0 kb. Lanes 5 to 9 show the DNA of the clones W1187 to W1191. All but one of these mutant clones (W1190, lane 8) lack the 2.0 kb wild type *CRK3* fragment but do contain the 4.1 kb and 4.8 kb fragments indicative of integration of the *CRK3::HYG* and *CRK3::BLE* targeting constructs (lanes 5, 6, 7, and 9). These clones also contain the 5.0 to 10 kb bands due to the presence of the pGL100 episome. These results suggests that these clones lack a genomic copy of *CRK3* due to disruption of both alleles, and that *CRK3* is being expressed from the pTEX episome. Clone W1190 still contains the 2.0 kb wild type allele, even though both the *CRK3::HYG* and *CRK3::BLE* constructs are present (lane 8). This clone is diploid (see Fig 3.9, panel E) so the presence of an 'extra' *CRK3* allele is not due to ploidy changes such as those discussed previously (section 3.2.1 and 3.2.2), but may be due to a recombination event or the duplication of an individual chromosome.

3.2.5 Expression of an inactive CRK3 kinase followed by gene disruption results in changes in ploidy

In order to aid the analysis of the *CRK3* gene we generated a cell line expressing a modified, epitope tagged CRK3 protein. The epitope tag consists of six histidine residues. This epitope can be recognised by a specific monoclonal antibody and also allows purification of the recombinant protein by affinity chromatography with Nickel NTA agarose. This system has been used to express a his-tagged version of the *L.*

mexicana CRK1 gene, allowing purification and analysis of kinase activity (Mottram *et al.,* 1993). It was thought that the $p13^{suc1}$-binding kinase activity of *Leishmania* was encoded by the *CRK3* gene. Production of a cell line expressing only epitope tagged CRK3 would allow the verification of this hypothesis. To do this a pTEX based construct containing a *CRK3*his sequence (pGL320) was introduced into the *CRK3::HYG* heterozygote cell line, W583. The *CRK3*His sequence consists of the *CRK3* ORF fused to a short sequence encoding six histidine residues which would allow the production of a his-tagged CRK3 fusion protein. 20μg of the pTEX*CRK3*his construct, pGL320, was introduced into the W583 heterozygote mutant by the standard electroporation method, and clones were selected on solid media containing 50 μg ml^{-1} hygromycin B and 25 μg ml^{-1} Geneticin. Of the clones isolated, one was selected, grown in liquid medium, prepared and transfected with the *CRK3::BLE* targeting construct. Transfectants were selected for resistance to 50 μg ml^{-1} hygromycin and 10 μg ml^{-1} phleomycin. Clones derived on solid media were transferred to liquid media and tested for resistance to 50 μg ml^{-1} Geneticin. Two independent clones were prepared as before for DNA content analysis by FACS. Both clones were resistant to all three antibiotics and FACS analysis indicated that they were both aneuploid (Fig 3.12 panels B and C) and therefore retain functional copies of the *CRK3* gene. Analysis of purified *CRK3*his from the W1033 cell line showed that the modified kinase was inactive (Dr. K.M. Grant, personal communication). Subsequent sequence analysis of the plasmid indicated that rearrangement had occurred. The results described in 3.2.4 and 3.2.5 prove that the ability to disrupt both *CRK3* alleles relies on prior introduction of an episome expressing a functional CRK3 enzyme.

3.2.6 Expression of a modified *CRK3*His gene and generation of null mutants using a pX-based construct

As a second attempt to generate a null mutant cell line expressing CRK3his, a new construct, pGL89 (generated by Dr. K.M. Grant), based on the pX vector was used. The pGL89 construct consists of the modified *CRK3*his gene, cloned into the pX vector. This shuttle vector is a *Leishmania* expression vector containing the neomycin phosphotransferase gene for selection and maintenance of the construct in transfected parasites (LeBowitz *et al.,* 1990). The construct also contains 5' and 3' flanking sequences derived from the dihydrofolate reductase thymidilate synthase (*DHFR-TS*) gene of *L. major*. These sequences flank the multiple cloning site and provide the necessary trans-splicing and polyadenylation signals which allow the production of mature mRNA molecules. A map of this construct is shown in Fig. 3.13.

The pGL96 construct had been used previously to express recombinant, his-tagged *CRK3* in *L. mexicana* wild type cells. The recombinant protein can be purified and has kinase activity against Histone H1 (Grant *et al.,* 1998). An attempt was made to produce a cell line containing this construct in a null background allowing the exclusive expression of his-tagged CRK3, as was previously achieved for his-tagged CRK1 (Mottram *et al.,* 1996a).

20 μg of pGL96 plasmid was transfected into 4 x 10^7 mid-log promastigotes of clone W583 and transfectants were selected on solid medium containing 50 μg ml^{-1} hygromycin and 50 μg ml^{-1} Geneticin. Ten colonies were picked and grown in liquid culture in the presence of 50 μg ml^{-1} Geneticin to confirm the presence of the pGL96 episome. Clone W925H was selected and prepared for a second round of transfection in order to knockout the remaining *CRK3* allele. W925H cells were transfected with 10 μg

of gel purified *CRK3::BLE* targeting fragment. Clones were isolated on solid medium supplemented with 50 μg ml^{-1} hygromycin and 10 μg ml^{-1} of phleomycin. Six clones (W945, W946, W947, W948, W949 and W950) were transferred to liquid culture, grown and prepared for FACS analysis to assess their DNA content. All six clones were diploid and resistant to Geneticin (Fig.3.14, Panels B to G). Panel A corresponds to wild type cells. DNA was prepared from four of these clones for Southern blot analysis. The Southern blot analysis was performed as described previously. In all the clones the 2.0 kb fragment containing the wild type *CRK3* gene was present (Fig 3.15 lanes 1 to 7). The 4.1 kb fragment expected to be present due to integration of the *CRK3::BLE* targeting fragment was not found to be present in any of the clones (lanes 4 to 7). The most likely explanation for this is that the *CRK3::BLE* fragment has integrated into the episome. This is possible because of the 874 bp of shared sequence between the episome and the targeting fragment. Both contain *DHFR-TS* sequence as well as *CRK3* sequence (see Fig. 3.16). As it is the termini of the introduced DNA fragment that are involved in the homologous recombination process (Tobin *et al.,* 1991), then any nuclease activity which removes some of this *CRK3* sequence from either end of the DNA fragment will expose the *DHFR-TS* sequence, allowing the possibility of integration into the episome. This process is most likely to occur at the 3'end of the *CRK3::BLE* fragment where there is only 339 bp of *CRK3* sequence. In addition it is likely that there are multiple copies of the pGL96 episome, possibly as many as 100 copies. There is therefore a greater chance of integration into the episome in preference to integration at the *CRK3* allele, which is present only as a single copy in the W925H heterozygote mutant cell line used in this experiment (see Table 3.1 for descriptions of mutant clones and their genotypes). This problem was not encountered previously when the same procedure was used to generate null mutants expressing *CRK3* from the pTEX

episome (see section 3.2.2). This may be because pTEX does not contain *Leishmania DHFR-TS* sequence but contains *T. cruzi GAPDH* sequence to provide the trans-splicing and polydenylation signals. There is therefore less homology between the pTEX based construct and the *CRK3::BLE* targeting fragment, and consequently, less chance of integration into the episome.

3.2.7 Co-transfection of an episomal vector expressing CRK3his with the *CRK3::BLE* targeting fragment

To avoid preferential integration into the pGL96 episome rather than into the genomic locus, the episome was introduced simultaneously with the *CRK3::BLE* targeting construct into the W583 heterozygote cell line. Clones were selected on solid agar containing 50 μg ml^{-1} hygromycin and 10 μg ml^{-1} phleomycin. Geneticin was not added to the medium for the initial selection to avoid any problems associated with combined selection with three antibiotics, as mentioned in section 3.2.2. Twenty independent clones were grown and prepared for DNA content analysis by FACS (W991-W1006 and NS1-NS4). These clones were also tested for resistance to Geneticin at a concentration of 50 μg ml^{-1}. Eight of the twenty clones were sensitive to Geneticin (W991-W994 and NS1-NS4). FACS analysis indicated that four of these clones were diploid (NS1, NS2, NS3 and NS4) (Fig. 3.17 panels B, C, D and E); whilst the remaining four clones (W991, W992, W993 and W994) were aneuploid (Fig. 3.16 panels F, G, H and I). Panel A corresponds to wild type cells. The four aneuploid clones have probably arisen through the same mechanism as the aneuploid clones described in section 3.2.1. These aneuploid clones were not analysed further. DNA was prepared from the diploid, Geneticin sensitive clones (NS1-4) for Southern blot analysis. The results (Fig. 3.18)

indicated that the wild type *CRK3* gene was still present and that the 4.1 kb band, indicative of the integrated *CRK3::BLE* construct, was not present (Lanes 4, 5, 6, and 7). This suggests that these cells have arisen through a genomic rearrangement event similar to those described in section 3.2.3. The remaining 12 clones (W995-W1006) were resistant to Geneticin, indicating the presence of the pGL96 episome. FACS analysis also indicated that all twelve clones were diploid (Fig. 3.19, panels B to M). Panel A corresponds to wild type cells. DNA was prepared from these clones for Southern blot analysis. The results of this analysis are shown in Fig. 3.20. The 2.0 kb fragment characteristic of an intact *CRK3* locus was present in all clones, lanes 4 to 16. It is unclear whether the *CRK3::HYG* and *CRK3::BLE* targeting constructs have correctly integrated (N.B Lanes 1 to 3 correspond to wild type, W583 and W585 clones respectively). What is clear however is that these clones are not null at the *CRK3* locus and have retained a functional copy of *CRK3* either by integration of the *CRK3::BLE* targeting construct at an incorrect locus, or by undergoing a genomic rearrangement. A more extensive Southern blot analysis using a variety of restriction enzyme digests of genomic DNA, and the use of pulse field gels to separate whole chromosomes, should help to answer these questions. However as the aim of this study was to generate null mutants expressing an epitope tagged version of *CRK3*, a detailed analysis of the nature of such presumed genomic rearrangements falls outwith the scope of this work.

3.3.1 DISCUSSION

The experiments described in this chapter provide evidence that the *CRK3* gene is essential to *L. mexicana* promastigotes. This may be expected for a gene that has a possible role in cell cycle progression or growth control. The evidence that the gene is

essential is that attempts to disrupt both wild type alleles invariably fail. This is not due to any effect of the construction of the gene disruption constructs as either the *CRK3::HYG* or the *CRK3::BLE* constructs can be easily and reproducibly integrated at the correct locus, as determined by Southern blot analysis (see section 3.2.1 and Fig. 3.3 lanes 2 and 3). Neither is the difficulty in generating *CRK3* null mutants due to incompatibility of the two drug resistance genes used (*HYG* and *BLE*) as these genes have previously been shown to be compatible in combination (Freedman and Beverley, 1993). The only way that both wild type alleles could successfully be disrupted, was if the *CRK3* gene was introduced on an episome prior to disruption of the second wild type allele (section 3.2.4). It should also be noted however that careful consideration must be given to the drug selection protocol, as initial selection of transfectants with all three antibiotics (hygromycin B, Geneticin and phleomycin) repeatedly failed (section 3.2.2). Only when Geneticin selection was omitted when selecting for the second integration event, was the procedure successful (section 3.2.4). Geneticin selection in this instance would not be required if the *CRK3* gene, present on the plasmid, is essential, as the presence of the plasmid should be sufficient to provide a selection pressure for maintenance of the episome. It is unclear exactly why the triple drug selection protocol proved to be problematic. This effect however, has been reported from a number of laboratories. The fact that both hygromycin B and Geneticin belong to the same class of compounds which affect protein synthesis, and that the *HYG* and *NEO* resistance genes both encode phosphotransferase enzymes (Laban *et al.,* 1990; Lee and Van der Ploeg, 1991) suggests that the problem may be due to cross reactivity between the drugs and the drug resistance enzymes, or possibly due to a synergistic effect between these components. It is unclear however why this combination of drugs can be used to select for double gene disruption events in some cases (Cruz *et al.,* 1991;

Mottram *et al.,* 1993; Souza *et al.,* 1994; Webb and McMaster, 1994; Dumas *et al.,* 1997) but not in others.

Other evidence that *CRK3* encodes an essential activity is that attempts at generation of null mutants frequently produced genetic changes in the parasite. Such as changes in the overall ploidy of the parasites, or presumed gene amplification events that allow retention of one copy of the wild type *CRK3* gene, whilst integrating both targeting fragments. Ploidy changes resulting from double targeted gene disruption experiments have been accepted as positive evidence that the gene being targeted is essential (Cruz *et al.,* 1993). Further evidence that *CRK3* is essential is that ploidy changes can be avoided and both alleles of the *CRK3* gene can be successfully disrupted by expressing the gene from an episome.

The mechanism behind the generation of aneuploid parasites upon sequential disruption of essential genes in *Leishmania* is unknown. It may be that spontaneous generation of aneuploid cells occurs at a low level in axenically grown *Leishmania*, but such cells would be at a disadvantage in a more rapidly dividing diploid population. Any aneuploid parasites present in a culture which is prepared for transfection may integrate the introduced construct just as diploid cells would. If this integration event occurs during the second round of transfection then such cells would contain both drug resistance markers and would grow upon double drug selection, whilst retaining two copies of the wild type gene. Diploid cells, however, that have integrated both of the drug resistance markers will lack the wild type gene and will be null at the target locus. If the gene being targeted is essential to parasite survival then the only cells which could possibly grow upon double drug selection would be aneuploid cells containing both drug resistance markers whilst retaining two functional copies of the essential gene. As the only clones that could be derived from a second round of disruption of the *CRK3*

gene were aneuploid then we can conclude that this is an essential gene. Because any aneuploid cells present in the culture of the heterozygote mutant W583 would be at a low level then the probability of integration of the *CRK3::BLE* targeting fragment into such cells would be very low, this may explain the observed low efficiency of the second round transfection where only two viable clones were obtained compared to first round transfection experiments which regularly produce greater than 50 independent clones.

The transfection procedure itself may cause diploid heterozygote cells to fuse, generating aneuploid cells. Subsequent integration of the second targeting fragment would generate a clone that contains both targeting constructs, but still retains a copy of the wild type gene.

Another alternative explanation is that at the moment of transfection a proportion of cells will have gone through S-phase, but not yet divided. Such cells would have a 4N DNA content. If, due to the extreme conditions of electroporation, these cells failed to divide and re-enter DNA synthesis phase, then they may integrate the second targeting fragment. After the next cell division at least one daughter cell would be aneuploid and contain both the integrated disruption constructs.

Using a similar procedure to that used to generate null mutants expressing *CRK3* from the pGL100 episome, an attempt was made to generate a cell line expressing a modified hexa-histidine tagged version of the *CRK3* gene in a null background. The pX vector used in this case was however different from the pTEX vector used to express the unmodified gene. Because the pX vector contains genuine *L. major* sequence derived from the *DHFR-TS* locus the targeting construct used to disrupt the second copy of the *CRK3* gene integrated preferentially into the episome rather than at the genomic locus. This possibility is more likely because the episome is present in multiple copies whereas

there is of course only one copy of the wild type gene in the W583 heterozygote cell line used in these experiments.

Attempts were made therefore to co-transfect the heterozygote with both the targeting fragment and the episome. In this instance none of the clones analysed were null at the *CRK3* locus. One explanation for this is that integration of the *CRK3::BLE* targeting fragment and simultaneous uptake of the pX*CRK3*his episome is a rare event. This however is clearly not the case as 40% of clones obtained in this experiment were resistant to hygromycin, phleomycin and Geneticin, indicating the presence of both gene disruption constructs and the pX*CRK3*his episome (pGL96). It is possible that some critical aspect of CRK3 function is affected by the presence of the histidine tag. The histidine tagged protein however can be expressed in a wild type background and can be purified to give an active Histone H1 kinase (Grant *et al.*, 1998).

There is a complication in the interpretation of these results due to the use of different vectors for expression of the modified and unmodified genes. The change in use of vectors was due to initial problems encountered with the pTEX vector which did not generate resistant clones with high efficiency, this was thought to be due to the use of *T. cruzi* gapdh sequence for trans-splicing and polyadenylation of expressed mRNAs. For this reason it was decided to use the pX vector which uses *L. major DHFR-TS* sequence to provide trans-splicing and polyadenylation signals. The problem with using this vector however is that similar *DHFR-TS* sequence is present in the gene disruption constructs allowing integration of the targeting construct into the episome rather than at the *CRK3* locus. By using a pTEX based construct, and using the drug selection protocol outlined in section 3.2.4, the generation of a *CRK3* null mutant cell line, expressing hexa-histidine tagged CRK3 may still be possible. Hexa-histidine tagged CRK3 can be expressed in a wild type genetic background and the modified kinase is

active against an exogenous substrate in an in vitro assay. The tag does not therefore interfere with the enzyme activity (Grant *et al.,* 1998). Unless the tag interferes with some other aspect of CRK3 function, not directly related to it's kinase function then expression of tagged CRK3 in a null background should be possible. This would be a valuable tool for the purification and analysis of this enzyme and other interacting proteins such as cyclin partners or inhibitory molecules.

This study has indicated that the CRK3 kinase is essential in promastigotes of *L. mexicana*. Clearly therefore, although there are a number of other CRKs in *Leishmania*, they are not degenerate and may each have specific, non-overlapping roles. Both *CRK1* and *CRK3* are essential and have different patterns of stage-regulated activity. CRK1 may play a role in differentiation, as it's activity is detected only in log-phase and stationary phase promastigotes, and is absent from amastigotes. CRK3 is a good candidate to be the functional Cdc2 homologue in *Leishmania*. It binds with high affinity to $p13^{suc1}$, a protein that forms part of the active Cdc2 complex in fission yeast. CRK1 on the other hand does not bind to $p13^{suc1}$. CRK3 activity is present in the proliferative stages of the life-cycle and is absent from cell-cycle arrested metacyclics. If CRK3 does play a crucial role in cell cycle progression, then it is probable that the gene would be essential in amastigotes. This possibility cannot yet be tested as an efficient method for transfection of amastigotes has yet to be developed.

Fig. 3.1 Sequence of the 2.0 kb *Hin*dIII fragment derived from the *CRK3* locus

The complete sequence of the 2026 bp *Hin*dIII fragment derived from the *CRK3* locus, and cloned into pBluescript to give pGL89, is shown. The *CRK3* ORF and approximately 300 bp of upstream sequence was provided by Philomena Halford and Dr. Jeremy C. Mottram. Restriction sites are shown in purple, oligonucleotides are in red and the *CRK3* open reading frame is in blue.

*Hin*dIII
~~~~~~

```
  1  AAGCTTAGGA GGTGCTGGCG GTGTGCCACC GGCAACTTCG CAGGCTGCTT
     TTCGAATCCT CCACGACCGC CACACGGTGG CCGTTGAAGC GTCCGACGAA

 51  CGACTCCAGC CCTAGGAGCT GGTTTCAACC CTGCAATGTT TGCACCTCCT
     GCTGAGGTCG GGATCCTCGA CCAAAGTTGG GACGTTACAA ACGTGGAGGA

101  GTTCCAGAAG GTAATCCACG GGAGGTTTAT CGCGAGCAAC TGCAGCTGCT
     CAAGGTCTTC CATTAGGTGC CCTCCAAATA GCGCTCGTTG ACGTCGACGA

151  GCGAGATATG GGTTTTCCCA ATGAGGAAGC GAACATTGCT GCTCTTCAGC
     CGCTCTATAC CCAAAAGGGT TACTCCTTCG CTTGTAACGA CGAGAAGTCG

            OL382
       ==================
201  AGGCTCAGGG CAATGTTCAG TTCGCATTGG AGCGGCTTCT TGGTGCATGA
     TCCGAGTCCC GTTACAAGTC AAGCGTAACC TCGCCGAAGA ACCACGTACT

251  TATATTGTGC TTTGCTTCCA CTTACTTCTT CCCCCTATTC TTACTCGTGC
     ATATAACACG AAACGAAGGT GAATGAAGAA GGGGGATAAG AATGAGCACG

301  TTTCGATCTT CAGAACTGAT CGTGGGAAGG GGAAGCCCTT GTGCTTTACG
     AAAGCTAGAA GTCTTGACTA GCACCCTTCC CCTTCGGGAA CACGAAATGC

351  AGGTAAGAGT ACGTTTCTTG GTGCTGGTGG GCCGATTTTA GTGAGTTTAC
     TCCATTCTCA TGCAAAGAAC CACGACCACC CGGCTAAAAT CACTCAAATG

401  AGTACAGAGG ATACTCTATG AACCCAAACA CTAGTCAAAA AGACGGTGGC
     TCATGTCTCC TATGAGATAC TTGGGTTTGT GATCAGTTTT TCTGCCACCG

451  TGTAACTGGC AGCAGCGATT TGGCAGGGGT GCTGCTCATG CGAGTGACAG
     ACATTGACCG TCGTCGCTAA ACCGTCCCCA CGACGAGTAC GCTCACTGTC

501  TAAAGCAAAG GTAGAGGATG CCGTTTTGCT GGATTTAAAC GCCAAGCACA
     ATTTCGTTTC CATCTCCTAC GGCAAAACGA CCTAAATTTG CGGTTCGTGT

551  AGAAGCGAGC AAGTGCATGC TATCAGATAC TTCTAAATGA AAGTCAGCTA
     TCTTCGCTCG TTCACGTACG ATAGTCTATG AAGATTTACT TTCAGTCGAT

601  CTCTCCTCTT TTTTTGGCAT CATCTTTTTT TCTCCTCTGT CCCTCATCTT
     GAGAGGAGAA AAAAACCGTA GTAGAAAAAA AGAGGAGACA GGGAGTAGAA

651  GTTTCCTTCC GATCAGAAGT GATAACGCTT CATAGTGAAG GCGTGCATTT
     CAAAGGAAGG CTAGTCTTCA CTATTGCGAA GTATCACTTC CGCACGTAAA

701  GCCAAGTGTG AAACTTTTTT TTCGACTCAC GATCTAGAAC TTGTCGGAAA
     CGGTTCACAC TTTGAAAAAA AAGCTGAGTG CTAGATCTTG AACAGCCTTT

751  AAAAAAGTAG CATTTAAAAA AGCATACACA CACTTACACA AATATCTTTT
     TTTTTTCATC GTAAATTTTT TCGTATGTGT GTGAATGTGT TTATAGAAAA
```
~~~~~~

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                                                                  OL383
                                                                  ==========
801  TCTTTTCCCC TTTAGCGTCT CTGCTGTTGC ACGTTTCTGG CCCTTTCTCC
     AGAAAAGGGG AAATCGCAGA GACGACAACG TGCAAAGACC GGGAAAGAGG

     =========
851  GTGCTACAAC ACACACACAC ACAGATGCCA AAGCACTAGC TTCTGGTGCC
     CACGATGTTG TGTGTGTGTG TGTCTACGGT TTCGTGATCG AAGACCACGG

                                                  EcoRI
                                                  ~~~~~~~
901  TGATTTTTTG TTGTTTGTAA GGAGAGGAGA CGAAGGAATT CCTTTCTTTT
     ACTAAAAAAC AACAAACATT CCTCTCCTCT GCTTCCTTAA GGAAAGAAAA

951  TTGAAGGTTT CGGAGCTTTT GCTTTGAACA AAGTTTCTCC CTGCAACATT
     AACTTCCAAA GCCTCGAAAA CGAAACTTGT TTCAAAGAGG GACGTTGTAA

                         OL322
                    ====================
1001 TCAAATGTCT TCGTTTGGCC GTGTGACCGC CCGCAGCGGC GACGCTGGGA
     AGTTTACAGA AGCAAACCGG CACACTGGCG GGCGTCGCCG CTGCGACCCT

1051 CCCGTGACAG TCTTGACCGG TACAATCGCT TGGATGTTTT GGGAGAGGGA
     GGGCACTGTC AGAACTGGCC ATGTTAGCGA ACCTACAAAA CCCTCTCCCT

                                    SalI
                                    ~~~~~~
1101 ACGTACGGCG TTGTGTATCG TGCGGTCGAC AAAATCACTG GACAGTACGT
     TGCATGCCGC AACACATAGC ACGCCAGCTG TTTTAGTGAC CTGTCATGCA

1151 TGCTCTCAAG AAAGTGCGAC TCGATCGCAC TGAGGAGGGT ATTCCGCAGA
     ACGAGAGTTC TTTCACGCTG AGCTAGCGTG ACTCCTCCCA TAAGGCGTCT

1201 CTGCGCTGCG CGAGGTGTCA ATTCTGCAAG AGTTCGACCA CCCCAACATT
     GACGCGACGC GCTCCACAGT TAAGACGTTC TCAAGCTGGT GGGGTTGTAA

1251 GTGAACTTGC TTGATGTCAT TTGCTCGGAC GGGAAGCTCT ACCTTGTCTT
     CACTTGAACG AACTACAGTA AACGAGCCTG CCCTTCGAGA TGGAACAGAA

1301 CGAGTATGTG GAGGCGGACC TGAAAAAGGC GATTGAAAAG CAAGAGGGCG
     GCTCATACAC CTCCGCCTGG ACTTTTTCCG CTAACTTTTC GTTCTCCCGC

1351 GCTACTCTGG AATGGATCTG AAGCGGCTTA TTTATCAGCT TTTAGACGGC
     CGATGAGACC TTACCTAGAC TTCGCCGAAT AAATAGTCGA AAATCTGCCG

1401 CTTTACTTTT GCCACCGCCA TCGCATCATC CACCGTGATC TGAAGCCAGC
     GAAATGAAAA CGGTGGCGGT AGCGTAGTAG GTGGCACTAG ACTTCGGTCG

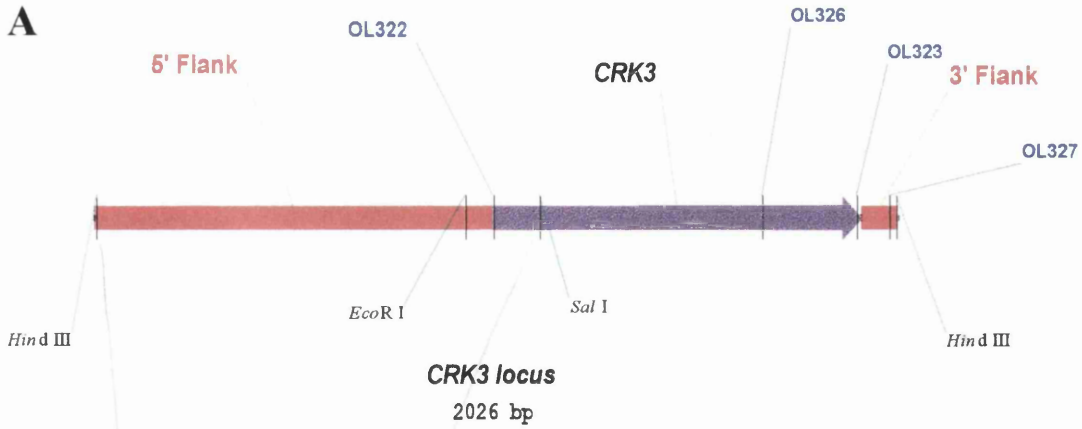
1451 CAACATCCTC CTGACATCAG GGAACGTCCT TAAATTGGCT GATTTCGGTC
     GTTGTAGGAG GACTGTAGTC CCTTGCAGGA ATTTAACCGA CTAAAGCCAG

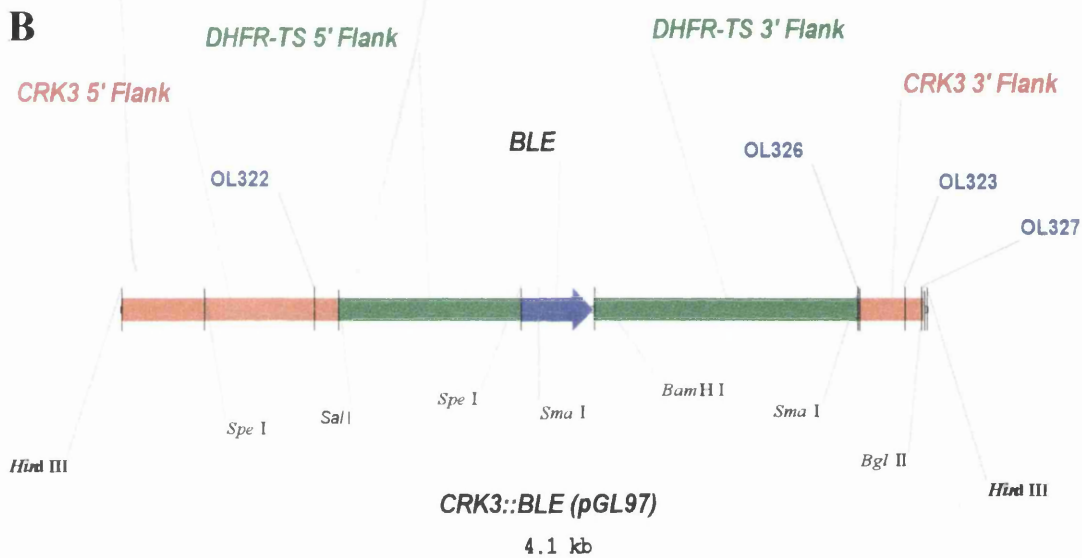
1501 TCGCCCGTGC GTTTCAAGTG CCCATGCACA CCTACACGCA CGAGGTGGTT
     AGCGGGCACG CAAAGTTCAC GGGTACGTGT GGATGTGCGT GCTCCACCAA
```

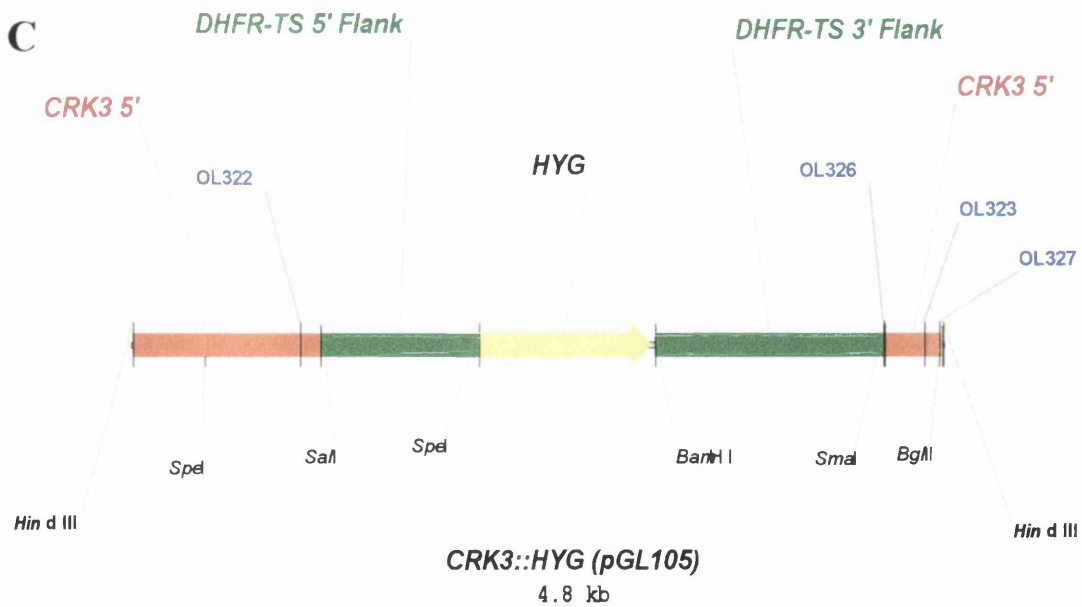

1551 ACGCTGTGGT ACCGTGCCCC TGAGATCCTC CTCGGTGAGA AGCACTACGC
TGCGACACCA TGGCACGGGG ACTCTAGGAG GAGCCACTCT TCGTGATGCG
1601 TCCTGCTGTG GATATGTGGA GTGTCGGCTG CATTTTCGCC GAGCTAGCAC
AGGACGACAC CTATACACCT CACAGCCGAC GTAAAAGCGG CTCGATCGTG
OL326
1651 GCCGAAAGGT TCTTTTCCGC GGCGATAGCG AAATCGGGCA GTTGTTTGAG
CGGCTTTCCA AGAAAAGGCG CCGCTATCGC TTTAGCCCGT CAACAAACTC
1701 ATTTTTCAAG TGTTGGGGAC TCCGACGGAC ACCGAGGGGT CCTGGCCTGG
TAAAAAGTTC ACAACCCCTG AGGCTGCCTG TGGCTCCCCA GGACCGGACC
1751 TGTGTCGCGG CTTCCTGATT ACCGCGACGT ATTTCCCAAG TGGACCGCAA
ACACAGCGCC GAAGGACTAA TGGCGCTGCA TAAAGGGTTC ACCTGGCGTT
1801 AGCGGCTGGG GCAGGTACTA CCAGAACTTC ATCCAGACGC TATTGATCTT
TCGCCGACCC CGTCCATGAT GGTCTTGAAG TAGGTCTGCG ATAACTAGAA
1851 CTCTCCAAGA TGCTCAAGTA CGATCCACGG GAGCGCATAT CAGCCAAGGA
GAGAGGTTCT ACGAGTTCAT GCTAGGTGCC CTCGCGTATA GTCGGTTCCT
OL323
1901 GGCCCTACAG CACCCGTGGT TCAGCGACCT TCGTTGGTAG TGGAAAAGGC
CCGGGATGTC GTGGGCACCA AGTCGCTGGA AGCAACCATC ACCTTTTCCG
1951 ATGACTGAAT ACAGCCTTCT GACGCGTTGA ACGATGGAGG ATTTGTTTTT
TACTGACTTA TGTCGGAAGA CTGCGCAACT TGCTACCTCC TAAACAAAAA
OL327 HindIII
2001 CTGAGGGTGC TTTGTTAAGG AAGCTT
GACTCCCACG AAACAATTCC TTCGAA

Fig. 3.2 Map of *CRK3* locus and gene targeting constructs

Panel A. Map of the *CRK3* gene. The 2.0 kb *Hin*dIII fragment was cloned into pBluescript to give plasmid pGL89. Panels B and C show the *CRK3::HYG* and *CRK3::BLE* disrupted alleles. Restriction sites are indicated. Regions of homology are colour coded. The green regions present in the targeting constructs correspond to the *L major* dihydrofolate reductase thymidylate synthase sequences that allow expression of the drug resistance gene. The 5' flank of both targeting constructs consists of the 1096bp *Hin*dIII/*Sal*I fragment from the *CRK3* locus. The 3' flank consists of a 339 bp fragment amplified from the 3' end of the *CRK3* locus by PCR with primers OL326 and OL327.


A
OL322
OL326
5' Flank
CRK3
OL323
3' Flank
OL327
EcoR I
Sal I
Hind III
Hind III
CRK3 locus
2026 bp


B
DHFR-TS 5' Flank
DHFR-TS 3' Flank
CRK3 5' Flank
CRK3 3' Flank
BLE
OL326
OL322
OL323
OL327
Spe I
Sal I
Spe I
Sma I
BamH I
Sma I
Bgl II
Hind III
Hind III
CRK3::BLE (pGL97)
4.1 kb


C
DHFR-TS 5' Flank
DHFR-TS 3' Flank
CRK3 5'
CRK3 5'
HYG
OL322
OL326
OL323
OL327
SpeI
SalI
SpeI
BamHI
SmaI
BglII
Hin d III
Hin d III
CRK3::HYG (pGL105)
4.8 kb

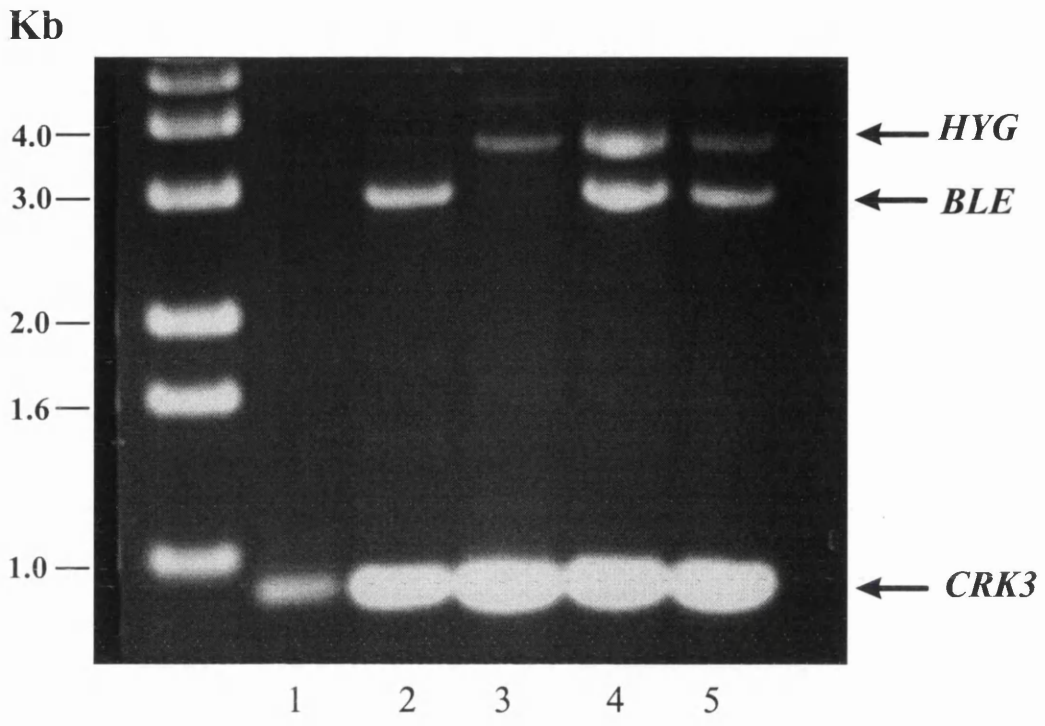


Fig. 3.3 PCR analysis of transgenic cell lines

Approximately 50ng DNA from wild type *L. mexicana* and *CRK3* mutants was used as the template for a PCR reaction with primers OL322 and OL323 which anneal to the 5' and 3' sequence of *CRK3*. This sequence is also present in the *CRK3::HYG* and *CRK3::BLE* disruption cassettes (Fig. 3.1). Molecular weight markers are 1kb ladder (Gibco BRL)

Lane 1. Wild type

Lane 2. W585

Lane 3. W583

Lane 4. W625

Lane 5. W626

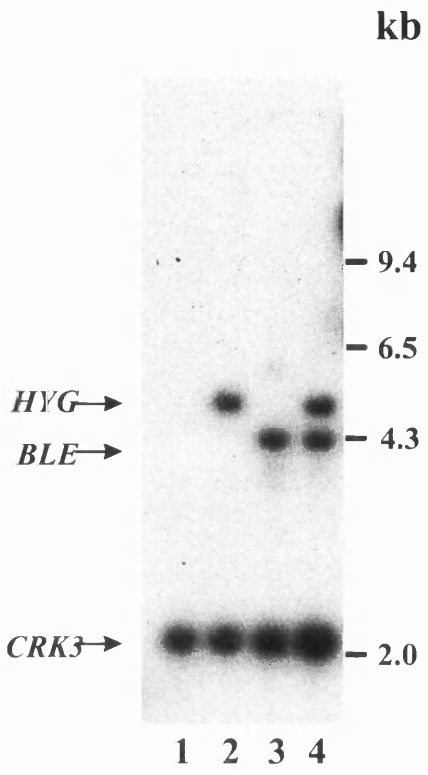


Fig. 3.4 Southern blot analysis of transgenic cell lines

5μg DNA from wild type *L. mexicana* or *CRK3* mutants was digested with *Hin*dIII, separated by agarose gel electrophoresis, and transferred to a positively charged nylon membrane. The membrane was then probed with the 2.0 kb *Hin*dIII fragment from plasmid pGL89, containing the complete *CRK3* gene. Molecular weight markers are λ *Hin*dIII digest

Lane 1. Wild type

Lane 2. W583

Lane 3. W585

Lane 4. W625

Fig. 3.5 DNA content analysis of transgenic cell lines by fluorescence activated cell sorting (FACS).

The DNA content of wild type *L. mexicana* or *CRK3* mutants was analysed by FACS. Mid-log promastigotes were fixed and stained with propidium iodide, then sorted with an Epics/XL flow cytometer (Coulter). A total of 10,000 cells were analysed for each sample.

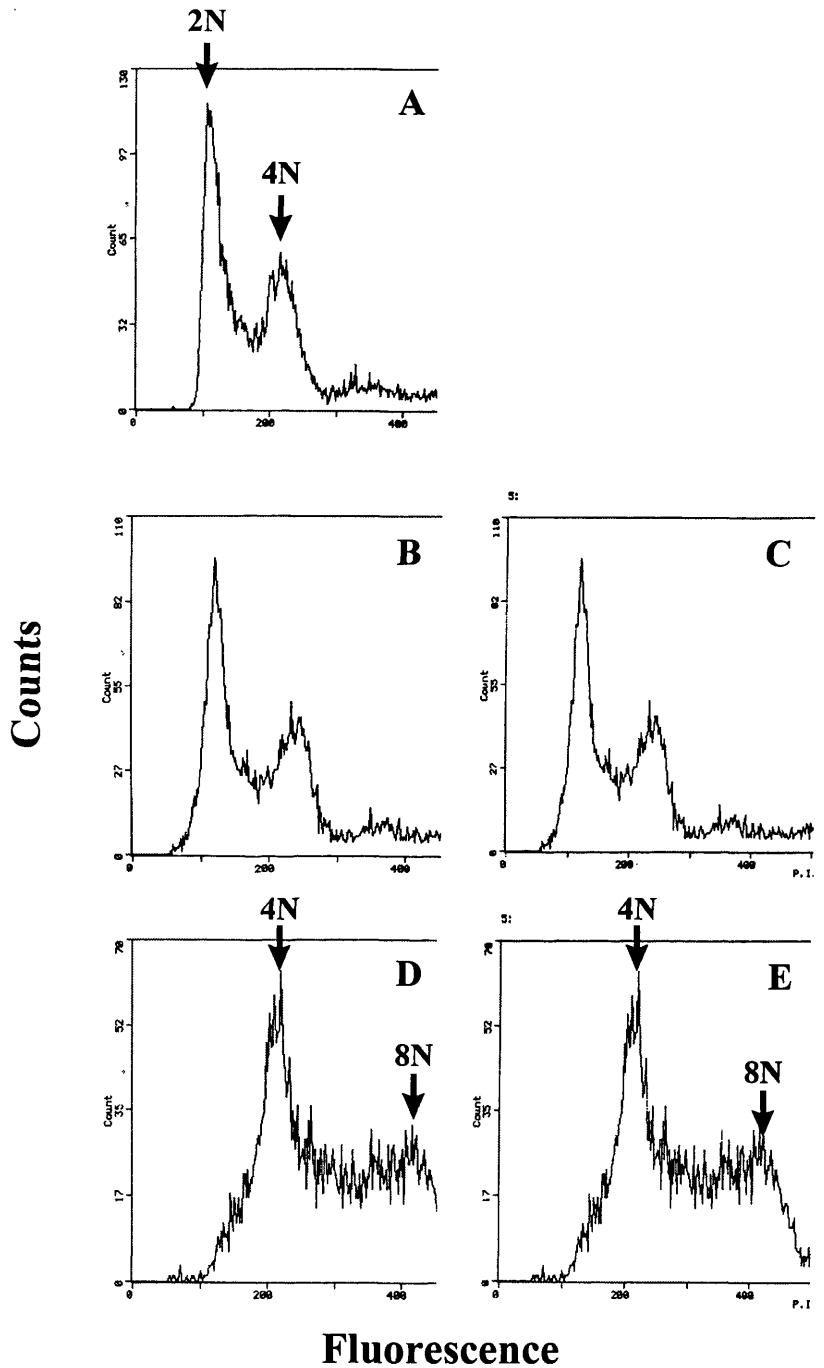
Panel A Wild type

Panel B W583

Panel C W585

Panel D W625

Panel E W626


2N
A
4N
B
C
4N
D
8N
4N
E
8N
Counts
Fluorescence

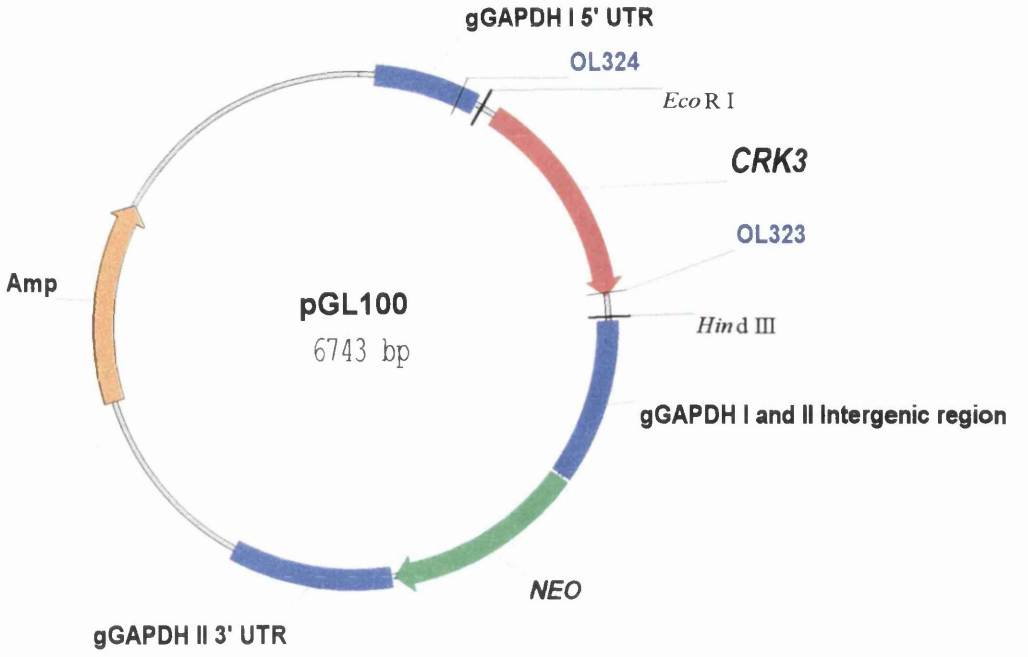


Fig. 3.6 Map of pTEX*CRK3* episomal construct, plasmid pGL100.

The plasmid used to express the *CRK3* gene in *L. mexicana* is based on the pTEX shuttle vector (Kelly *et al.*, 1992). This vector contains both the Amp^r gene, conferring resistance to Ampicillin, and the *NEO* gene, allowing selection and maintenance of the plasmid in *E. coli* and *Leishmania*. The *Trypanosoma cruzi* g*GAPDH* flanking and intergenic sequences, provide the signals needed for trans-splicing and polyadenylation of the *CRK3* and *NEO* mRNAs in *Leishmania*. The position of the OL323 and OL324 oligonucleotides is shown.

Fig. 3.7 DNA content analysis of clones, derived from co-transfection experiment 3.2.2.

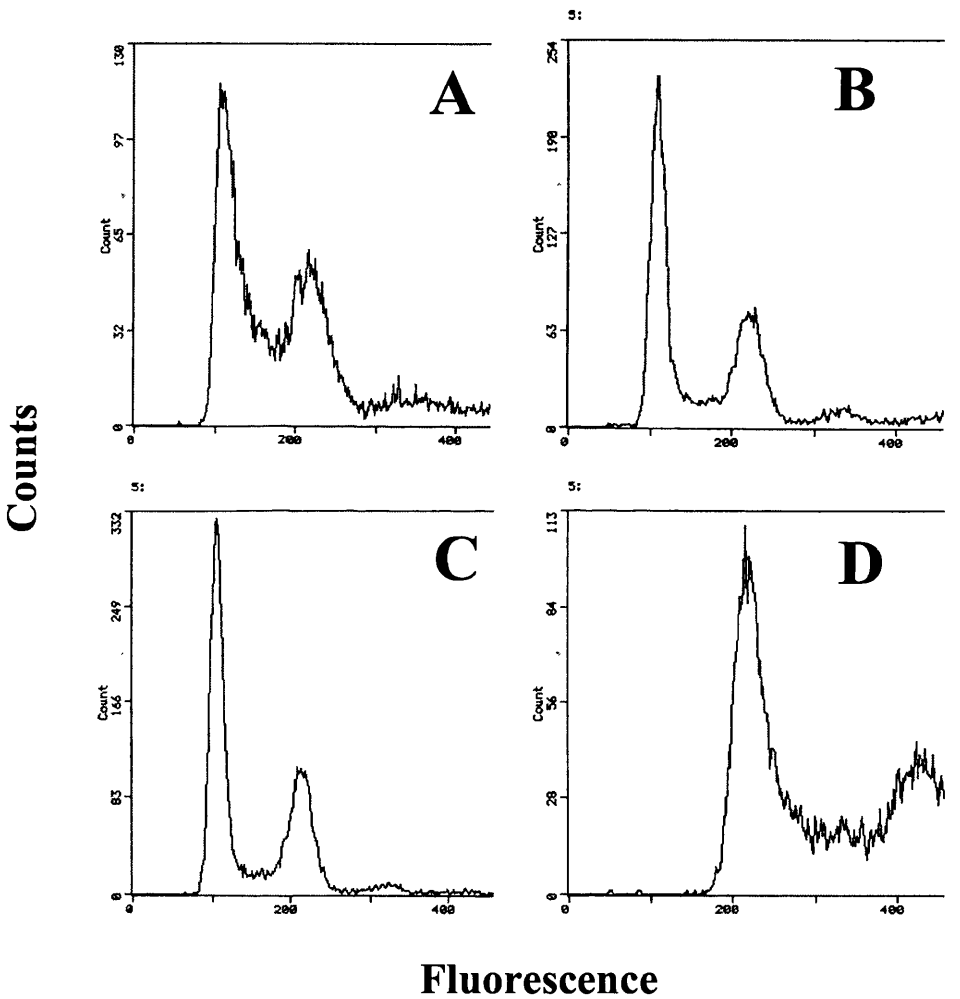
Clonal cell lines derived from the co-transfection experiment described in section 3.2.2, in which H1 heterozygote mutants were transfected with both the pGL100 episome (Fig. 3.5) and the *crk3::ble* targeting fragment, were analysed by FACS to determine their overall DNA content. Cells were fixed, stained with propidium iodide and analysed on a Epics/XL flow cytometer (Coulter). A total of 10,000 cells were analysed for each sample.

Panel A. Wild type, $G418^S$

Panel B. W635, $G418^R$

Panel C. W633

Panel D. W634


A
B
C
D
Counts
Fluorescence
Count

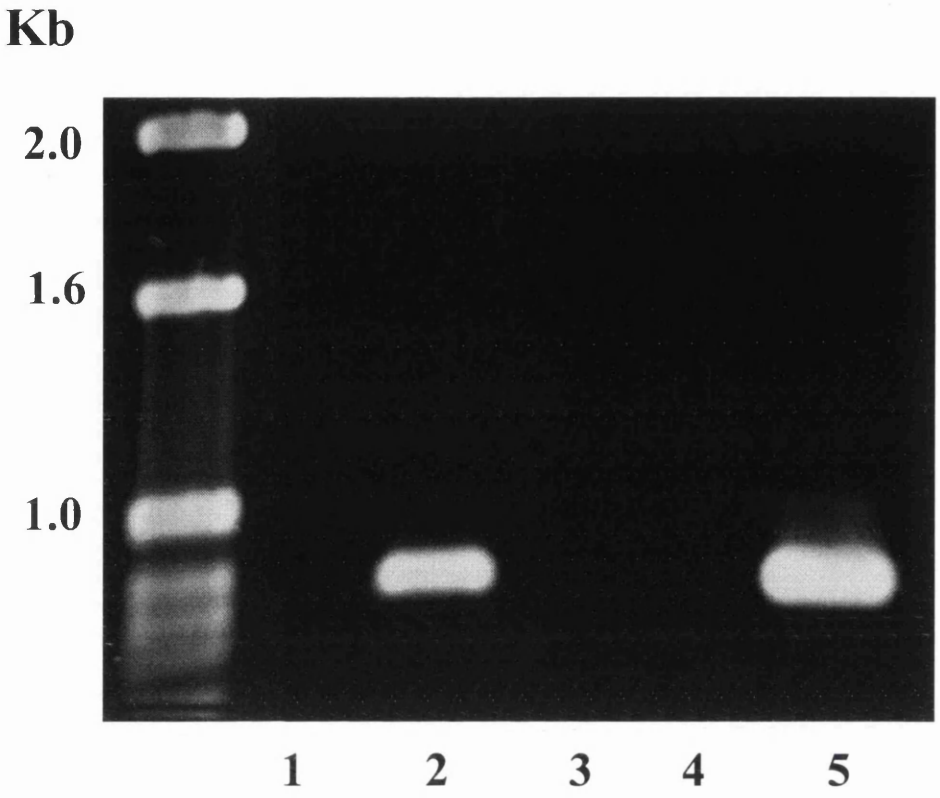


Fig.3.8 PCR analysis of clones derived from co-transfection experiment 3.2.2.

DNA from wild type *L. mexicana*, or clonal cell lines derived from experiment 3.2.2 were analysed by PCR. 50ng of DNA was used as the template in a PCR reaction with the OL324 (pTEX-specific) and OL323 (*CRK3*-specific) primers. Samples were electrophoresed on an 0.8% agarose gel, stained with ethidium bromide and imaged under UV illumination. Molecular weight marker was 1kb ladder (Gibco BRL)

Lane 1. Wild type ($G418^S$)

Lane 2. W635 ($G418^R$)

Lane 3. W632 ($G418^S$)

Lane 4. W633 ($G418^S$)

Lane 5. Plasmid pGL100

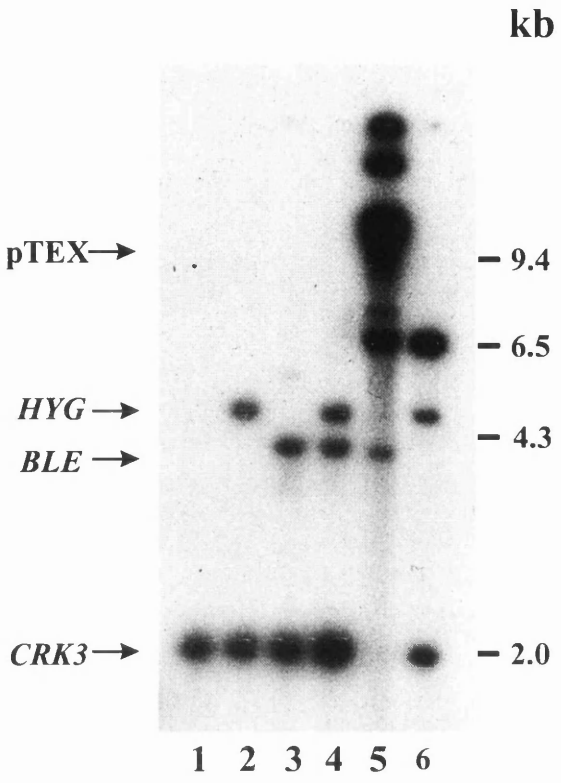


Fig. 3.9 Southern blot analysis of clones derived from co-transfection experiment 3.2.2.

5μg of genomic DNA was digested with *Hin*dIII, separated by gel electrophoresis and transferred to a positively charged nylon membrane. The membrane was probed with the 2.0 kb *Hin*dIII fragment of pGL89, which contains the full *CRK3* sequence (see Fig. 3.1)

Lane 1. Wild type

Lane 2. W583

Lane 3. W585

Lane 4. W625

Lane 5. W635

Lane 6. W633

Fig. 3.10 DNA content analysis of clones derived from experiment 3.2.4.

The DNA content of clones from experiment 3.2.4 was analysed by FACS. Cells were fixed, stained with propidium iodide and analysed on a Epics/XL flow cytometer (Coulter). A total of 10,000 cells were analysed for each sample.

Panel A. Wild type

Panel B. W638

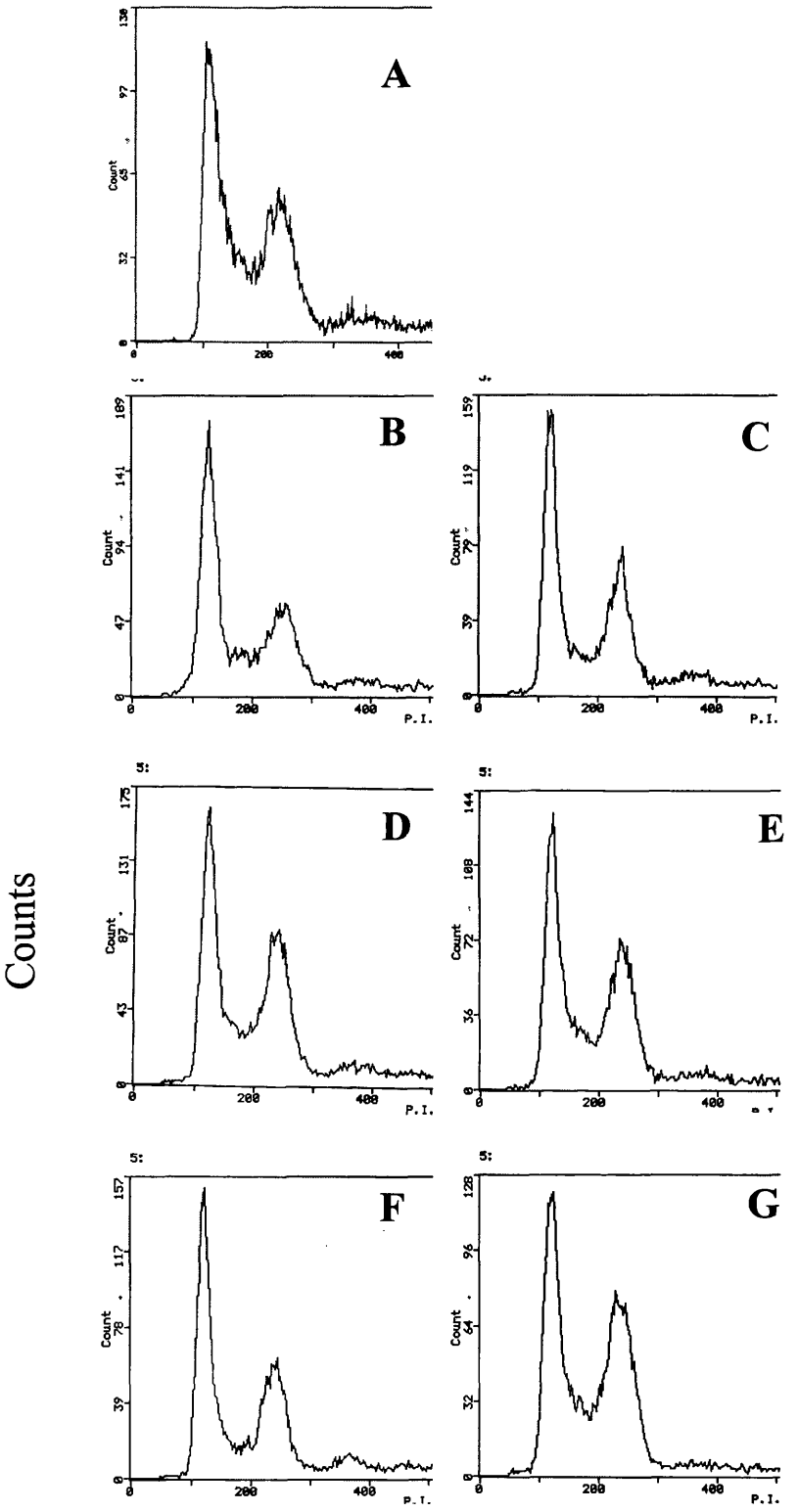
Panel C. W1187

Panel D. W1188

Panel E. W1189

Panel F. W1190

Panel G. W1191


A
B
C
D
E
F
G
Count
P.I.
Counts
Fluorescence

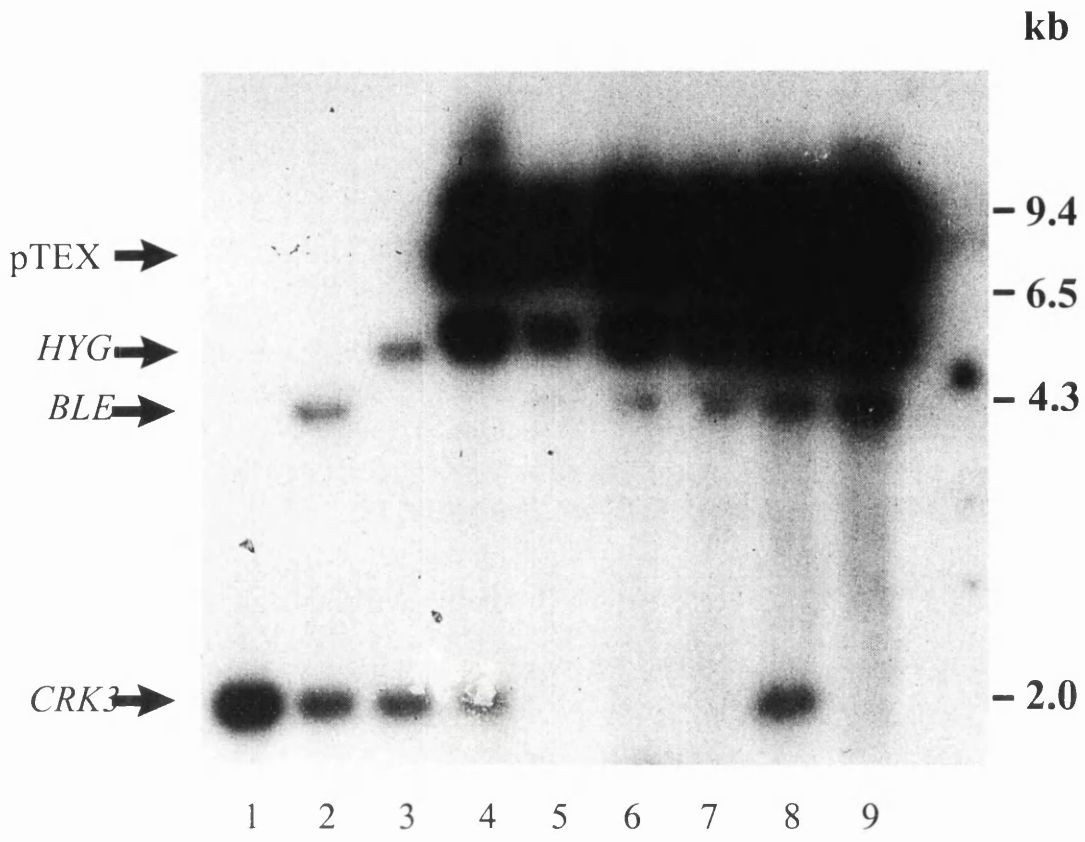


Fig. 3.11 Southern blot analysis of transfectants from experiment 3.2.4.

5μg of genomic DNA from clones derived as described in section 3.2.4, was digested with *Hin*dIII, separated by gel electrophoresis and transferred to a positively charged nylon membrane. The membrane was probed with the 2.0 kb *Hin*dIII fragment of pGL89, which contains the full *CRK3* sequence (see Fig. 3.1)

Lane 1. Wild type

Lane 2. W583

Lane 3. W585

Lane 4. W638

Lanes 5. W1187

Lane 6. W1188

Lane 7. W1189

Lane 8. W1190

Lane 9. W1191

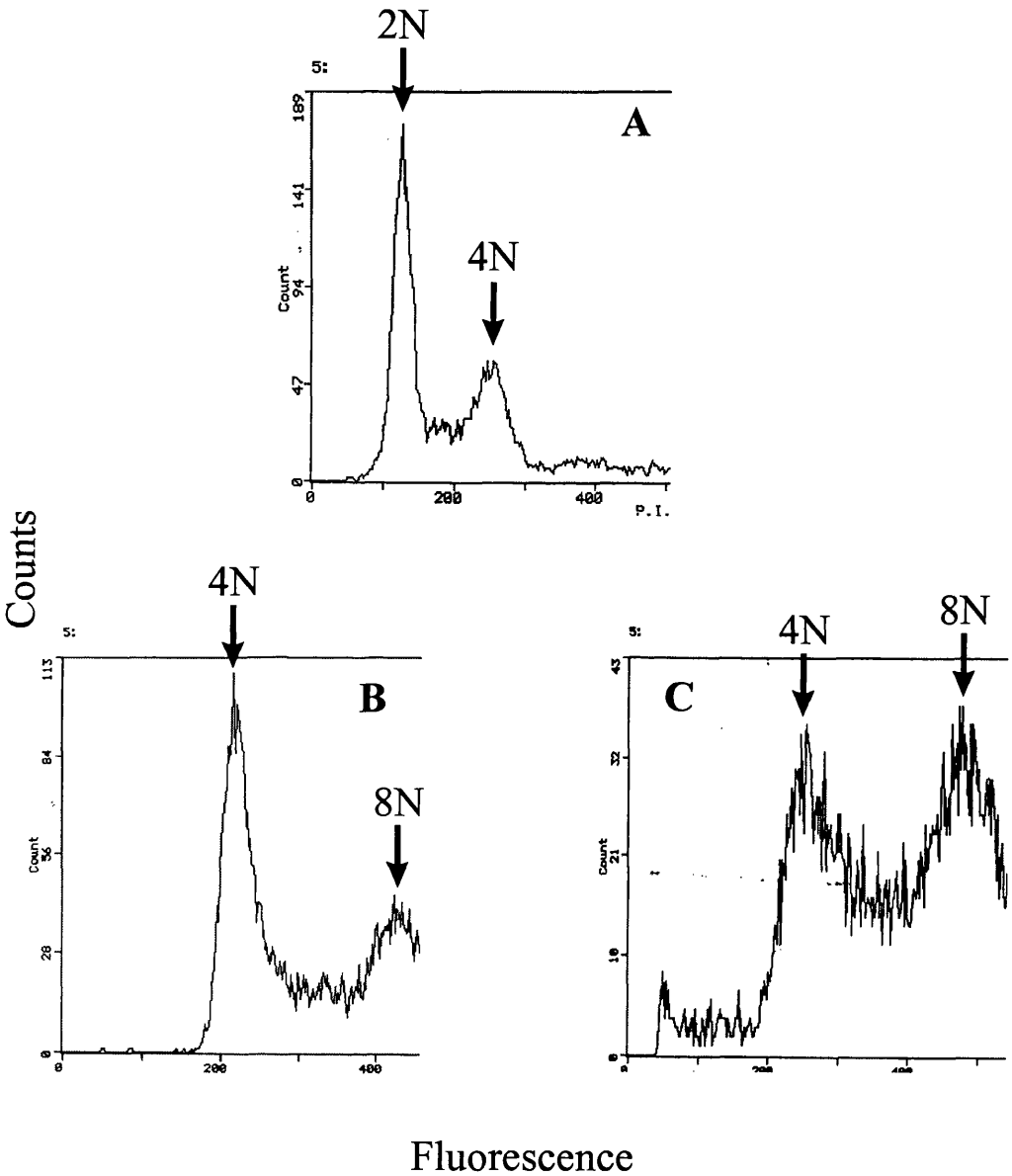
Fig. 3.12 DNA content analysis of clones derived from transfection experiment 3.2.4

Clones derived from experiment 3.2.4 were analysed by FACS in order to determine their overall DNA content. Cells were fixed and stained with propidium iodide and 10,000 cells were analysed on a Epics/XL flow cytometer (Coulter).

Panel A. Wild type

Panel B. W669

Panel C. W669b


2N
4N
A
Count
P.I.
Counts
4N
8N
B
C
Fluorescence

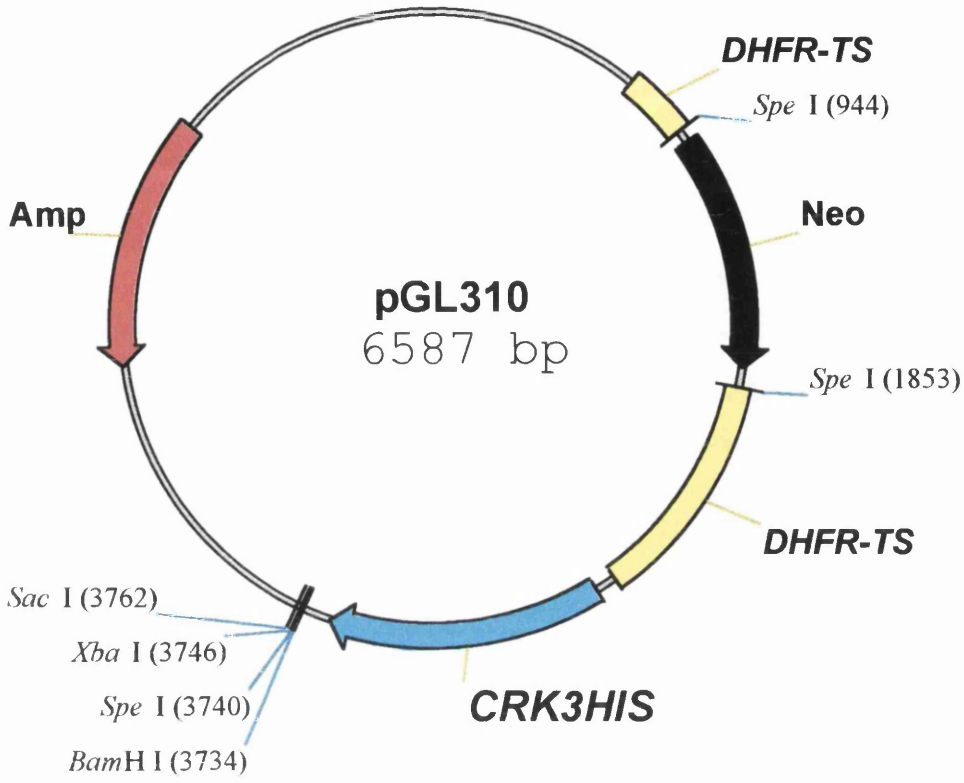


Fig. 3.13 Restriction map of the pGL96 (pX*CRK3HIS*) episome

The modified, hexahistidine tagged, *CRK3* gene can be expressed from the pX episome in *L. mexicana* to produce active kinase. This episome contains both the Amp^r and the *NEO* genes, allowing selection in either *E. coli* or *Leishmania*. The plasmid also contains *L. major* DHFR-TS flanking sequence which provides trans-splicing and polyadenylation signals.

Fig. 3.14 DNA content of clones generated as described in section 3.2.5

The DNA content of the clones derived from experiment 3.2.5 was assessed by FACS analysis. Cells were fixed and stained with propidium iodide and 10,000 cells were analysed on a Epics/XL flow cytometer (Coulter).

Panel A. Wild type

Panel B. W925H

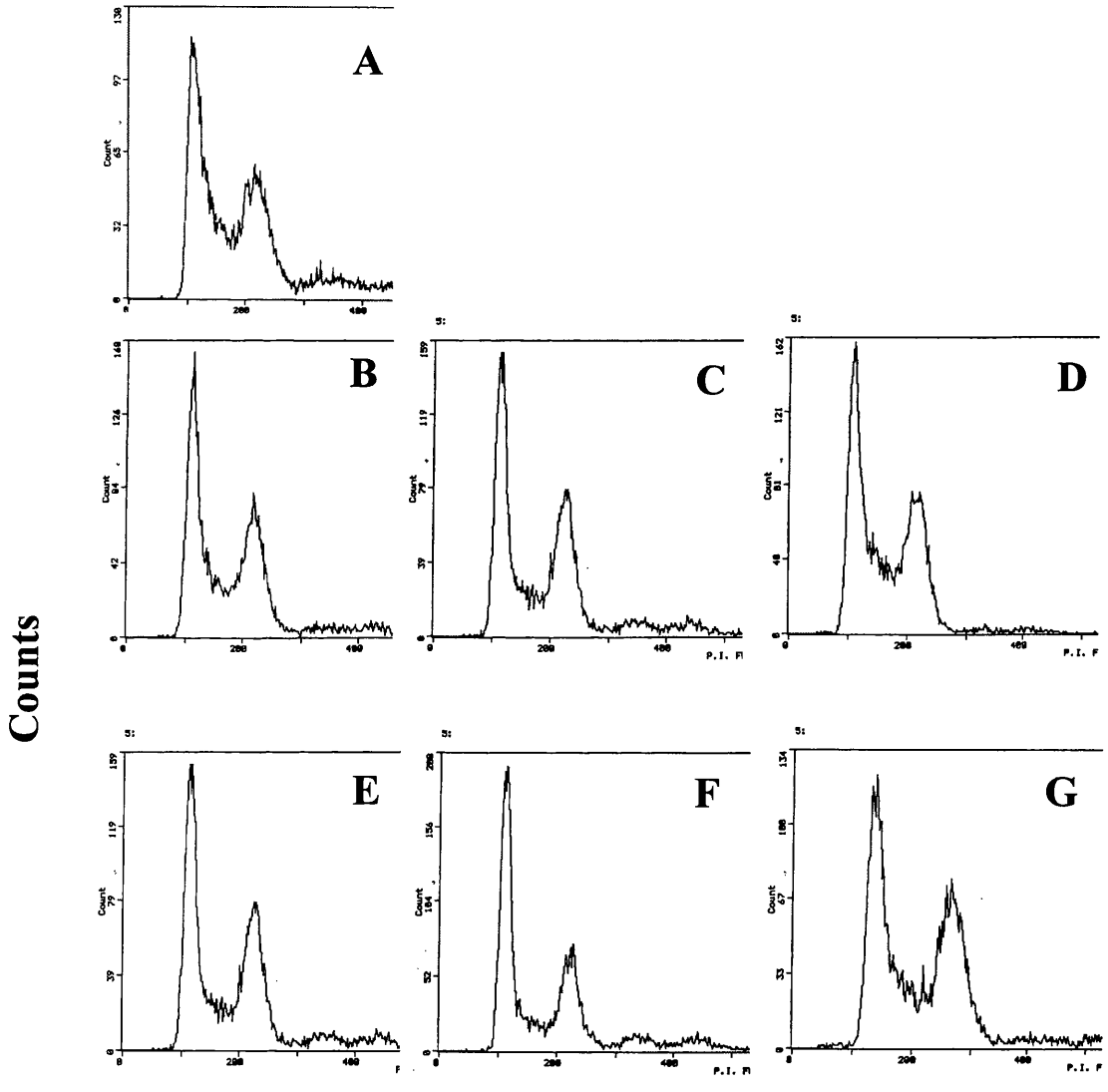
Panel C. W945

Panel D. W946

Panel E. W947

Panel F. W948

Panel G. W949


A
B
C
D
E
F
G
Counts
Fluorescence

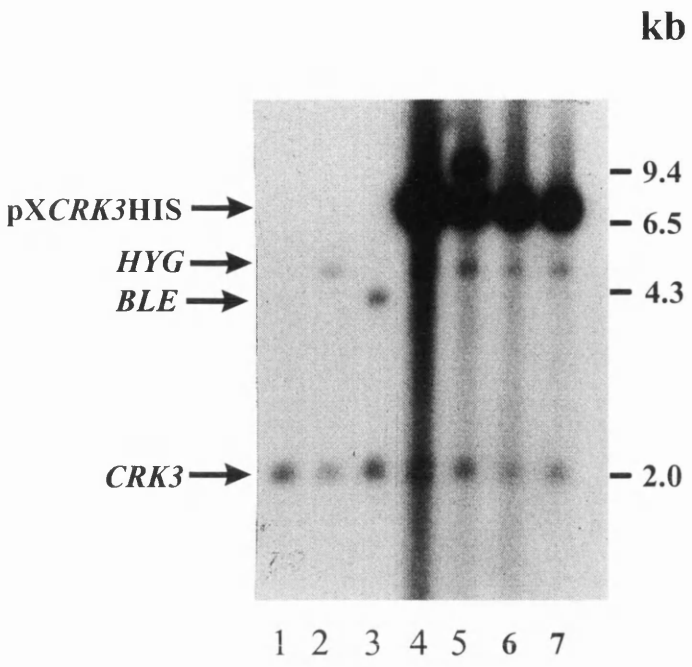


Fig. 3.15 Southern blot analysis of clones derived from experiment 3.2.4

5μg of genomic DNA was digested with *Hin*dIII, separated by agarose gel electrophoresis, transferred to positively charged nylon membrane and probed with the 2.0 kb *Hin*dIII fragment of plasmid pGL89, which contains the complete *CRK3* coding sequence.

Lane 1. Wild type

Lane 2. W583

Lane 3. W585

Lane 4. W945

Lane 5. W946

Lane 6. W947

Lane 7. W948

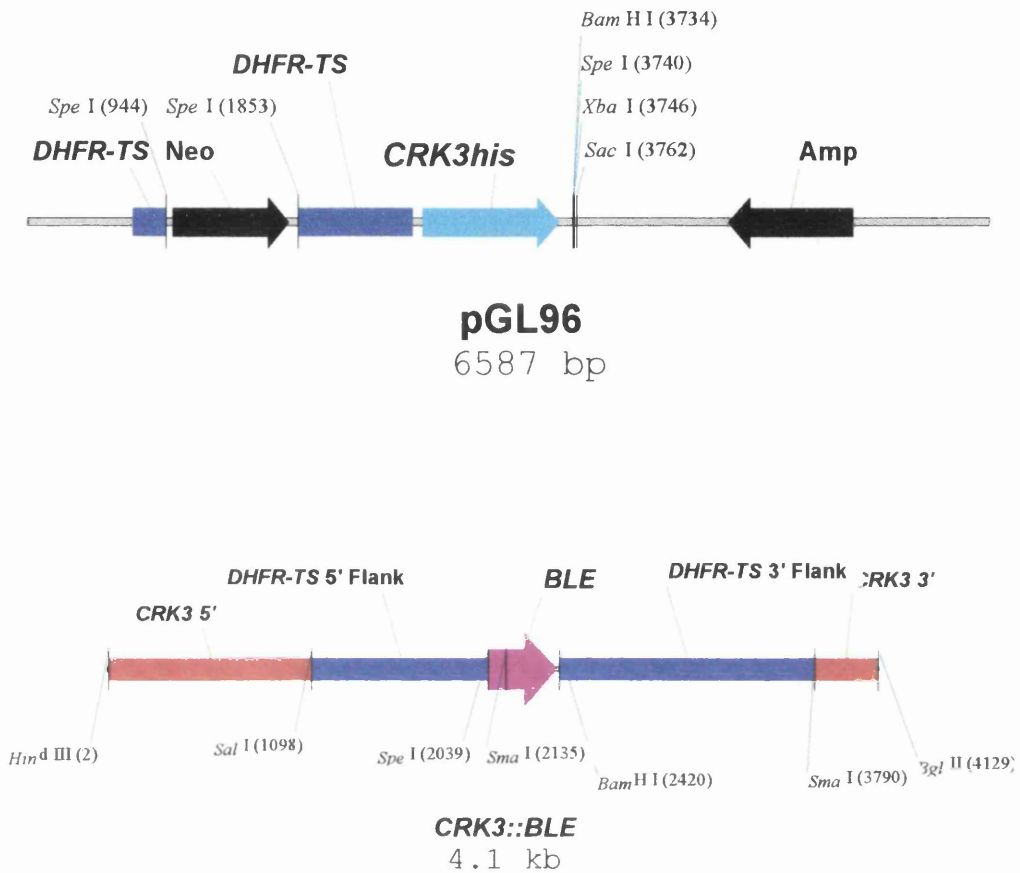


Fig. 3.16 Comparison of homology between the pGL96 and *CRK3::BLE* constructs

Both the pGL96 episome, and *CRK3::BLE* constructs both contain extensive regions of homology between the *DHFR-TS* sequence present in both constructs (blue regions). Degradation of the prepared *CRK3::BLE* targeting fragment could conceivably expose the *DHFR-TS* sequence. This may allow homologous recombination to occur between the targeting fragment and the episome.

Fig. 3.17 DNA content analysis of geneticin sensitive clones derived from experiment 3.2.6

The DNA content of the Geneticin sensitive mutant clones described in section 3.2.6 was determined by FACS analysis. Cells were fixed and stained with propidium iodide and analysed on a Epics/XL flow cytometer (Coulter). A total of 10,000 cells were analysed.

Panel A. Clone W991

Panel B. Clone W991

Panel C. Clone W992

Panel D. Clone W993

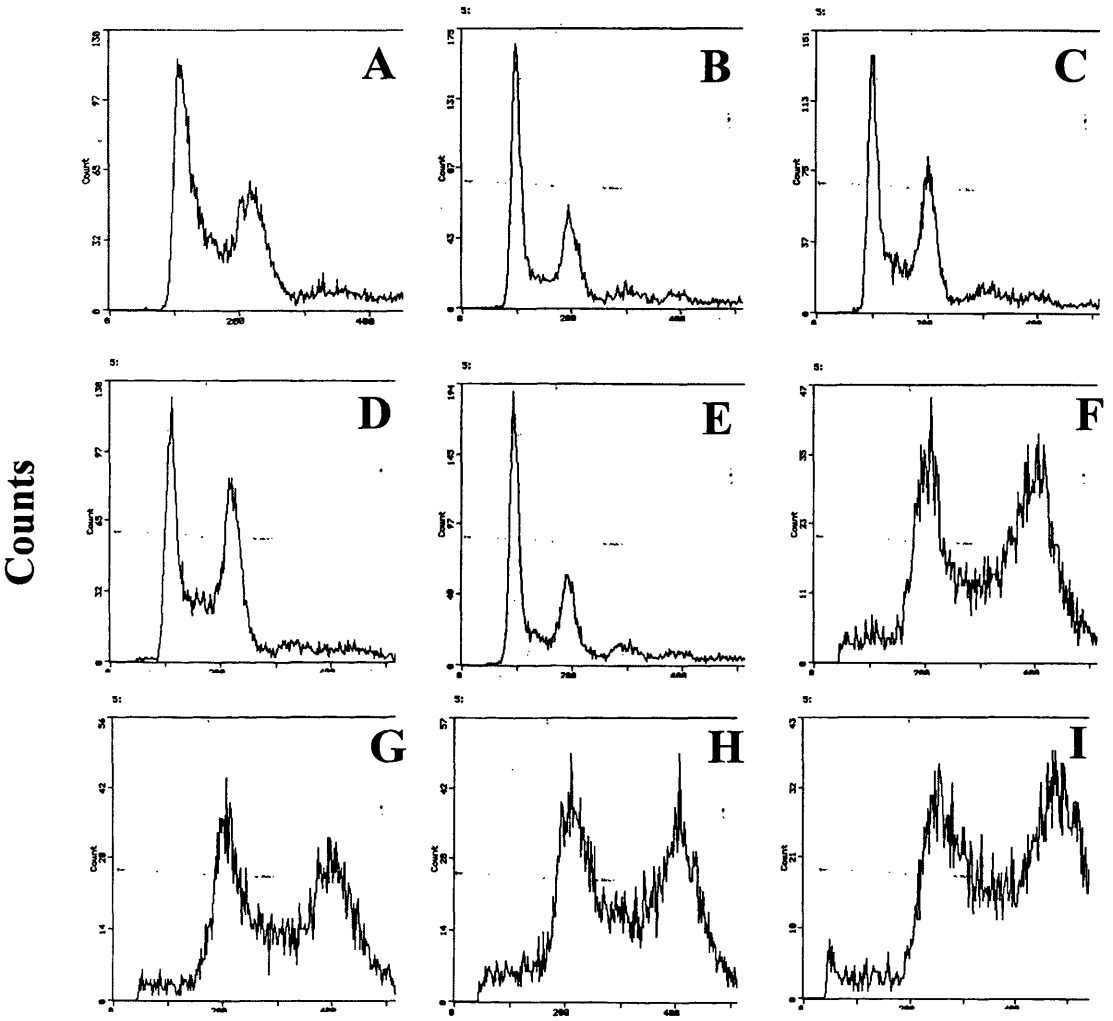
Panel E. Clone W994

Panel F. Clone NS1

Panel G. Clone NS2

Panel H. Clone NS3

Panel I. Clone NS4


A
B
C
D
E
F
G
H
I
Counts
Fluorescence

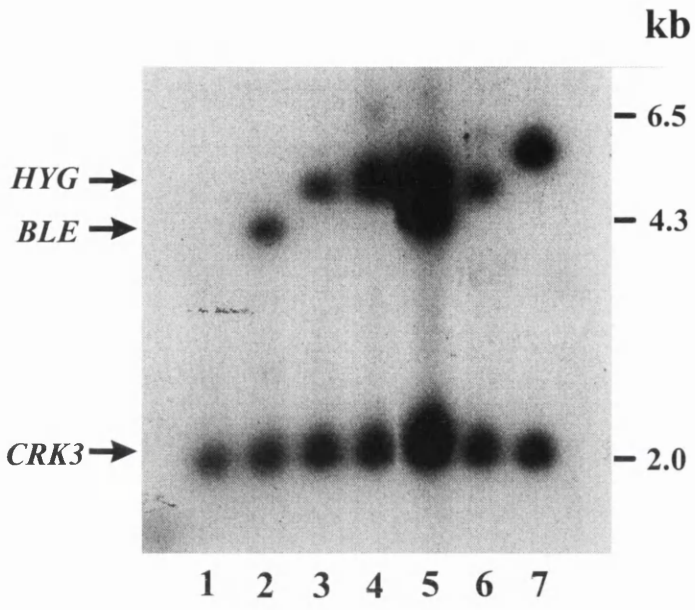


Fig. 3.18 Southern blot analysis of diploid, Geneticin sensitive clones derived from experiment 3.2.6

5µg of genomic DNA was digested with *Hin*dIII, separated by agarose gel electrophoresis, transferred to a positively charged nylon membrane and probed with the 2.0 kb *Hin*dIII fragment of plasmid pGL89. This fragment contains the complete *CRK3* sequence.

Lane 1. Wild type

Lane 2. W583

Lane 3. W585

Lane 4. NS1

Lane 5. NS2

Lane 6. NS3

Lane 7. NS4

Fig. 3.19 DNA content analysis of clones derived from experiment 3.2.6

The DNA content of clones derived from experiment 3.2.6 was analysed by FACS. Cells were fixed and stained with propidium iodide and analysed on a Epics/XL flow cytometer (Coulter). A total of 10,000 cells were analysed.

Panel A. W583

Panel B. W995

Panel C. W996

Panel D. W997

Panel E. W998

Panel F. W999

Panel G. W1000

Panel H. W1001

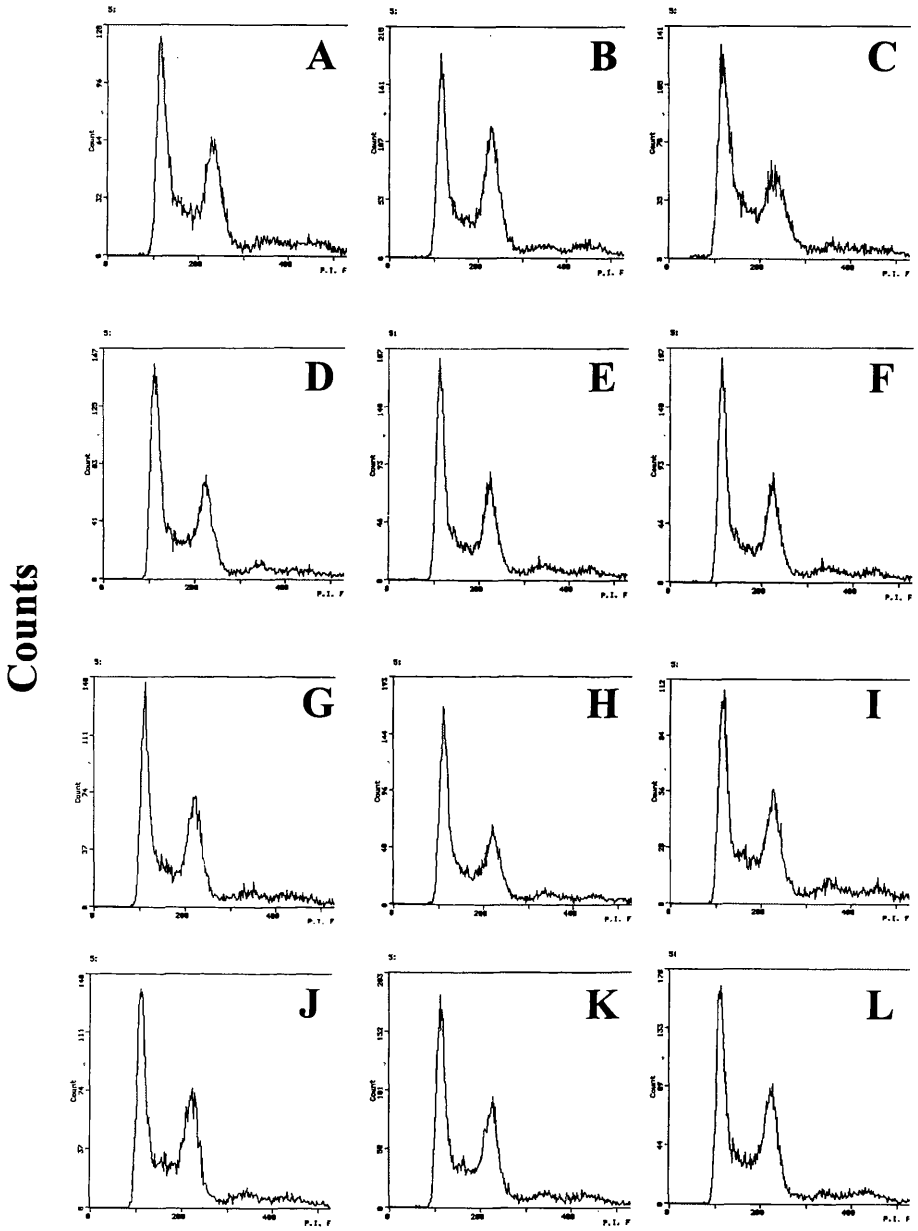
Panel I. W1002

Panel J. W1003

Panel K. W1004

Panel L. W1005

Panel M. W1006


A
B
C
D
E
F
G
H
I
J
K
L
Counts
Fluorescence

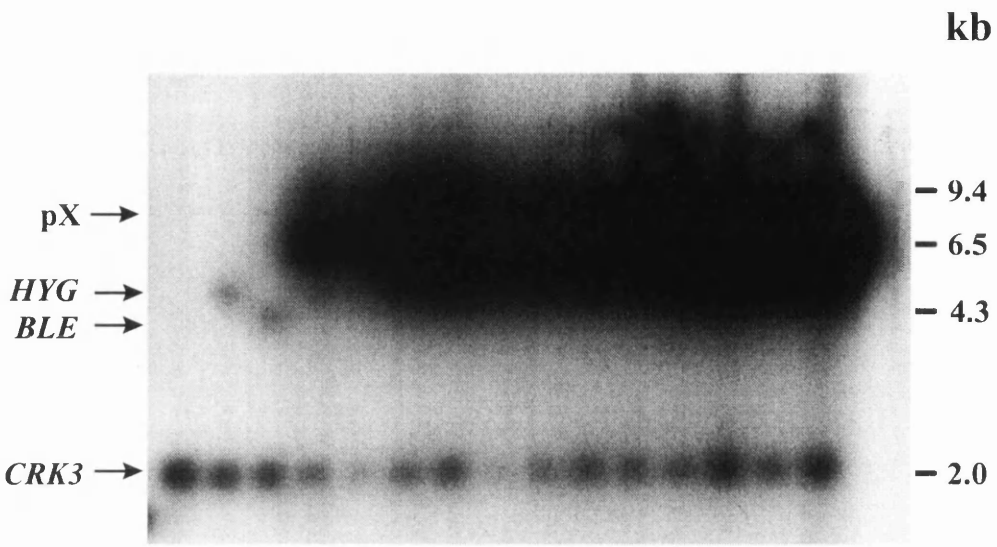


Fig. 3.20 Southern blot analysis of clones derived from experiment 3.2.6

5µg of genomic DNA was digested with *Hin*dIII, separated by agarose gel electrophoresis, transferred to positively charged nylon membrane and probed with the 2.0 kb *Hin*dIII fragment of plasmid pGL89. This fragment contains the complete *CRK3* sequence.

Lane 1. Wild type

Lane 2. W583

Lane 3. W585

Lane 4. W995

Lane 5. W996

Lane 6. W997

Lane 7. W998

Lane 8. W999

Lane 9. W1000

Lane 10. W1001

Lane 11. W1002

Lane 12. W1003

Lane 13. W1004

Lane 14. W1005

Lane 15. W1006

Table 3.1 Nomenclature and genotype of *L. mexicana* clones derived from transfection experiments carried out in this study

The clones derived from the experiments described in sections 3.2.1 to 3.2.6 are listed above along with details of their observed drug resistance, ploidy and deduced genotype.

(A) Name of clone and experiment from which it was derived

(B) Drug resistance assessed by *in vitro* growth in the presence of 50μg ml^{-1} hygromycin B, 10μg ml^{-1} Geneticin (G418) or 10μg ml^{-1} phleomycin.

(C) Ploidy as assessed by FACS

(D) Genotype with respect to *CRK3* locus. Those indicated by an asterisk are predicted from the available data, others were verified by experimental evidence

(A) Clone (Expt.)	(B) Drug Resistance	(C) Ploidy	(D) Genotype
W583 (3.2.1)	Hyg	Diploid	*CRK3/CRK3::HYG*
W585 (3.2.1)	Ble	Diploid	*CRK3/CRK3::BLE*
W625 (3.2.1) W626	Hyg and Ble	Aneuploid	*CRK3/CRK3/CRK3::HYG/CRK3/BLE* *
W638 (3.2.2)	Hyg and Neo	Diploid	*CRK3/CRK3::HYG* [pTEX *CRK3*]
W635 (3.2.3)	Hyg, Ble and Neo	Diploid	*CRK3/CRK3::HYG/CRK3::BLE* [pTEX *CRK3*]
W632 (3.2.3) W633	Hyg and Ble	Diploid	*CRK3/CRK3::HYG/CRK3::BLE*
W1187 (3.2.4) W1188 W1189 W1191	Hyg, Ble and Neo	Diploid	*CRK3::HYG/CRK3::BLE* [pTEX *CRK3*]
W1190 (3.2.4)	Hyg, Ble and Neo	Diploid	*CRK3/CRK3::HYG/CRK3::BLE* [pTEX *CRK3*]
W1191 (3.2.4)	Hyg, Ble and Neo	Diploid	*CRK3::HYG/CRK3::BLE* [pTEX *CRK3*]
W669 (3.2.4) W669b	Hyg, Ble and Neo	Aneuploid	*CRK3/CRK3/CRK3::HYG/CRK3/BLE* [pTEX*CRK3*HIS] *
W925H (3.2.5)	Hyg and Neo	Diploid	*CRK3/CRK3::HYG* [pX*CRK3* HIS]
W945 (3.2.5) W946 W947 W948 W949 W950	Hyg, Ble and Neo	Diploid	*CRK3/CRK3::HYG* [pX*CRK3::BLE* HIS]
W991 (3.2.6) W992 W993 W994	Hyg Ble and Neo	Aneuploid	*CRK3/CRK3/CRK3::HYG/CRK3/BLE* [pTEX*CRK3*HIS] *
W995 (3.2.6) W996 W997 W998 W999 W1000 W1001 W1002 W1003 W1004 W1005 W1006	Hyg Ble and Neo	Diploid	*CRK3/CRK3::HYG/CRK3::BLE* [pX*CRK3* HIS]
NS1 (3.2.6) NS2 NS3 NS4	Hyg and Ble	Diploid	*CRK3/CRK3::HYG/CRK3::BLE*

Chapter 4

The effects of direct and indirect inhibition of CRK3 on cell cycle progression in *Leishmania mexicana*

4.1.1 Introduction

Cyclin dependent kinases (CDKs) are key regulators of the cell cycle thought to be ubiquitous throughout all eukaryotic organisms. Members of the CDK family have a role in cell cycle control through conserved mechanisms that are similar in all eukaryotes (Nigg, 1995). This is not surprising, as most cell types need to fulfil the same set of operations in order to survive. They need to integrate environmental signals, eg. cytokine or growth factor signals, availability of nutrients etc., with intracellular signals controlling cell growth, DNA replication, and cell division (Hartwell, 1995). These signalling events impinge on cell cycle control via the activity of CDKs that in turn phosphorylate a number of target proteins to control progression through the cell cycle (Nigg, 1993).

Members of the cyclin dependent kinase family of proteins control transition points between the G1/S and G2/M phases of the cell cycle. Such kinases are not constitutively active but have their activity regulated in a number of ways. Firstly; they require the binding of a cyclin partner protein to achieve full activity (Meijer *et al.,* 1989; Brizuela *et al.,* 1989). Monomeric CDKs have almost no kinase activity (Lees, 1995). CDKs are also regulated by phosphorylation and dephosphorylation of a number of critical residues. Phosphorylation of Thr 161 is required for full activity whereas phosphorylation of Thr 15 has an inhibitory effect on the kinase (Gu *et al.,* 1992).

Interference with ATP binding, or inhibition of the dephosphorylation of the inhibitory phospho-Thr 15 can therefore result in inactivation of CDKs. The CKI family of cyclin dependent kinase inhibitor molecules, inhibit CDK activity by binding to the cyclin/CDK complex, causing conformational changes and blocking the ATP binding site (Russo *et al.,* 1996).

CDK activity can also be inhibited by a number of structurally unrelated chemical compounds that act through the same mechanism; competitive binding to the ATP binding site (Meijer, 1995). Chemical inhibitors of CDKs are of interest not only because they can be used as tools to analyse aspects of cell cycle control by CDKs, but also because they may have therapeutic value in the treatment of cancer. The most promising inhibitor of CDKs in this respect is flavopiridol, a semi-synthetic flavone, structurally related to a natural alkaloid, originally purified from the bark of *Dysoxylum binectariferum*, a plant indigenous to India (Sedlacek *et al.,* 1996). Flavopiridol was found to be a potent inhibitor of a number of tumour cell lines, and reduced the growth of human tumour xenografts in nu/nu mice (Sedlacek *et al.,* 1991; Czech *et al.,* 1995). Flavopiridol can inhibit cdk1 (Losiewicz *et al.,* 1994), and cdk2 and 4 (Carlson *et al.,* 1996), and blocks cells in either G1 (Kaur *et al.,* 1992), or G2 (Worland *et al.,* 1993). It has also been shown to affect S-phase progression in *Plasmodium falciparum* (Graeser *et al.,* 1996). The structure and inhibitory properties of flavopiridol against a number of protein kinases is shown in Figure 4.1. Flavopiridol has undergone phase I clinical trials and is currently undergoing phase II trials for the treatment of therapy-resistant progressive tumour diseases (Sedlacek *et al.,* 1996; Stadler *et al.,* 1998). In this study flavopiridol was tested for its effect on *L. mexicana* promastigote growth, and for its inhibitory effect against CRK3, as a method to analyse cell cycle progression of *L. mexicana* promastigotes in greater detail.

Synchronisation of mammalian cells by microtubule drugs, such as nocodazole (Zieve *et al.,* 1980) or aphidicolin, an inhibitor of DNA polymerase α (Pedrali-Noy *et al.,* 1980), has aided the biochemical analysis of cell cycle progression in mammalian cells. An efficient method for synchronising cultures of *Leishmania* would aid the analysis of the biochemistry of cell cycle progression. Many genes involved in cell cycle control, such as cyclins, may be present only at certain times in the cell cycle, as is true for most other eukaryotes. Synchronisation would also allow a more detailed analysis of the roles of CRK1 and CRK3 in cell cycle progression.

A number of methods to synchronise *Leishmania* and *T. brucei* have been used previously without success. Aphidicolin and hydoxyurea result in a cell cycle arrest of bloodstream form *T. brucei*, with cells accumulating in G2 phase of the cell cycle with two kinetoplasts and one nucleus. After release from inhibition there is a 24-hour lag period before cells begin to divide. Re-entry into the cell cycle occurs asynchronously (Mutomba and Wang, 1996). Hydroxyurea has been used to successfully synchronise *Crithidia fasciculata* (Cosgrove *et al.,* 1979) and *Leishmania tarentolae* (Simpson and Braly, 1970). However attempts to use hydroxyurea to synchronise *L. mexicana* promastigotes did not work effectively (J.C. Mottram, personal communication). The use of anti-microtubule agents does not result in a cell cycle block but causes disruption of organelle segregation and cytokinesis, resulting in cell division and formation of a nucleated cell and an anucleate, flagellated cell termed a zoid (Robinson *et al.,* 1995). Inhibition of purine salvage by the purine analogue (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine ((S)-HPMPA) in *T. brucei* results in arrest during S-phase. Again it was not possible to synchronise cells using this inhibitor, as there was a significant lag period before all cells had re-entered the cell cycle (Kaminsky *et al.,* 1998). This chapter describes the use of two independent methods to attempt to

synchronise *Leishmania* in order to study in more detail the function of CRK3 in cell cycle progression.

4.2 Inhibition of *Leishmania* growth by the protein tyrosine phosphatase inhibitor bpV(phen)

A study carried out in parallel, and recently published, has shown that growth of *L. major* amastigotes was inhibited by the protein tyrosine phosphatase inhibitor bpV(phen) (Olivier *et al.,* 1998). bpV(phen) is potent phosphotyrosine phosphatase inhibitor and acts as an insulin mimetic *in vivo* by blocking the dephosphorylation of the insulin growth factor receptor (Posner *et al.,* 1994). bpV(phen) also arrests the growth of cultured mammalian cells (Faure *et al.,* 1995). This growth inhibition is due to inhibition of cell cycle progression, blocking cells in the G2 phase of the cell cycle. Growth arrest of *L. major* by bpV(phen) was also shown to be due to inhibition of cell cycle progression. Cells incubated in the presence of bpV(phen) accumulated in the G2 phase of the cell cycle, as assessed by FACS. It was proposed that this cell cycle block resulted from inhibition of the *Leishmania* homologue of the fission yeast Cdc25 phosphotyrosine phosphatase (Olivier *et al.,* 1998). In fission yeast and human cells the Cdc25 phosphatase has an important role in regulating the function of Cdc2/cdk1. Phosphorylation of the Tyr 15 residue of Cdc2/cdk1 by the protein tyrosine kinase Wee1 results in inactivation of the Cdc2/cdk1 kinase complex (Parker and Piwnica-Worms, 1992). Dephosphorylation of Tyr 15 by Cdc25 at the end of the G2 phase of the cell cycle results in restoration of Cdc2 activity and triggers entry into mitosis (Hoffman and Karsenti, 1994). Temperature sensitive mutants of *cdc25* arrest in late G2, prior to mitosis (Moreno *et al.,* 1989; Booher *et al.,* 1989). As this arrest phenotype is similar to

the cell cycle arrest of *Leishmania* caused by bpV(phen), it was proposed that this was due to inhibition of a phosphotyrosine phosphatase activity in *L. major* that is homologous to the *S. pombe* Cdc25 tyrosine phosphatase. It was shown that a 35 kDa protein in *Leishmania* cross-reacts with a monoclonal antibody raised against the highly conserved PSTAIR epitope of human cdk1 (the human homologue of *S. pombe* cdc2). This 35 kDa protein was immunoprecipitated from cell extracts of parasites arrested by incubation with bpV(phen). Western blot experiments on immunoprecipitated material, with an antibody raised against the phosphorylated Tyr 15 residue of cdk1, showed that the putative *L. major* cdk1 homologue cross reacted with this antibody, suggesting that the corresponding residue in the *Leishmania* CDK1 homologue is phosphorylated in arrested cells (Olivier *et al.*, 1998).

The experiments outlined in this section were performed in order to determine whether bpV(phen) inhibited CRK3 in *L. mexicana*, and also to test the applicability of bpV(phen) mediated cell cycle arrest as a method for studying cell cycle progression in *Leishmania*. bpV(phen) was initially provided as a kind gift by Dr. M. Olivier, Laval University, Quebec, Canada, and was later obtained from Alexis corporation.

4.2.1 Incubation of *Leishmania* promastigotes with bpV(phen) results in a reduction of CRK3 activity

Cultures of *L. mexicana* promastigotes were seeded at a cell density of $1x10^7$ cells ml^{-1} and incubated for 12 or 24 hours in the presence or absence of 10 μM bpV(phen). Cells were harvested by centrifugation, washed in PBS and used for analysis of $p13^{suc1}$ binding kinase activity. $p13^{suc1}$ binding kinase activity had previously been shown to be CRK3 (Grant *et al.*, 1998).

Cell pellets were lysed in lysis buffer containing glycerol (LSG) and the extracts were incubated with either $p13^{suc1}$ bound Sepharose beads or with control Sepharose beads. The beads were pre-incubated in BSA and then washed with a high salt buffer (HSLS), to remove non-specifically binding components. Kinase activity, bound to the beads, was assessed by resuspending the samples in kinase assay mix (KAM) containing radiolabelled ^{32}P-γ-ATP and histone H1 substrate. Kinase reactions were incubated at 30°C for 20 minutes before the reaction was stopped by addition of SDS PAGE sample buffer. Reactions were boiled and separated by electrophoresis on a 12% SDS PAGE gel. Gels were dried, wrapped in cellophane and exposed to photographic film. Relative kinase activity was assessed by phosphorimaging. The results of the autoradiograph and phosphorimaging analysis are shown in Fig. 4.2. The structure of bpV(phen) is shown in panel A. Panel B shows the results of the autoradiograph. Lanes labelled p13 correspond to samples incubated with $p13^{suc1}$ beads and lanes labelled C correspond to samples incubated with control beads. Panel C shows the relative values obtained from the phosphorimaging analysis. Incubation of *L. mexicana* for 12 hours with 10 μM bpV(phen) does not result in any loss of $p13^{suc1}$ binding kinase activity (compare panel B, lane 5 with lane 1 and panel C, column 5 and column 1), however, incubation for 24 hours results in a complete reduction of $p13^{suc1}$ binding kinase activity to background levels (compare panel B, lane 7 with lanes 3 and 4, and panel C, column 7 with columns 3 and 4). The results of cell cycle analysis, by FACS, of cells incubated with 10 μM bpV(phen) for 12 or 24 hours are shown in Fig. 4.3. Panel A corresponds to a control sample of cells incubated for 12 hours in the absence of bpV(phen). Panel B indicates that cell cycle progression is not affected by a 12-hour incubation with 10 μM bpV(phen), whereas panel D shows that incubation for a further 12 hours resulted in an accumulation of cells in the G1/S phase of the cell cycle. Such an accumulation of cells

in G1 is not observed in the control sample after the same incubation time (panel C). The cell cycle arrest caused by 24 hour incubation with bpV(phen) is coincident with the loss of $p13^{suc1}$ binding kinase activity as shown in Fig. 4.2, lane 7. These results together suggest that bpV(phen) may indeed inhibit the as yet unidentified *Leishmania* homologue of Cdc25. Loss of such activity would be predicted to result in the retention of the inhibitory phosphorylation at the Tyr 34 residue of CRK3, the *Leishmania* equivalent of the Cdc2 Tyr 15 residue, which is dephosphorylated by Cdc25 (Millar *et al.,* 1991). To test whether the Tyr 34 residue of CRK3 was phosphorylated, western blot analysis was performed using an antibody raised against the phosphorylated Tyr 15 epitope of human cdk1. CRK3 kinase from cells incubated for 24 hours with 10 μM bpV(phen), was purified by affinity binding to $p13^{suc1}$. Purified kinase was run on a 12% SDS-PAGE gel and transferred to PVDF membrane, which was then blocked and probed with the anti-phospho-Tyr 15 antibody. No signal was detected using this antibody. It is possible that this antibody is unable to cross-react with the analogous epitope on the *L. mexicana* CRK3 protein due to amino acid differences in this region of the protein. The antibody recognises the epitope, KIEKIGEGTY(P)GVVYKGRHK. The analogous epitope of *L. mexicana* CRK3, RLDVLGEGTY(P)GVVYRAVDK, differs by nine amino acids from the epitope recognised by the antibody.

4.2.2 Restoration of CRK3 activity and re-entry into the cell cycle upon release from bpV(phen) induced growth arrest.

Given that bpV(phen) causes a G1/S cell cycle arrest in *L. mexicana* we tested whether this arrest was reversible and could be used as a method of obtaining a synchronous population of *Leishmania*, progressing uniformly through the cell cycle. *L. mexicana*

promastigotes were seeded at a density of $1x10^7$ cells ml^{-1} in HOMEM medium containing 10 μM bpV(phen) and incubated at 25°C for 24 hours. Cells were then harvested, washed twice in cold PBS and resuspended in fresh complete medium lacking bpV(phen). Cells were incubated at 25°C and samples were taken at 2-hour time points for 12 hours and samples were prepared for FACS analysis and for determination of $p13^{suc1}$ binding kinase activity. Results of the $p13^{suc1}$ binding kinase assay are shown in Fig. 4.4. Panel A shows the autoradiography results and panel B shows the relative signal intensity as judged by phosphorimaging analysis. Lane 1 corresponds to the background activity binding to control beads. After 24 hours in the presence of bpV(phen) the $p13^{suc1}$ binding activity has been significantly reduced (lane 2). After washing to remove the inhibitor, this activity increases (lanes 2-7), reaching a peak after 10 hours (lane 7). FACS analysis indicates that the majority of cells incubated with bpV(phen) for 24 hours are arrested in the G1 and S-phases of the cell cycle (Fig. 4.5 panel B). Washing the cells to remove the inhibitor reverses this arrest, resulting in re-entry into and progression through the cell cycle (panels C to I). Re-entry into a normal cell cycle was, however, only partially synchronous. A significant lag period existed before some of the cells began to progress through S-phase, G2 and mitosis. Panel C, shows that the proportion of cells in G2 has increased, two hours after release from the bpV(phen) block. There is still a significant proportion of cells in G1 or S-phase. After 4 hours (panel D) the majority of cells are in S-phase or G2, but with a significant number still in G1. After the 6 hour time point, most cells are in G2, with far fewer cells in G1 or S-phase (panel E). From 8 to 12 hours the cell cycle profile of the population reverts back to the standard profile as the population becomes less synchronised. The apparent lag in cell cycle progression in a proportion of the population is probably due to the fact that the population was not uniformly arrested at one defined point in the cell cycle but

was arrested at various points throughout G1 and S-phase. Cells arrested in late G1 or S-phase will enter into mitosis before those cells arrested in early G1, which must progress through the remainder of G1 phase, through S-phase and G2 before entering mitosis. It appears that it is not therefore possible to use bpV(phen) as a chemical method of synchronising cultures of *Leishmania* to allow a more detailed biochemical analysis of cell cycle progression. The compound, however, could be useful in the analysis of regulatory phosphorylation of key residues of CRK3, such as Tyr 34. Cell labelling studies, using radiolabelled ^{32}P γ-ATP, in conjunction with the use of bpV(phen) may help to determine the role of phosphorylation and dephosphorylation of key residues of CRK3 in regulating its kinase activity throughout the cell-cycle and life cycle.

4.3 Inhibition of CDKs by chemical inhibitors

Because CDKs play a significant part in controlling the growth and division of cells they have been the subject of intensive research aimed at elucidating their precise roles. This research has led to the discovery of chemical inhibitors that may have therapeutic value in treating a number of diseases associated with uncontrolled cell proliferation, such as occurs during tumour development. Some of these compounds may also prove valuable in the treatment of infection with rapidly dividing eukaryotic pathogens (Graeser *et al.,* 1996).

A number of these CDK inhibitor compounds now exist (see chapter1, section 1.7). Most of these compounds, though structurally very different, inhibit CDKs in a similar way, by competitive binding to the ATP binding site of the kinase complex (Meijer, 1995).

A previous study has indicated that the CRK3 kinase of *Leishmania* shows a similar inhibition profile to Cdc2 with the inhibitor, olomoucine (Grant *et al.,* 1998). It has not proved possible to generate null or conditional mutants of *CRK3* to analyse the possible role of CRK3 in cell cycle control in *Leishmania* (see chapter 3). Therefore, attempts were made to use flavopiridol, a chemical inhibitor of CDKs to study the effects of inhibition of CRKs on cell cycle progression in *Leishmania*.

Flavopiridol is a potent inhibitor of CDKs (see Fig. 4.1 for the structure and properties of flavopiridol), most active against the cdk1, cdk2 and cdk4 kinases of mammalian cells (Losiewicz *et al.,* 1994; Carlson *et al.,* 1996).These are the kinases with a most important role in cell cycle control. Flavopiridol has been shown to inhibit the growth of a number of human tumour cell lines and is undergoing clinical trials as a potential anti-tumour agent (Carlson *et al.,* 1996).

4.3.1 Inhibition of *Leishmania* Promastigote Growth by the CDK Inhibitor Flavopiridol

Cultures of *Leishmania* promastigotes were seeded in triplicate at a density of 1.0x 10^6 cells ml^{-1} and incubated in the presence of a range of concentrations of flavopiridol. Cells were counted at 24 hour time points and the mean value of triplicate cell counts was plotted (Fig. 4.6). Growth of *Leishmania* was inhibited by flavopiridol in a dose dependent manner. A concentration of 1.0 μM resulted in complete arrest of promastigote growth, whereas lower concentrations only partially inhibited cell growth and higher concentrations resulted in cell death. 50% inhibition of cell growth was achieved at a concentration of approximately 250 nM. Because flavopiridol is a known chemical inhibitor of CDKs it is likely that the growth inhibition effect is due to the

inhibition of one or more of the *Leishmania* CRKs that are homologues of CDKs. It is possible that the growth inhibition effect is due to inhibition of other kinases involved in growth control, however this is unlikely as flavopiridol does not inhibit other classes of kinases to the same extent as CDKs (see Fig. 4.1, Panel B).

4.3.2 Flavopiridol inhibits CRK3 with an IC_{50} value of 100 nM.

CRK3his protein was purified by Nickel NTA affinity chromatography from the W1033 cell line that expresses native and his-tagged CRK3 (see Chapter 3, Table 3.1). Bound kinase was eluted from the beads with 100 mM EDTA and was then bound by affinity binding to $p13^{suc1}$ Sepharose beads. This protocol follows that used for the assay of inhibition by olomoucine (Grant *et al.,* 1998) and therefore provides a direct comparison of the inhibitory properties of olomoucine and flavopiridol against CRK3. It has been shown previously that active kinase complex from *Leishmania* cell extracts binds to $p13^{suc1}$ beads and that the CRK3 protein is the major catalytic sub-unit of this kinase activity (Grant *et al.,* 1998).

Samples of kinase bound to $p13^{suc1}$ beads were aliquoted and flavopiridol was added to each sample at a different concentration. Kinase activity bound to the beads was assessed using the previously described histone H1 kinase assay. The reaction was halted by the addition of SDS sample buffer and samples were boiled for 5 minutes prior to loading on a 12% SDS PAGE gel. Gels were dried, wrapped in cellophane and exposed to a phosphorimaging plate for analysis and quantification of kinase activity. The experiment was repeated three times. The results of a representative experiment are shown in Fig. 4.7 The plotted results give an IC_{50} value of 100 nM that is comparable to IC_{50} values for a number of human CDKs (other experiments gave IC_{50} values of 80 and

130 nM). These results indicate that flavopiridol is a potent *in vitro* inhibitor of CRK3, the $p13^{suc1}$ binding kinase activity of *L. mexicana*. It is not yet known however, what effect, if any, flavopiridol has on other CRKs in *Leishmania* and it cannot be concluded from these results alone that inhibition of CRK3 activity by flavopiridol is responsible for inhibition of promastigote growth *in vivo*.

4.3.3 Flavopiridol blocks promastigote growth by causing cells to arrest in G2 phase of the cell cycle.

Given that CRK3 is a putative controller of cell cycle progression and is inhibited *in vitro* by flavopiridol then it is probable that the inhibition of promastigote growth caused by flavopiridol is due to disruption of normal cell cycle progression. Flavopiridol has been shown to disrupt G1 to S-phase progression in *Plasmodium falciparum* (Graeser *et al.*, 1996) and causes G1/S and G2/M cell cycle arrests in various human cell lines (Kaur *et al.*, 1992; Worland *et al.*, 1993)

Cultures of *Leishmania* were set up at a density of $1x10^6$ cells ml^{-1} in the presence of 0, 1.0, 2.5 or 5.0 μM flavopiridol. 1ml samples were taken at 0, 12, 18 and 24 hour time points. These samples were fixed, stained with propidium iodide and analysed by FACS to determine the overall DNA content and to gain information on the cell cycle distribution of the cell samples.

The results of the FACS analysis of all the samples analysed is shown in Fig. 4.8. At a concentration of 2.5 μM flavopiridol leads to an accumulation of cells with a 4N DNA content after a period of 12 hours (panel J). As these cells are not dividing and have 4N DNA content, they must be arrested in the G2 phase of the cell cycle, after DNA replication, but before cell division. Cells blocked with flavopiridol for a longer time

period, or at higher concentrations of flavopiridol, begin to die resulting in a wider spread of fluorescence intensity signals as their nuclei break apart (Fig. 4.8, panels G, H, and P. At lower concentration the block is not complete, panel F.

Quantitative analysis of the FACS results using the ModFit LT software package allows the determination of the proportion of cells in each cell cycle phase. The results of such analysis are shown in Fig. 4.9. Panel A shows the results of FACS analysis. Panel B shows the results of ModFit analysis of the FACS histogram. Column 1 shows that approximately 45% of cells in this sample, from a log-phase promastigote culture, are in the G1 phase of the cell cycle. 20% of cells are in S-phase (column 2), and approximately 36% are in G2 phase (column 3). The cell cycle distribution of cells arrested by a 12-hour incubation with 2.5 μM flavopiridol is very different (panel C). Less than 5% of cells are in G1 (Panel D, column 1) and approximately 17% are in S-phase (column 2). The majority of cells, greater than 75%, in this population have been arrested in G2-phase of the cell cycle (column 3). These results are confirmed by fluorescence microscopy of cells stained with the DNA binding dye 4',6-diamidino phenylindole (DAPI). In an untreated log-phase culture dividing cells with two kinetoplasts and 2 nuclei represent approximately 12% of cells. Such cells are shown in Fig. 4.10. Panel A shows a phase contrast image and panel B shows the corresponding DAPI image. Cells blocked with flavopiridol are predominantly single cells with one kinetoplast and one nucleus (Panel D). Such cells make up approximately 76% of the total number of cells.

This cell cycle arrest is likely to be due to the *in vivo* inhibition of CRK3 because of it's presumed role as the *Leishmania* Cdc2 homologue. This experiment does not however rule out the possibility that flavopiridol inhibits other Cdc2 homologues in *L. mexicana* that may also contribute to proper control of cell cycle progression. However, given that

CRK3 is strongly inhibited by flavopiridol *in vivo*, and that other evidence strongly suggests that CRK3 is the functional homologue of Cdc2 in *Leishmania* (Grant et al., 1998), it seems likely that the observed G2/M block, resulting from incubation of *L. mexicana* promastigotes in the presence of flavopiridol, is due primarily to inhibition of CRK3.

4.3.4 Release from flavopiridol induced cell cycle arrest

To test whether the G2/M phase arrest caused by flavopiridol was reversible, log phase *L. mexicana* promastigotes were seeded at a density of $1x10^6$ cells ml^{-1} (-12 hr time point) and incubated for 12 hours in the presence of 2.5 μM flavopiridol. Cells were then washed twice in complete medium and resuspended in fresh, complete HOMEM minus flavopiridol (0 hr time point). Cells were incubated at 25°C and samples were prepared for cell cycle analysis by FACS at 2, 4 and 6 hours. The results of this analysis are shown in Fig. 4.11. Panel A shows the FACS results and panel B shows the results of ModFit analysis. At the -12Hr timepoint the sample shows the normal cell cycle distribution of logarithmically growing promastigotes. At time 0 hr, 95% of cells are blocked with a 4N DNA content, presumably in the G2 phase of the cell cycle. At time 0, cells were washed and resuspended in fresh medium to release from the flavopiridol-induced growth inhibition. 2 hours after release approximately 65% of cells still remain in G2 and 30% are in G1. After a further 2 hours (4 hr time point) approximately 70% of cells are in S-phase and approximately 10% are in G2 and a further 10% in G1. At the 6 hr time point 65% of cells are in G2, 17% in G1 and 18% in S-phase. These results show that washing the cells to remove the inhibitor reversed the G2/M arrest due to flavopiridol. After washing, cells divided and progressed through a full cell cycle.

Under these conditions however, cells are not uniformly released from the block. A significant sub-population of cells, approximately 65%, still remain in G2 phase after 2 hours when a large proportion of the cells, 30%, have divided and progressed into the G1 phase of the cell cycle. After 4 hours 70% of cells are progressing through S-phase, whilst 10% of cells remain in G1. These G1 cells may be the slow dividing sub-population that had not divided by 2 hours after release from the flavopiridol block. It is not clear why there should be a sub-population of cells that do not divide as promptly as the majority of cells do. It is possible that the G2 block induced by flavopiridol is not entirely uniform and that cells are blocked at different stages through G2. This may explain why some cells enter mitosis more rapidly than others upon release from the block. Those cells that are arrested in late G2 can divide very quickly, as soon as the block is released, however cells that were arrested in early G2 must progress through the remainder of the G2 phase before dividing.

To test the possibility that serum components are signalling cells to divide whilst they are being washed the experiment was repeated, this time washing the cells in serum free medium. The results of the FACS analysis (Fig. 4.11) were broadly similar to the results obtained for complete medium washed cells. A large proportion of cells, 72%, still remained in G2 at the 2 hr time point and divided within the next 2 hours to result in a population of cells mainly in G1 (41%) and S-phase (55%) at the 4 hr time point. The same experiment was also repeated using a PBS wash rather than complete or serum-free medium. The results (see Fig. 4.12) again do not show a completely synchronised re-entry into the cell cycle, however there is a larger proportion, 68%, of cells in the G1 phase of the cell cycle 2 hours after release from the block, and only 23% remain in G2. After a further 2 hours (4 hr time point), 70% of cells are in S-phase, whilst 12% and

18% are in G1 and G2 phase respectively. At the 6 hr time point the majority of cells, 61%, are in G2, whilst 21% and 18% are in G1 and S-phase respectively.

Although release from flavopiridol inhibition is not completely synchronous this method could certainly be used to enrich samples for cells in specific cell cycle stages. Samples taken at 2, 4, and 6 hours after release from the block are enriched for cells in G1 (68%), S-phase (70%), and G2 phase (61%) respectively. Such populations, enriched for cells at a particular stage of the cell cycle, could be used to purify and analyse cell-cycle regulated proteins.

4.3.5 Flavopiridol inhibits the growth of bloodstream and procyclic forms of *T. brucei*

The ability of flavopiridol to inhibit the growth of both bloodstream and procyclic culture forms of the monomorphic *T. brucei* strain STIB 427 was assessed. Triplicate cultures of procyclic or long slender bloodstream form trypanosomes were seeded at a density of $1x10^6$ and $1x10^5$ cells ml^{-1} respectively, in SDM-79 medium (Brun and Schonenberger, 1979) or HMI-9 medium (Hirumi and Hirumi, 1989). Cultures were incubated with a range of concentrations of flavopiridol at 27°C (for procyclics) or 37°C (bloodstream form cells). Cells were counted at various time intervals and the mean values of triplicate counts were plotted against time (Figs. 4.13 and 4.14). Flavopiridol affects the growth of both *T. brucei* bloodstream and procyclic forms. However there is a difference in the concentrations of inhibitor needed to result in a complete block of growth. Growth of procyclic trypanosomes is inhibited by 50% at a concentration of approximately 100 nM flavopiridol, whilst a concentration of 250 nM is required to inhibit the growth of the bloodstream form. This concentration is similar to that needed

to achieve 50% inhibition of *L. mexicana* promastigote growth (Fig. 4.6), however the starting density of the *T. brucei* bloodstream form cultures was tenfold less than that of the *L. mexicana* promastigote cultures or the procyclic cultures. Procyclics of *T. brucei* are therefore more sensitive to flavopiridol than the bloodstream form, and their growth inhibition resembles that of *L. mexicana* promastigotes, both of which are insect-adapted life cycle stages. However *T. brucei* procyclics treated with flavopiridol do not arrest exclusively at the G2/M transition but appear to arrest at both G1/S and G2/M. Fig. 4.16 shows the results of DNA content analysis of flavopiridol treated *T. brucei* procyclics. Panel A shows the FACS histogram of an untreated population. The proportions of cells in each phase of the cell cycle is shown in Panel B. 51% of cells are in G1, 33% in G2, and 16% in S-phase. In cells treated with 2.5 μM flavopiridol for 12 hours (Panel C) the cell cycle distribution of cells is as follows; 66% in G1, 33% in G2/M, and only 1% in S-phase (Panel D). The reasons for the difference in sensitivity to flavopiridol between procyclic and bloodstream trypanosomes may be due to structural differences in the target enzyme thereby altering the binding properties of flavopiridol, or may be due to a difference in uptake of the drug. It is not known whether flavopiridol diffuses passively into the cell or whether it specifically enters via receptor mediated endocytosis. The culture conditions of bloodstream trypanosomes are quite different from that of procyclic trypanosomes or *Leishmania* promastigotes. Differences in chemical composition of the media used, or differences in incubation temperature (37°C for bloodstream trypanosomes, 25°C and 27°C for procyclic trypanosomes and *Leishmania* promastigotes respectively) may affect the stability and half-life of the drug. None of these possibilities have currently been tested.

4.4 Discussion

The experiments described in this chapter provide evidence that *CRK3* has a role in cell cycle progression in *L. mexicana*. Inhibition of *L. mexicana* growth by the phosphotyrosine phosphatase inhibitor bpV(phen) caused arrest in the G1 and S-phases of the cell cycle. Purified CRK3 from such blocked cells is inactive. Activity of CRK3 recovers after release from the block as cells re-enter the cell cycle. It is not known however whether other CRKs such as CRK1 are also inhibited by this method. It is assumed that the cell cycle arrest observed after incubation with bpV(phen) is due to inhibition of the *Leishmania* homologue of the *Schizosaccharomyces pombe* Cdc25 protein tyrosine phosphatase. In *S. pombe* Cdc25 plays a role in regulating cell cycle progression by removing the inhibitory phosphate at the Tyr 15 residue of the Cdc2 kinase (Millar *et al.,* 1991). As CRK3 contains a homologous residue (Tyr34), it is likely that phosphorylation and dephosphorylation at this residue also regulates CRK3 activity. Inhibition of the Cdc25 homologue in *Leishmania* by bpV(phen) would therefore be expected to lead to inhibition of the activity of the *Leishmania* Cdc2 homologue, resulting in cell cycle arrest. Because bpV(phen) does lead to inhibition of cell cycle progression, and this inhibition correlates with inhibition of CRK3 kinase activity, this suggests that CRK3 does play a role in cell cycle progression in *Leishmania*. However it is also possible that the cell cycle arrest is due to inhibition of some other phosphatase with a role in growth control. Tyrosine kinase activity plays an important role in cell signalling events in other organisms and is likely to play a role in growth and differentiation of trypanosomatids (Parsons *et al.,* 1990; Dell and Engel, 1994). The use of okadaic acid, a serine threonine phosphatase inhibitor, resulted in defects in cell division in *T. brucei* (Das *et al.,* 1994). The action of kinases and phosphatases is clearly important in signalling events in the trypanosomatids, just as

they are in other organisms. It is therefore possible that bpV(phen) is acting against other phosphatase targets in the experiments performed in this study. Without confirmation that *Leishmania* does indeed possess a Cdc25 homologue, it is not possible to rule out this prospect.

Inhibition of *L. mexicana* growth by the CDK inhibitor flavopiridol results in an accumulation of cells with a 4N DNA content (Fig. 4.1, Panel J). This is indicative of a cell cycle block in the G2 phase of the cell cycle, when cells have replicated their DNA but are blocked prior to mitosis. Purified CRK3 kinase complex can be inhibited by flavopiridol at nanomolar concentrations *in vitro*. The IC_{50} value obtained was 100nM. This is slightly lower than the IC_{50} values obtained for inhibition of the human CDK1, CDK2 and CDK4 kinases by flavopridol, which are 400 nM (Losiewicz *et al.,* 1994; Carlson *et al.,* 1996). Because flavopiridol is a very potent inhibitor of CRK3 *in vitro*, and inhibits cell growth at a similar range of concentrations it is likely that the cell cycle arrest caused by flavopiridol is primarily due to inhibition of CRK3 *in vivo*. However it is not possible to rule out the possibility that flavopiridol inhibits other protein kinases such as CRK1, thereby contributing to the cell cycle arrest. However, given that CRK3 forms the majority of the $p13^{suc1}$ binding kinase activity in *L. mexicana* and is therefore likely to play a similar role in regulating cell cycle progression as *S. pombe* Cdc2, it is likely that the inhibition of *CRK3* by flavopiridol is directly affecting cell cycle progression. Because flavopiridol appears to be a more potent inhibitor of *L. mexicana* CRK3 than human CDK1, 2, or 4, it may be a suitable lead compound for rational drug design. The work described in this study was performed on promastigotes, or on CRK3 kinase purified from promastigotes. The effect of flavopiridol on the growth of amastigotes or on CRK3 purified from amastigotes has not yet been done. It is possible that a different CRK acts as the master regulator of cell cycle control in amastigotes.

The effect of flavopiridol on the growth of the bloodstream form of *T. brucei* was less potent than on the procyclic form. However, given that CRK3 in *Leishmania* is also active in amastigotes, it probably has a similar function in this life cycle stage. There may, however, be differences in other components of the kinase complex, a different cyclin partner for example, that may have an effect on the binding of flavopiridol. As flavopiridol exerts its effect by competitively binding to the ATP binding site of the kinase (De Azevedo *et al.,* 1996) however, this is unlikely. What may have a more important bearing on the effectiveness of flavopiridol on amastigotes is the way in which it enters the cell. It is also true that in an *in vivo* situation, where amastigotes reside inside the parasitophorous vacuole of infected macrophages, the drug must first enter the macrophage before it can begin to have any effect on the parasite. This may mean that doses required to kill the amastigotes will have toxic side effects. This of course, is a problem for all potential anti-leishmanial drugs. However, this is not the case for therapies acting against the bloodstream form of *Trypanosoma brucei* which does not infect host cells but lives free in the bloodstream of the host. Therefore flavopiridol, or derivatives of flavopiridol may be useful as anti-trypanosomal drugs.

Inhibition of cell cycle progression of *L. mexicana* promastigotes is reversible. Cells can be released from a G2 phase block by washing with PBS at 4°C, before resuspension in fresh medium. Although this does not result in a completely synchronous re-entry into the cell cycle, this method can still be used to obtain samples enriched for cells in a particular phase of the cell cycle. This may be useful to study the levels of cell cycle regulated transcripts, proteins, or for the analysis of components of the cell cycle machinery in *Leishmania*. For example, the role of regulatory phosphorylation of the Tyr34 residue of CRK3 throughout the cell cycle, or the binding of cyclin partners of

CRK3 at different points of the cycle could be analysed. The activity of CRK3 and other CRK complexes throughout the cell cycle could also be studied.

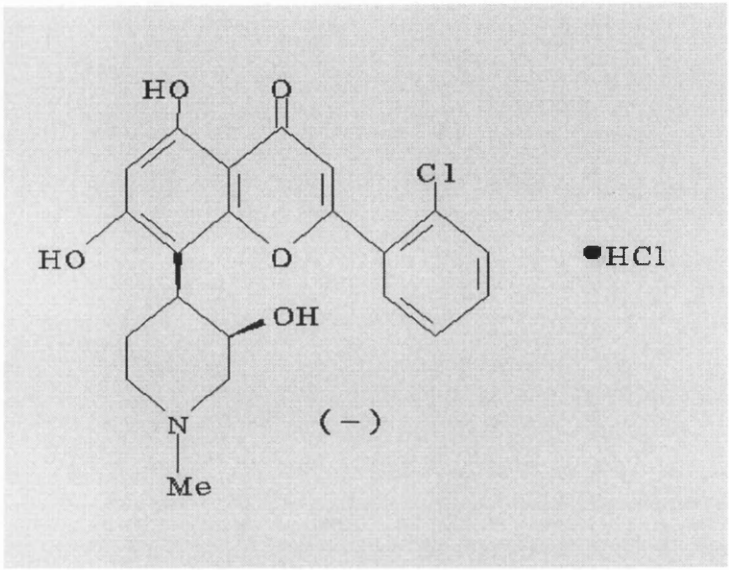
A number of methods that are used to synchronise various eukaryotic cell types, have been used to attempt to obtain synchronous populations of *Leishmania*. Hydroxyurea, an inhibitor of ribonucleotide reductase, causes inhibition of cell cycle progression by reducing the availability of deoxyribonucleotides, thereby inhibiting DNA synthesis (Yarbro, 1992). Hydroxyurea has been used to synchronise populations of *Leishmania tarentolae* and *Crithidia fasciculata* (Simpson and Braly, 1970; Cosgrove *et al.,* 1979). Attempts to use hydroxyurea to synchronise *L. mexicana* however, were unsuccessful (J.C. Mottram, personal communication). Other methods that have been used in the trypanosomatids include the use of aphidicolin, an inhibitor of DNA polymerase α which arrests cells at the G1/S-phase boundary (Feher and Mishra, 1994), to synchronise *Trypanosoma brucei* procyclic and bloodstream form cells (Mutomba and Wang, 1996). This method did not result in a synchronous population of cells as there was a significant lag period before cells re-entered the cell cycle upon release from the block. A similar problem was encountered in a study of the use of (S)-9-(3-hydroxy-2-phosphoenolmethoxypropyl)adenine ((S)-HPMPA), a purine analogue which arrests *T. brucei* in S-phase, to synchronise African trypanosomes (Kaminsky *et al.,* 1998).

The results presented in this study on the use of flavopiridol to synchronise *L. mexicana* promastigotes, currently appears to be the best available method for doing so. However, flavopiridol cannot be used to synchronise African trypanosomes as the effect of flavopiridol on both procylic and bloodstream stages of *T. brucei* is to cause a block at both G1/S and G2/M.

Fig. 4.1. Structure and inhibition properties of flavopiridol

The chemical structure (Panel A) and IC_{50} values of flavopiridol against a number of protein kinases is shown (Panel B). Table adapted from, Meijer, 1995.

(A)



(B)

Kinase	IC_{50} (μM)
EGF-receptor	21
Protein kinase A	122
Protein kinase C	6
CDK1/Cyclin B	0.3
CDK1/Cyclin A	0.3
CDK1/Cyclin E	0.3
CDK2/Cyclin A	0.1
CDK2/Cyclin E	0.1
CDK4/Cyclin D	0.4
CDK6/Cyclin D	0.4
CDK7/Cyclin H	0.4

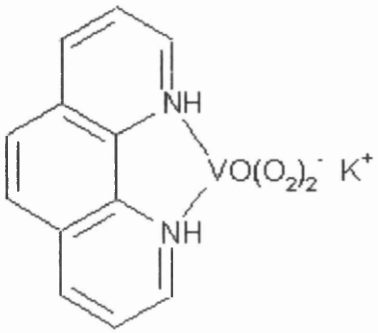
Fig. 4.2 Indirect inhibition of *L. mexicana* p13^{suc1} Binding Kinase (CRK3) by the protein tyrosine phosphatase inhibitor bpV(phen)

L. mexicana promastigotes were incubated at a density of 1x 10^7 cells ml^{-1} in the presence of 10 μM bpV(phen) for 12 or 24 hours. p13^{suc1} binding kinase was purified and assayed for ability to phosphorylate histone H1. Relative kinase activity was assessed by quantification on a phosphorimager (Fuji). (A) Structure of bpV(phen). (B) Autoradiograph results. (C) Results of Quantification by phosphorimaging.

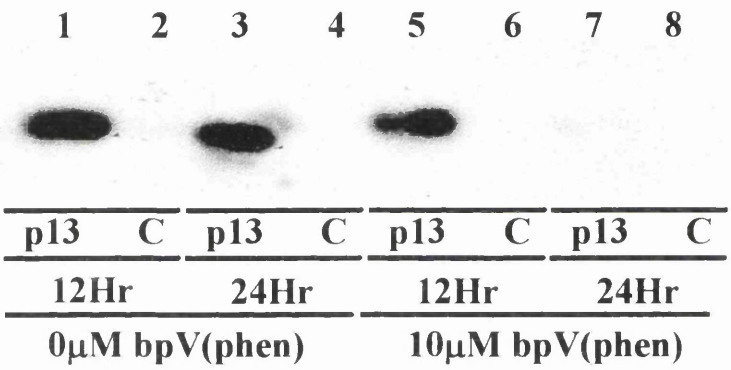
p13: p13^{suc1} beads

C: Control beads

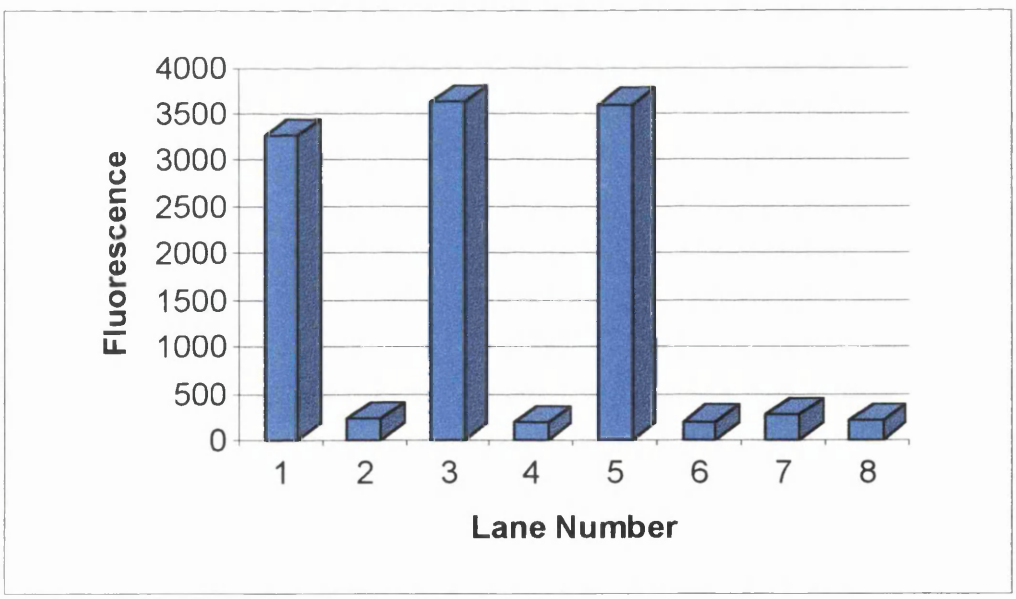
A


NH
$VO(O_2)_2^-$ K^+
NH

B


1 2 3 4 5 6 7 8
p13 C p13 C p13 C p13 C
12Hr 24Hr 12Hr 24Hr
0μM bpV(phen) 10μM bpV(phen)

C


Fluorescence
4000
3500
3000
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8
Lane Number

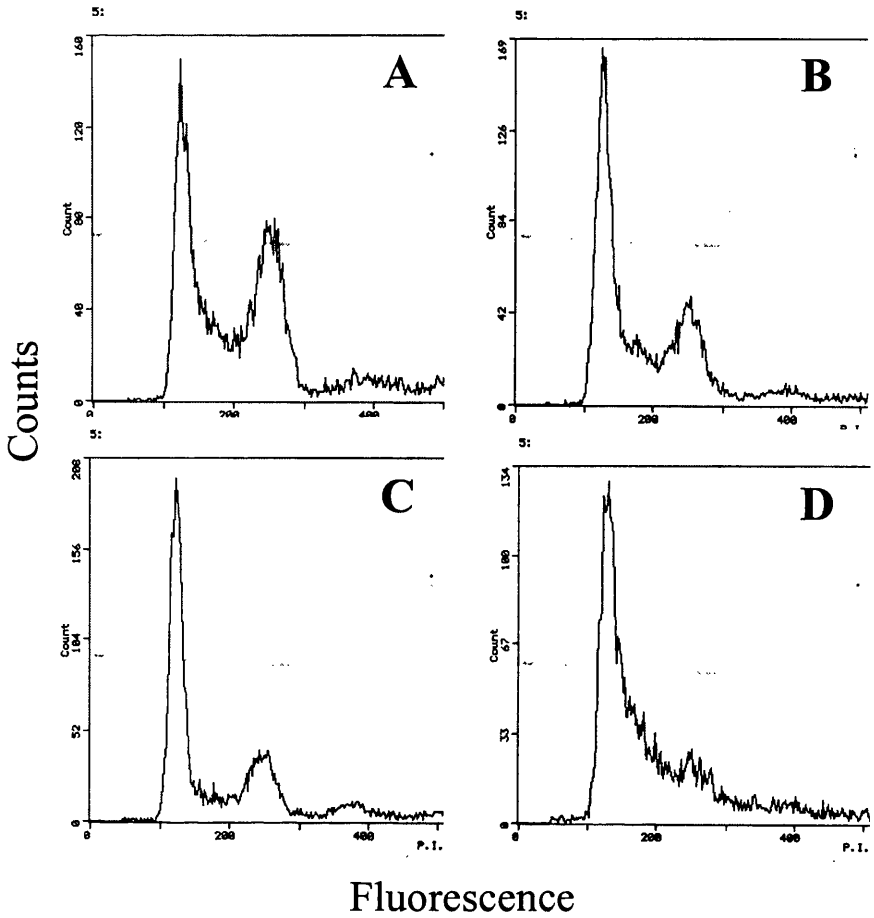


Fig. 4.3 FACS analysis of *L. mexicana* promastigotes incubated with bpV(phen)

L. mexicana promastigotes at a cell density of $1x10^7$ cells ml^{-1} were incubated for 12 or 24 hours in the presence of 10 μM bpV(phen). The samples were fixed and stained with 10 μg ml^{-1} propidium iodide, and DNA content was analysed by FACS. 10,000 cells were counted on an Epics/XL flow cytometer (Coulter).

Panel A: 12 hr, 0 μM bpV(phen)

Panel B: 12 hr, 10 μM bpV(phen)

Panel C: 24 hr, 0 μM bpV(phen)

Panel D: 24 hr, 10 μM bpV(phen)

Fig 4.4 Restoration of *L. mexicana* p13^{suc1} binding kinase activity upon release from bpV(phen) induced G1/S cell cycle arrest

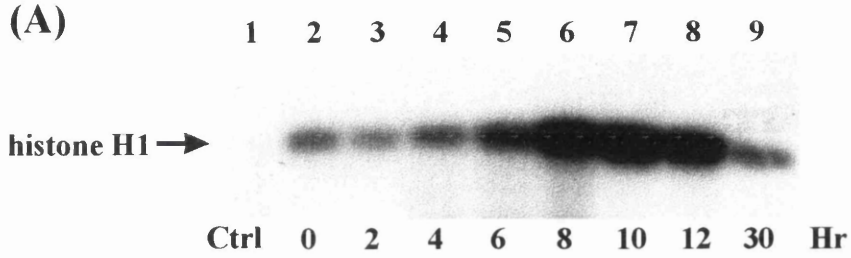
L. mexicana promastigotes at a cell density of $1x10^7$ cells ml^{-1} were incubated for 24 hours with 10 μM bpV(phen). Cells were then washed twice in cold PBS to remove bpV(phen) and resuspended in fresh medium minus bpV(phen). Samples were prepared for p13^{suc1} binding kinase assay after 24 hour incubation with bpV(phen) (lane 2) and at 2 hour time points after cells were released from bpV(phen) inhibition (lane 3-9). p13^{suc1} binding kinase activity was assessed as by phosphorylation of histone H1 as described previously.

Panel A Autoradiograph

Panel B Quantification by phosphorimager

Ctrl. Assay of kinase activity bound to control beads

(A)


1 2 3 4 5 6 7 8 9
histone H1
Ctrl 0 2 4 6 8 10 12 30 Hr

(B)

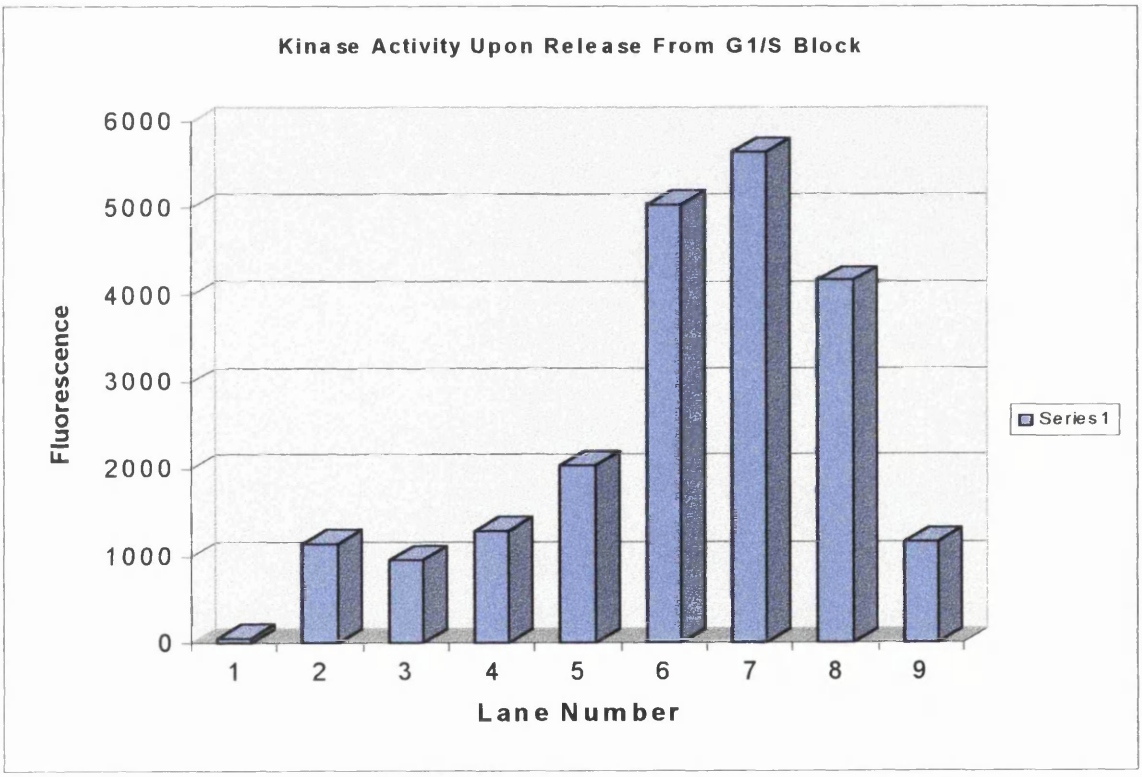

Kinase Activity Upon Release From G1/S Block
Fluorescence
6000
5000
4000
3000
2000
1000
0
1 2 3 4 5 6 7 8 9
Lane Number
Series1

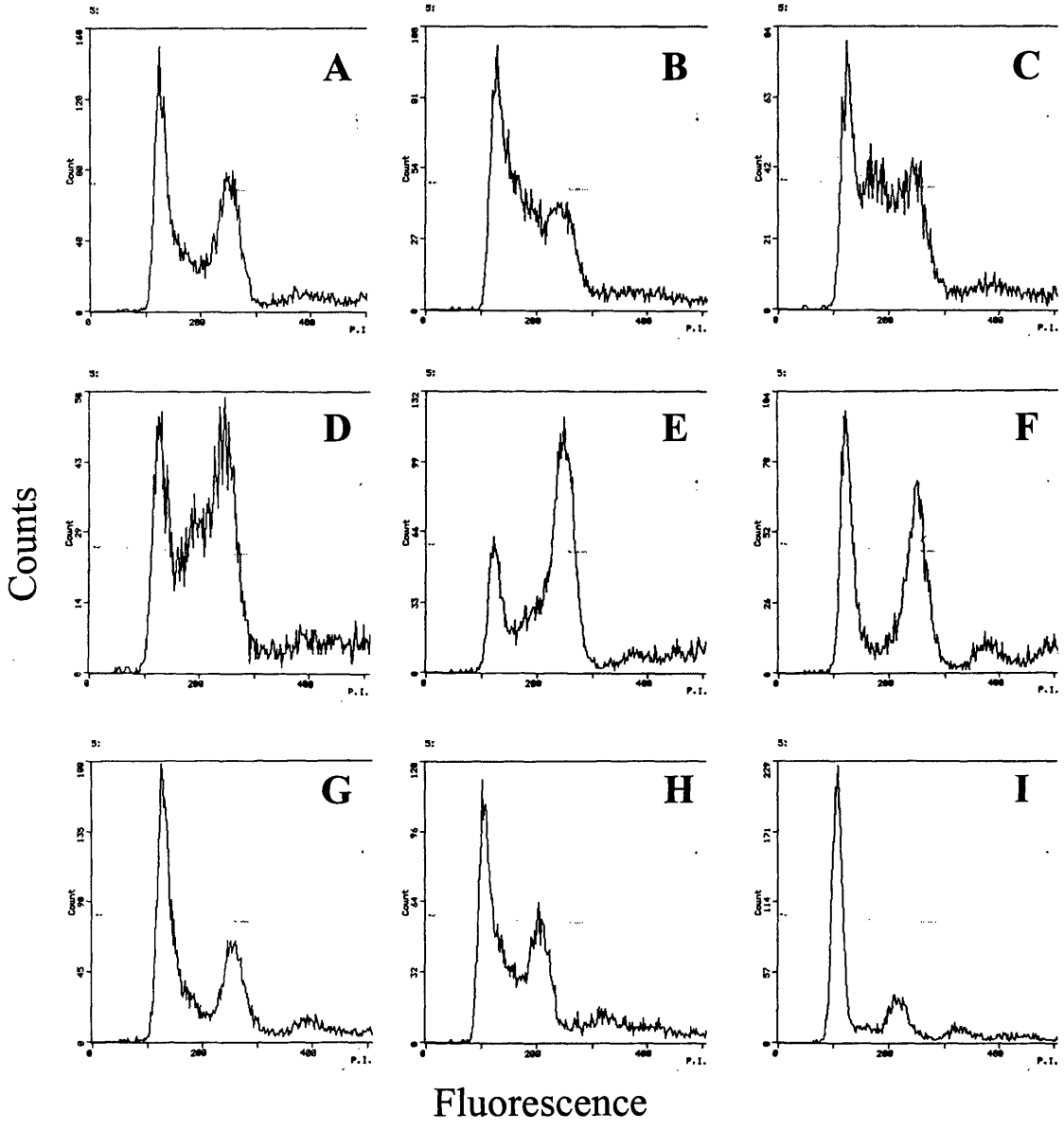
Fig. 4.5 FACS analysis of *L. mexicana* promastigotes after release from bpV(phen) induced G1/S-phase block

Samples of *L. mexicana* promastigotes inhibited with bpV(phen) and then released from inhibition were prepared and analysed for DNA content by FACS. Inhibition and release were as described in Figure 4.3. Cells were stained with 10μg ml^{-1} propidium iodide and analysed on an Epics/XL flow cytometer (Coulter). 10,000 cells were counted for each individual sample.

Panel A: 0 μM bpV(phen)

Panel B: 24 hr, 10 μM bpV(phen)

Panels C-I: Incubated for 24 hr with 10 μM bpV(phen), resuspended without bpV(phen) and incubated for 2, 4, 6, 8, 10, 12, and 30 hr respectively.


A
B
C
D
E
F
G
H
I
Counts
Fluorescence

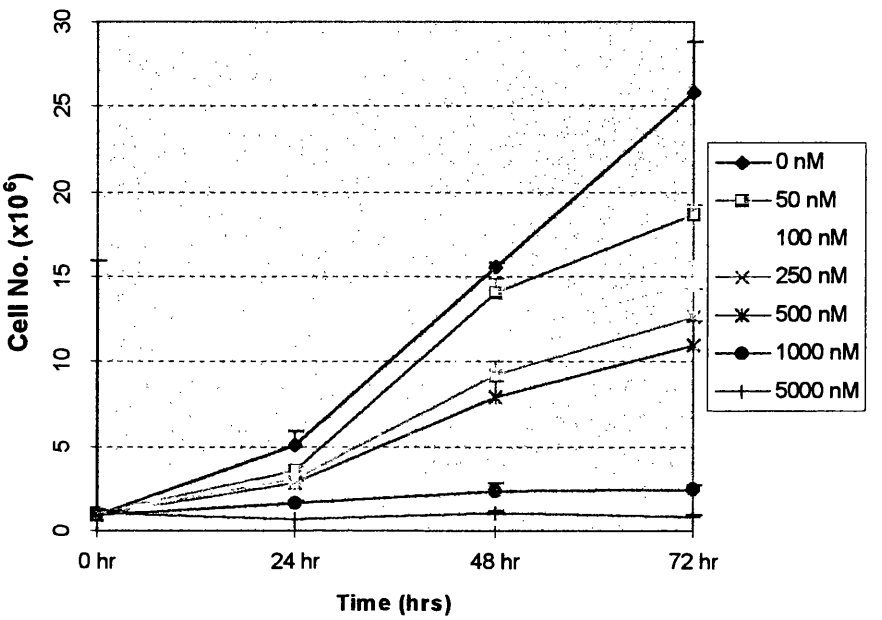


Fig 4.6 Growth inhibition of *L. mexicana* promastigotes by flavopiridol

Cultures of *L. mexicana* promastigotes were seeded at a density of $1x10^6$ cells ml^{-1} and incubated in the presence of 0, 50, 100, 250, 500 nM, 1.0 and 5 μM flavopiridol. Cell density was determined at 24 hr intervals and the mean result of triplicate values is plotted. Error bars show the standard deviation.

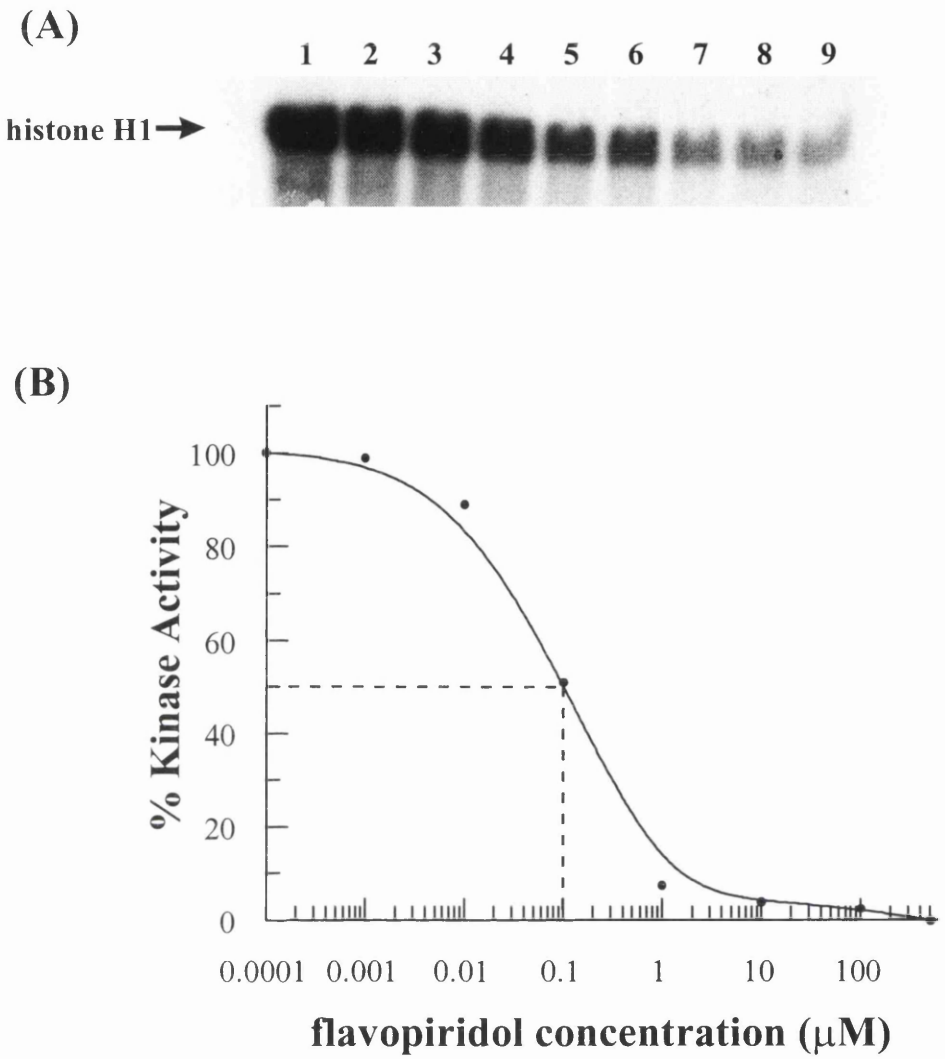


Fig 4.7 Flavopiridol inhibits CRK3 with an IC_{50} value of 100 nM

Active CRK3his was purified by Nickel NTA agarose affinity selection from the *L. mexicana* cell line, W1033. Histone H1 kinase activity was assayed in the presence of increasing concentrations of flavopiridol (Panel A), and the relative kinase activity was assessed by phosphorimaging analysis. The experiment was repeated three times giving IC_{50} values of 130, 100 and 80 nM respectively. A representative experiment is shown. Results are plotted as a percentage of uninhibited kinase activity (Panel B).

Fig 4.8 DNA content analysis of cells incubated with flavopiridol

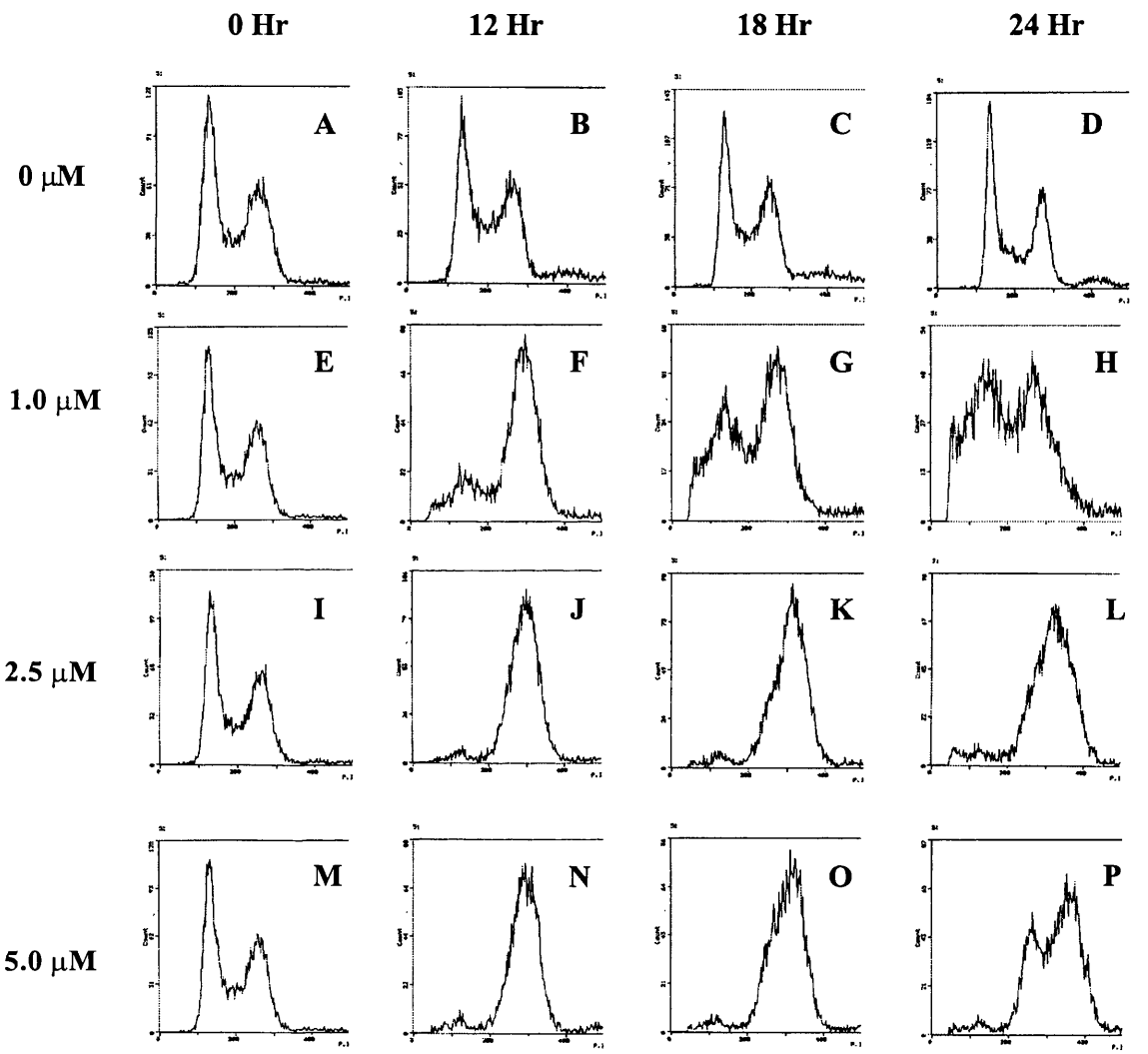
The total DNA content of *L. mexicana* promastigotes incubated in the presence of the CDK inhibitor, flavopiridol, at different concentrations and for differing time periods was assessed by FACS. Cells were fixed, stained with propidium iodide, and 10,000 cells were counted on a Coulter Epics/XL flow cytometer.

Panel A-D. Negative control culture with no inhibitor at 0, 12, 18 and 24 hours

Panel E-H. 1.0 μM flavopiridol at 0, 12, 18 and 24 hours

Panel I-L. 2.5 μM flavopiridol at 0, 12, 18 and 24 hours

Panel M-P. 5.0 μM flavopiridol at 0, 12, 18 and 24 hours


0 Hr
12 Hr
18 Hr
24 Hr
0 μM
1.0 μM
2.5 μM
5.0 μM
A
B
C
D
E
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H
I
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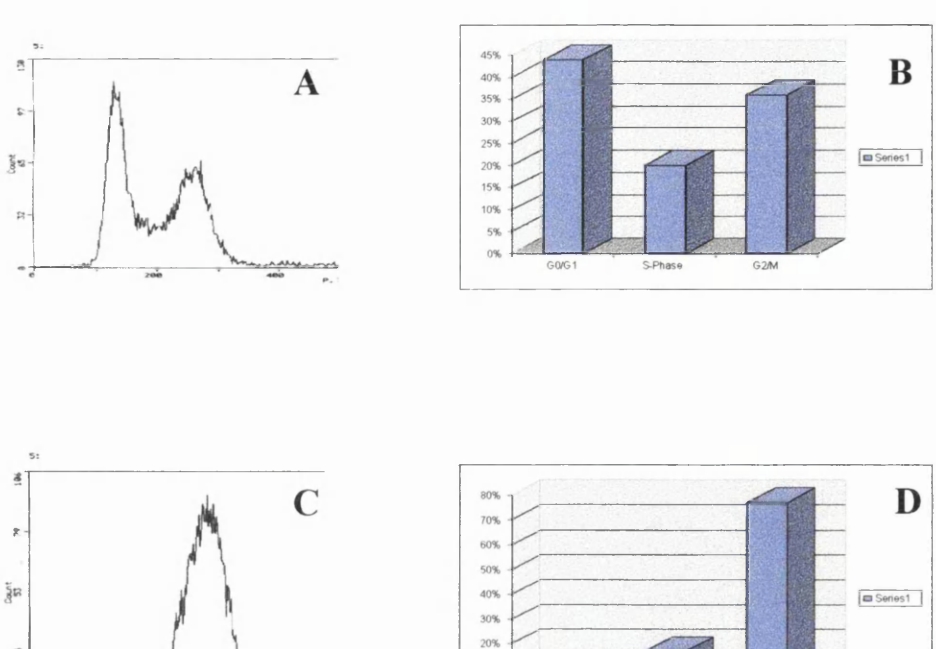


Fig 4.9 Analysis of the cell cycle distribution of *L. mexicana* promastigotes arrested with flavopiridol

L. mexicana promastigotes at a cell density of $1x10^6$ cells ml^{-1} were incubated with 2.5 μM flavopiridol for 12 hours. Cells were fixed and stained with propidium iodide and analysed on a Becton Dickinson FACScalibur flow cytometer. Cell cycle distribution was determined using the ModFit LT software package.

Panel A: *L. mexicana* promastigotes, no flavopiridol

Panel B: Cell cycle distribution of *L. mexicana* promastigotes minus flavopiridol

Panel C: *L. mexicana* promastigotes blocked with 2.5 μM flavopiridol for 12 Hrs

Panel D: Cell cycle distribution of *L. mexicana* promastigotes blocked with 2.5 μM flavopiridol for 12 Hrs

Fig 4.10 Fluorescence microscopy of flavopiridol treated *L. mexicana* promastigotes stained with DAPI

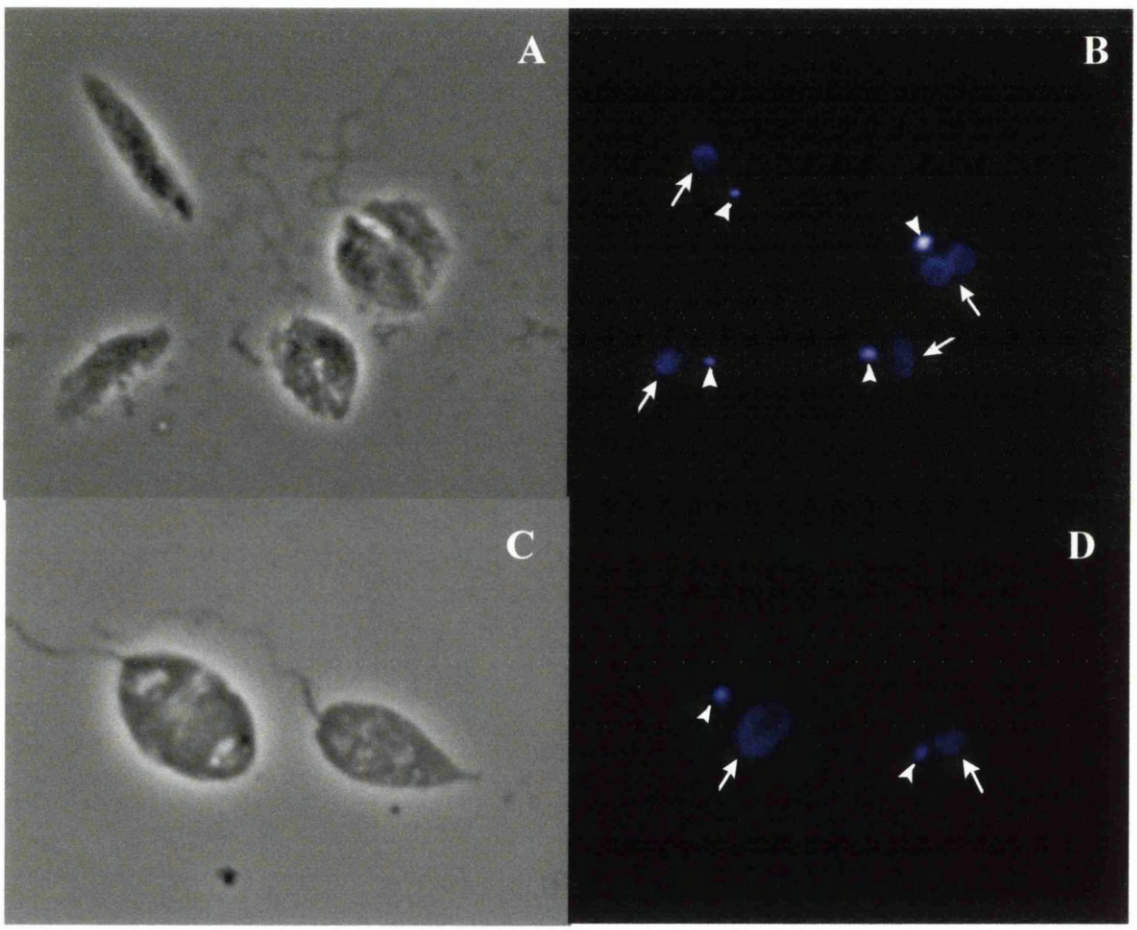
L. mexicana promastigotes were incubated in the presence or absence of 2.5 μM flavopiridol for 12 hours. Cells were smeared onto slides, fixed for 1 hour in 70% ethanol and then stained with 20 μg ml^{-1} DAPI. Slides were viewed under UV illumination using a Zeiss Axioplan microscope fitted with a CCD camera (Hamamatsu photonics). Images were captured on a Power Macintosh (Apple) using the OpenLab 2.0.2 software package (Improvision). Arrows indicate nuclei. Arrowheads indicate kinetoplasts.

Panel A. Untreated cells, phase contrast image

Panel B. Untreated cells, fluoresence image

Panel C. Flavopiridol treated cells, phase contrast image

Panel D. Flavopiridol treated cells, fluoresence image

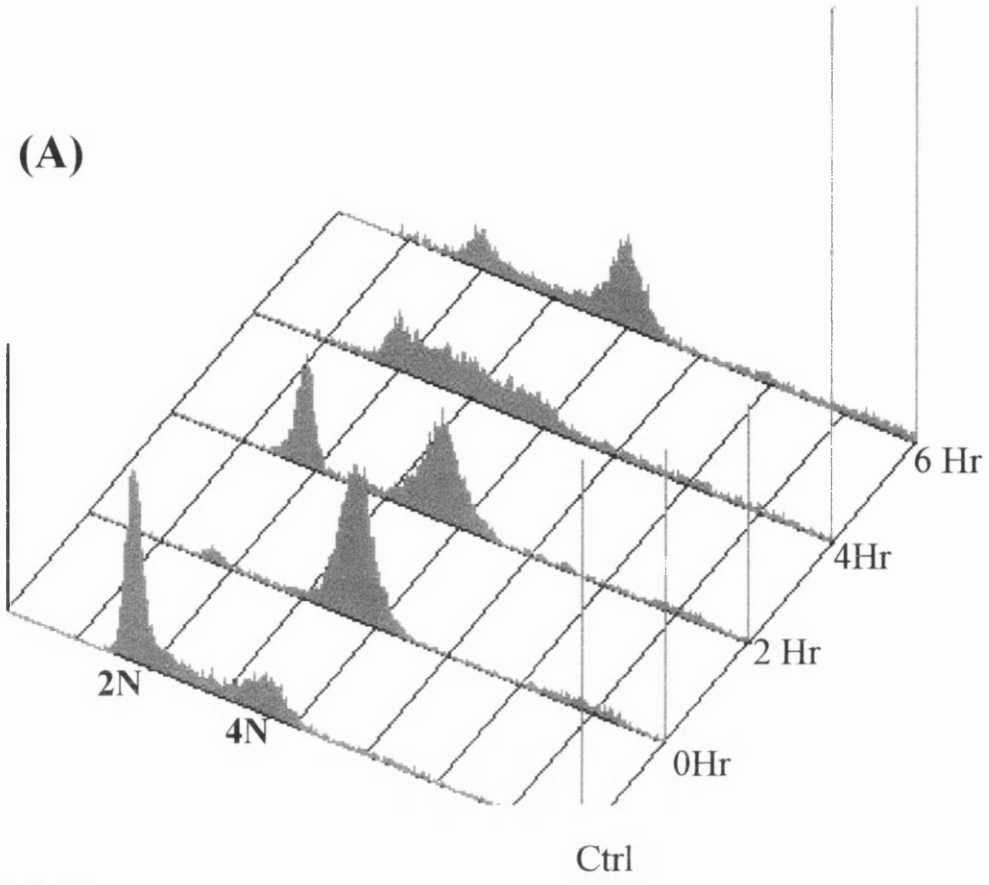


10 μm

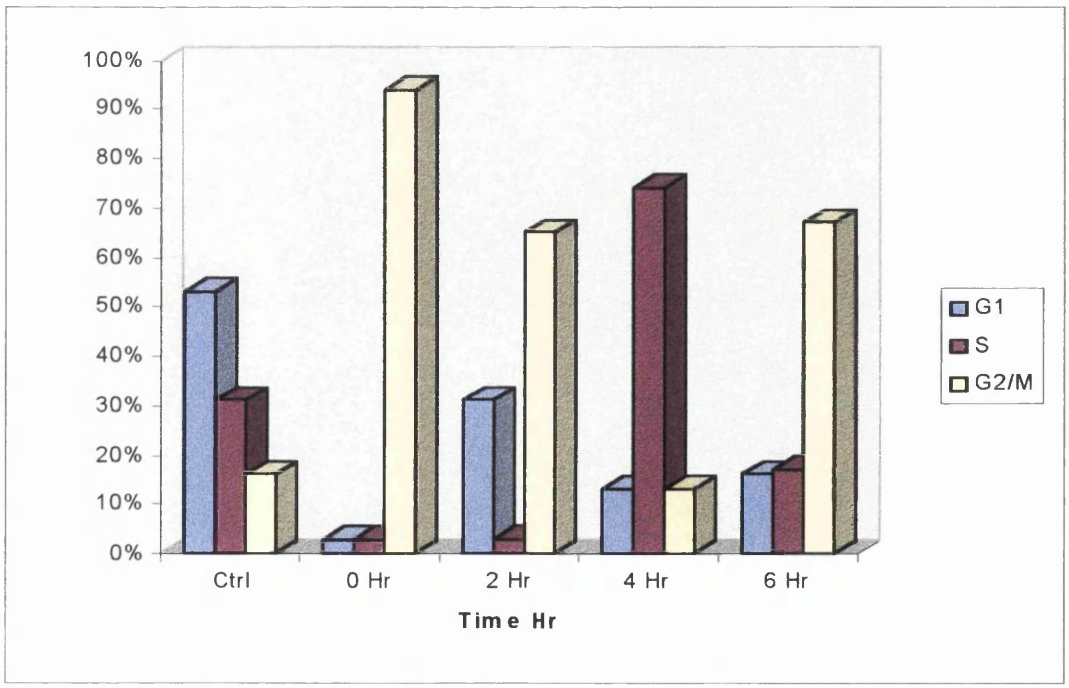
Fig 4.11 Release of *L. mexicana* promastigotes from flavopiridol induced cell cycle arrest

L. mexicana promastigotes at a density of $1x10^6$ cells ml^{-1} were incubated for 12 hours in the presence of 2.5 μM flavopiridol. Cells were washed twice in complete medium and resuspended in fresh complete medium. Cells were then incubated at 25°C and samples were removed at 2 hour time points, fixed and stained with propidium iodide and analysed on a FACScalibur flow cytometer (Panel A). 10,000 cells were analysed for each sample. Cell cycle distribution was determined using ModFit LT software (Panel B). Samples marked Ctrl correspond to untreated cells.

(A)


6 Hr
4Hr
2 Hr
0Hr
2N
4N
Ctrl

(B)


100%
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30%
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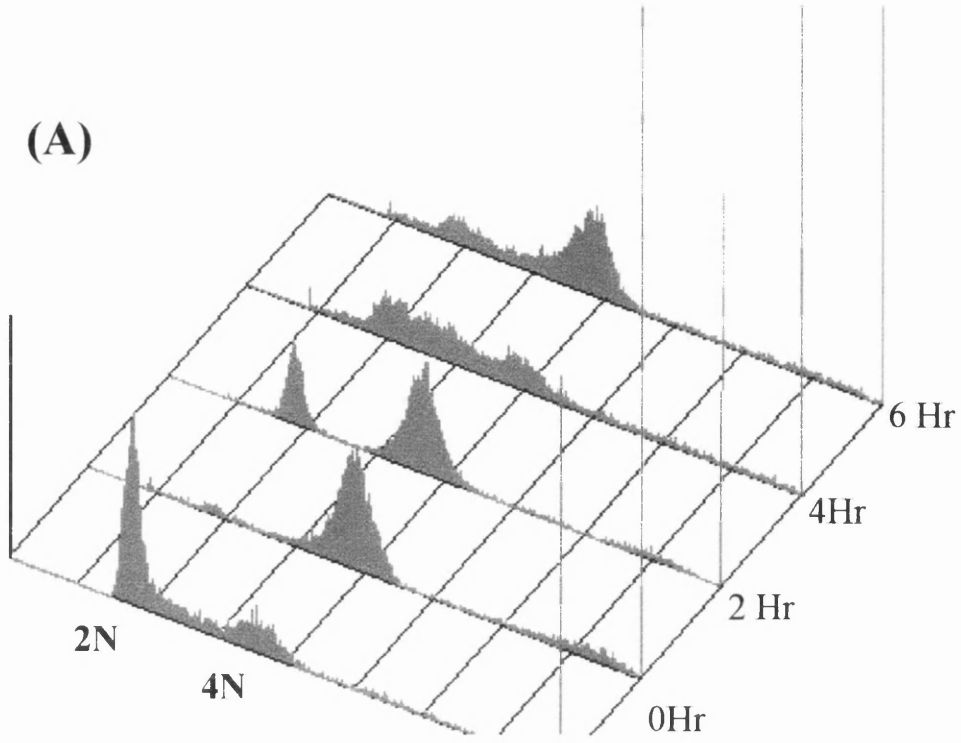
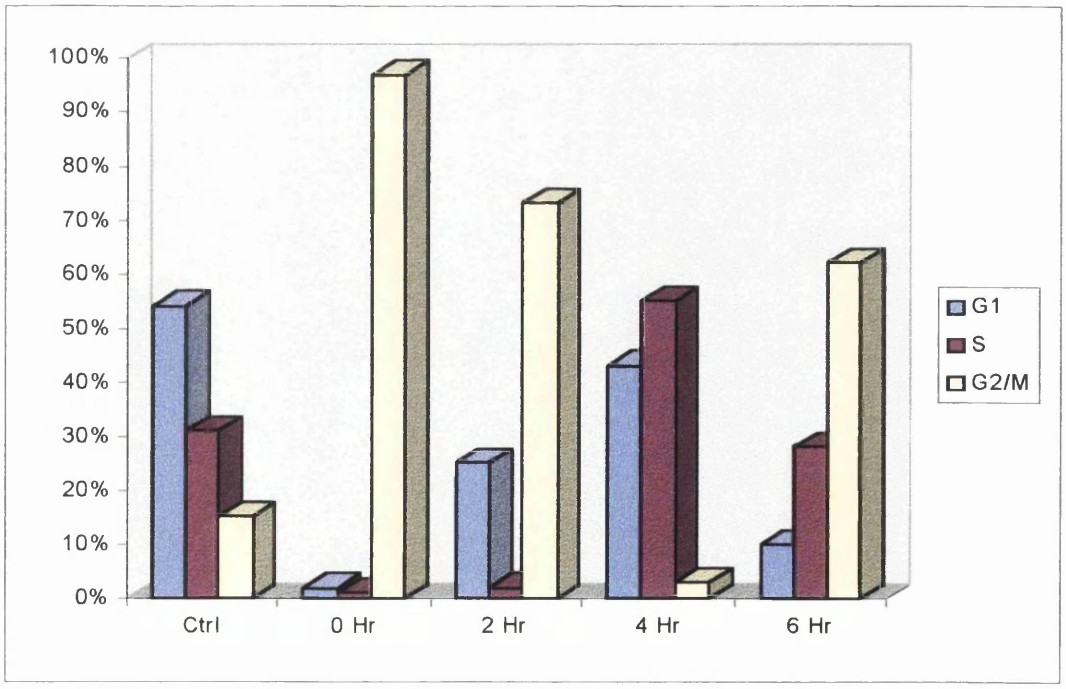
Ctrl
0 Hr
2 Hr
4 Hr
6 Hr
Time Hr
G1
S
G2/M

Fig. 4.12 Release of *L. mexicana* promastigotes from flavopiridol inhibition by serum-free medium wash

L. mexicana promastigotes at a density of $1x10^6$ cells ml^{-1} were incubated for 12 hours in the presence of 2.5 μM flavopiridol. Cells were washed twice in serum free medium and resuspended in fresh complete medium. Cells were then incubated at 25°C and samples were removed at 2 hour time points, fixed and stained with propidium iodide and analysed on a FACScalibur flow cytometer (Panel A). 10,000 cells were analysed for each sample. Cell cycle distribution was determined using ModFit LT software (Panel B). Samples marked Ctrl correspond to untreated cells.


(A)
6 Hr
4Hr
2 Hr
0Hr
2N
4N
(B)
Ctrl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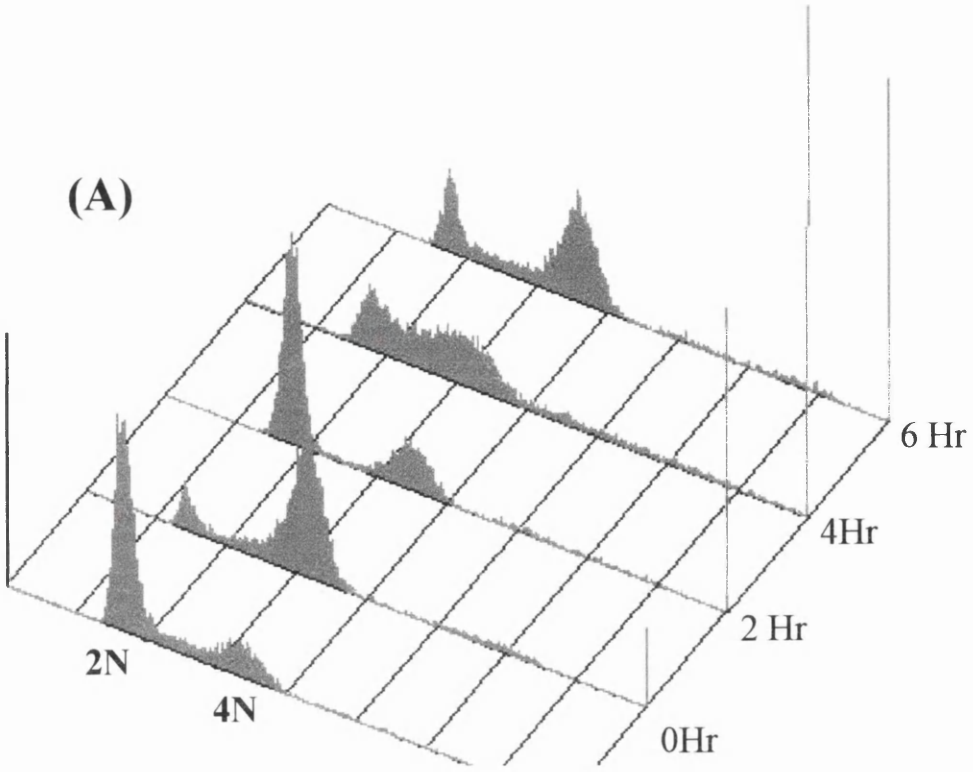
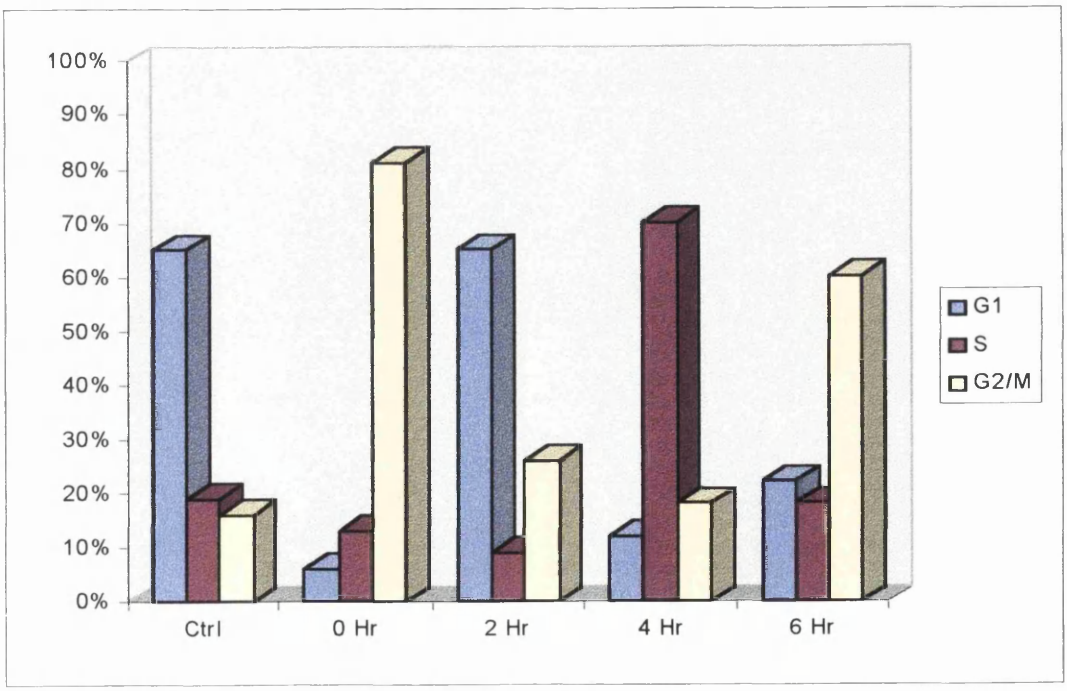
6 Hr
G1
S
G2/M

Fig. 4.13 Release of *L. mexicana* promastigotes from flavopiridol inhibition by PBS wash

L. mexicana promastigotes at a density of $1x10^6$ cells ml^{-1} were incubated for 12 hours in the presence of 2.5 μM flavopiridol. Cells were washed twice in PBS and resuspended in fresh complete medium. Cells were then incubated at 25°C and samples were removed at 2 hour time points, fixed and stained with propidium iodide and analysed on a FACScalibur flow cytometer (Panel A). 10,000 cells were analysed for each sample. Cell cycle distribution was determined using ModFit LT software (Panel B). Samples marked Ctrl correspond to untreated cells.


(A)
6 Hr
4Hr
2 Hr
0Hr
2N
4N
Ctrl

(B)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6 Hr
G1
S
G2/M

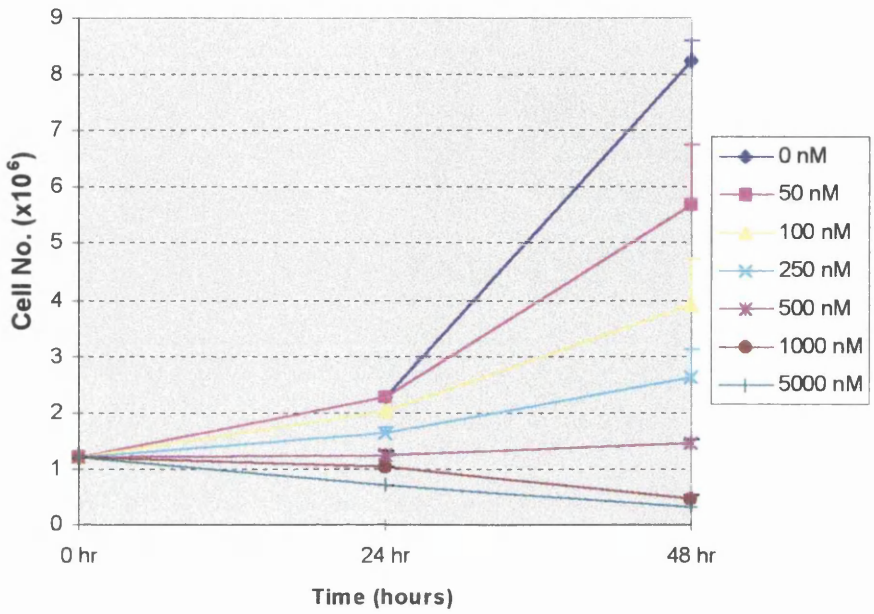


Fig. 4.14 Growth inhibition of procyclic form *T. brucei* by flavopiridol

Cultures of *T. brucei* procyclics were seeded at a density of $1x10^6$ cells ml^{-1} in the presence of 0, 50, 100, 250, 500 nM, 1.0 and 5.0 μM flavopiridol. Cell density was determined at 24 hour intervals and the mean result of triplicate values is plotted. Error bars show the standard deviation.

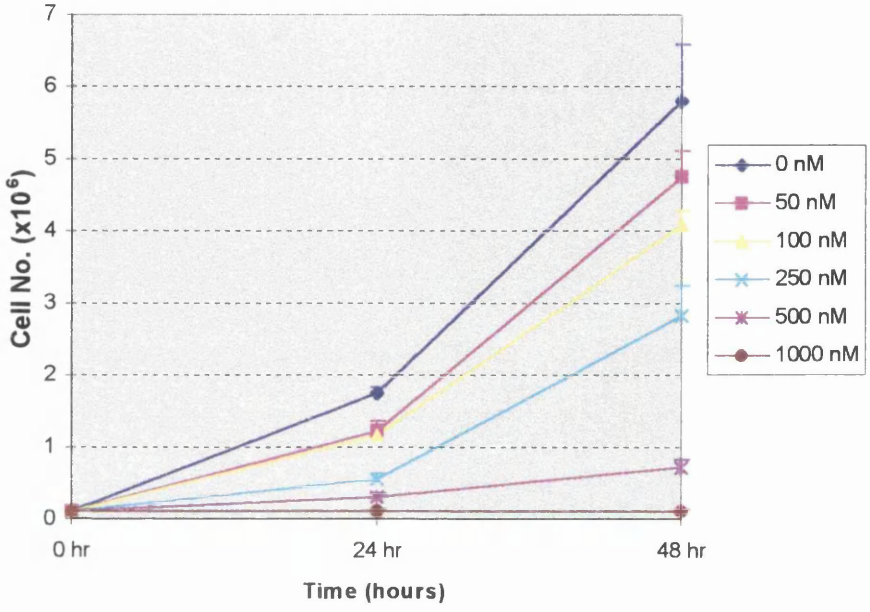


Fig. 4.15 Growth inhibition of bloodstream form *T. brucei* by flavopiridol

Cultures of *T. brucei* bloodstream form cells were seeded at a density of $1x10^5$ cells ml^{-1} in the presence of 0, 50, 100, 250, 500 nM and 1.0 μM flavopiridol. Cell density was determined at 24 hour intervals and the mean result of triplicate values is plotted. Error bars show the standard deviation.

Fig. 4.16 Cell cycle analysis of *T. brucei* procyclics incubated in the presence of flavopiridol

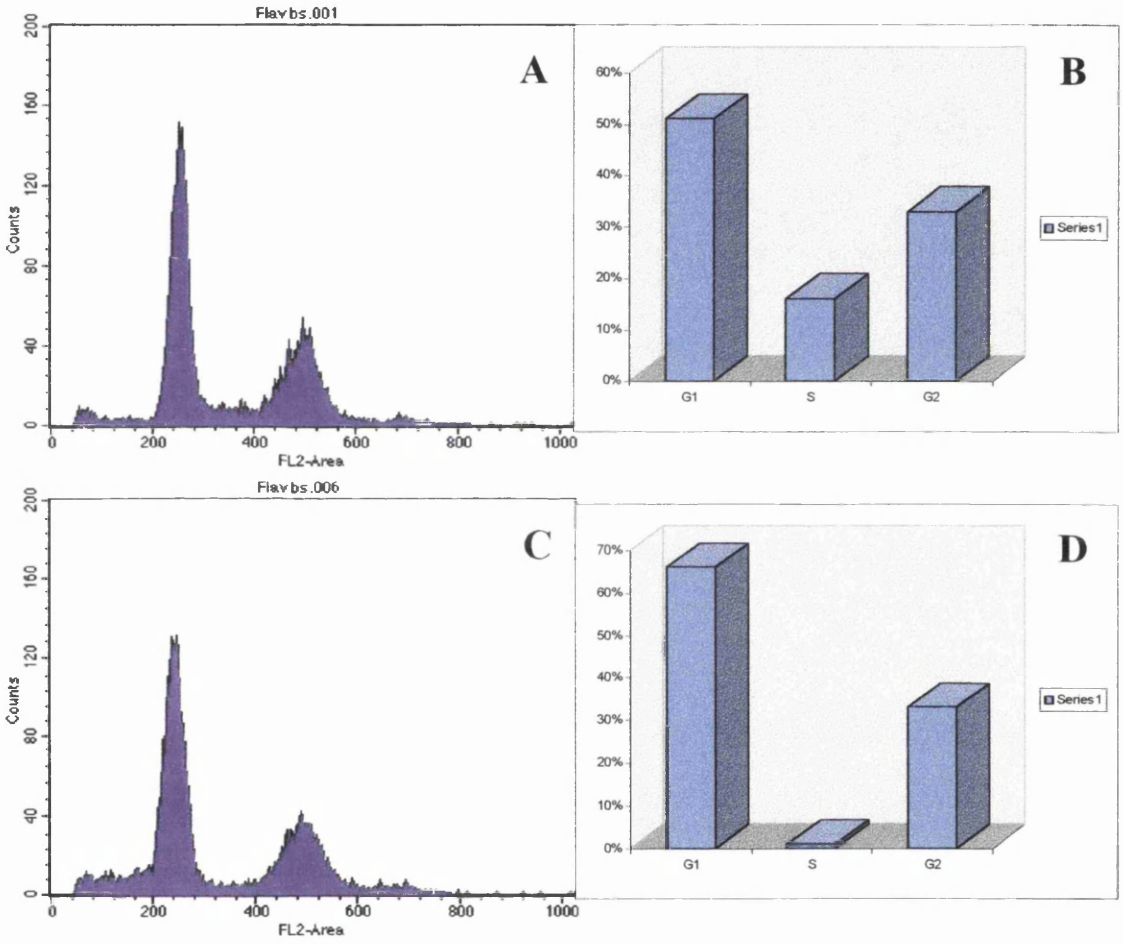
T. brucei procyclics at a cell density of $1x10^6$ cells ml^{-1} were incubated with 2.5 μM flavopiridol for 12 hours. Cells were fixed and stained with propidium iodide and analysed on a Becton Dickinson FACScalibur flow cytometer. Cell cycle distribution was determined using the ModFit LT software package.

Panel A: *T. brucei* procyclics, no flavopiridol

Panel B: Cell cycle distribution of *T. brucei* procyclics minus flavopiridol

Panel C: *T. brucei* procyclics blocked with 2.5 μM flavopiridol for 12 Hrs

Panel D: Cell cycle distribution of *T. brucei* procyclics blocked with 2.5 μM flavopiridol for 12 Hrs


Flavbs.001
A
B
Counts
FL2-Area
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40
80
120
160
200
0
200
400
600
800
1000
60%
50%
40%
30%
20%
10%
0%
G1
S
G2
Series1
Flavbs.006
C
D
Counts
FL2-Area
70%
60%
50%
40%
30%
20%
10%
0%
G1
S
G2
Series1

CHAPTER 5

5.1 Complementation experiments with *Saccharomyces cerevisiae cdc28^{ts}* mutants

The availability of a wide variety of temperature sensitive mutant strains of *S. cerevisiae* allow the identification of genes from other organisms that can complement for the loss of function of the gene bearing the conditional mutation. Many temperature sensitive mutants exhibit easily identifiable phenotypes at the restrictive temperature, eg. Changes in cell polarity (Rethinaswamy *et al.,* 1998), bud morphology (Zhang *et al.,* 1998), mitotic spindle formation (Spang *et al.,* 1996) or temperature dependent lethality (Uemura *et al.,* 1996). It is therefore possible to screen for homologous genes that, when expressed in such mutant strains at the restrictive temperature, are able to restore the phenotype to wild type (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.,* 1991). Many homologues of the *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae cdc2/CDC28* genes have been identified due to their ability to complement temperature sensitive mutants. Complementation of a *Schizosaccharomyces pombe cdc2* mutant led to the identification of a human (Lee and Nurse, 1987) and an alfalfa homologue of *cdc2* (Hirt *et al.,* 1991) and complementation of *Saccharomyces cerevisiae cdc28* mutants have led to the identification of human (Elledge and Spottswood, 1991; Ninomiya-Tsuji *et al.,* 1991), zea maize (Colasanti *et al.,* 1991), soybean (Setiady *et al.,* 1996), *Caenorhabditis elegans* (Mori *et al.,* 1994), and *Nicotinia tabacum* (Setiady *et al.,* 1996) homologues of CDC28. The identification of *cdc2/CDC28* homologues from such a diverse array of organisms by complementation illustrates the high degree of conservation of cell-cycle control mechanisms throughout the evolution of eukaryotes.

Complementation of temperature sensitive yeast mutants has been used successfully as a method to clone trypanosomatid homologues of various yeast genes. A *Trypanosoma cruzi* heat shock protein 90 gene (Palmer *et al.,* 1995), a *Trypanosoma brucei* profilin homologue (Williamina and Seebeck, 1997), *T. brucei* dolichol phosphate mannose synthase (Mazhari-Tabrizi *et al.,* 1996), and two *T. brucei* cyclin homologues (Neuville and Mottram, unpublished) have all been identified in this way. It is therefore possible to identify trypanosomatid homologues of yeast genes by complementation, despite the degree of evolutionary distance between these organisms. To determine whether the CRK3 kinase of *Leishmania mexicana* was a functional homologue of Cdc2/CDC28 the kinase was expressed in three separate temperature sensitive *cdc28 S. cerevisiae* mutant strains and tested for complementation.

It has been shown previously that some genes that can complement for loss of CDC28 G1/S kinase activity are not necessarily able to complement for loss of CDC28 G2/M kinase activity (Mori *et al.,* 1994) and *vice versa* (Hirt *et al.,* 1993). For this reason CRK3 was tested for the ability to complement three distinct mutant yeast strains; *cdc28-1N*^{ts} and *cdc28-4*^{ts}, which arrest at the G2/M transition and the *cdc28-13*^{ts} strain which arrests at the G1/S checkpoint (Lorincz and Reed, 1986). Expression levels of cell-cycle control genes are tightly regulated, it was therefore important that the *CRK3* gene was expressed over a range of levels to eliminate the possibility that overexpression of CRK3 may be toxic.

5.2.1 CRK3 fails to complement *S. cerevisiae cdc28-1N*^{ts}, *cdc28-4*^{ts} and *cdc28-13*^{ts}

The *CRK3* gene was excised from plasmid pGL89 by digestion with *Eco*RI and *Xho*1. The resulting fragment was purified from an agarose gel and ligated into the

pRS416MET yeast expression plasmid (Mumberg *et al.,* 1994) to give plasmid pGL120 (Fig. 5.1). This plasmid contains the *S. cerevisiae MET25* promoter upstream of the multiple cloning site. In the absence of methionine the *MET25* promoter is maximally active, and is repressed when methionine is present. This allows regulation of expression of cloned genes by altering the concentration of methionine in the medium (Mumberg *et al.,* 1994). The plasmid also contains the *URA3* gene allowing selection for cells bearing the plasmid when grown on medium lacking uracil. The *cdc28-1N*^{ts}, *cdc28-4*^{ts}, and *cdc28-13*^{ts} yeast strains were transformed with the pGL120 plasmid using the lithium acetate procedure (Ito *et al.,* 1983). Five independent clones per transformation were selected and patched onto duplicate plates. Plates were sealed with parafilm and incubated either at the permissive temperature of 25°C, or at the restrictive temperature, 37°C. Plates were incubated for 4 days to allow colonies to form. The results of the complementation test are shown in Fig. 5.2. Colonies formed at the permissive temperature of 25°C (Fig. 5.2, Panel B) but did not form at the restrictive temperature (Panel A). To determine whether CRK3 was expressed in these clones western blot analysis of cell lysates was performed. 10ml overnight cultures were harvested and cells were washed and resuspended in breaking buffer. Samples were incubated on ice and an equal volume of acid-washed glass beads was added. Samples were alternately vortexed and incubated on ice for 30 seconds. This procedure was repeated eight times. The resultant clarified supernatant was removed and mixed with SDS-PAGE sample loading buffer. Samples were separated by SDS-PAGE and transferred to PVDF membrane (NEN™ Life Sciences) by electroblotting. Samples were run in duplicate so that the membrane could be split into two identical halves. Both halves of the membrane were blocked and probed with a rabbit polyclonal antibody raised against a 10 amino acid peptide corresponding to the extreme C-terminus of

CRK3. One half of the membrane was probed with the anti-CRK3 peptide antibody alone and the other was probed in the presence of 2 μg ml^{-1} competing peptide.

The blot was probed with a goat HRP-conjugated anti-rabbit secondary antibody, exposed to enhanced chemiluminescence (ECL) reagents, then exposed to medical X-ray film. The resulting autoradiograph is shown in Fig. 5.3. Lanes 1 to 3 correspond to lysates from the *cdc28-1N*^{ts}, *cdc28-4*^{ts} and *cdc28-13*^{ts} strains transformed with the pGL120 plasmid and probed with anti-CRK3 peptide antibody in the presence of competing peptide. No signal is detected for any of these samples. Lanes 4 to 6 correspond to lysates from the *cdc28-1N*^{ts}, *cdc28-4*^{ts} and *cdc28-13*^{ts} probed with the anti-CRK3 antibody in the absence of competing peptide. A clear signal is detected in all three samples. The predicted molecular weight of the protein to which the antibody has bound, is approximately 35kDa. These results indicate that CRK3 is being expressed in all three yeast strains, but complementation is not occurring.

To test whether complementation might occur by altering the expression levels of CRK3, all three transformed yeast strains were patched onto YNB-agar plates containing a range of concentrations of methionine. The presence of methionine should repress the *MET25* promoter, reducing the expression levels of CRK3. As a control the *cdc28-13*^{ts} strain was transformed with a plasmid that allows expression of the *Caenorhabditis elegans ncc1* gene. *C. elegans* NCC1 is a homologue of CDC28 and has previously been shown to complement a *cdc28-1N*^{ts} mutant (Mori *et al.*, 1994). Plates were incubated at the restrictive temperature (37°C) for four days to allow colonies to form. The results are represented in Fig. 5.4. Panel A corresponds to the control plate incubated at 25°C. Panels B to F correspond to plates containing 1000, 500, 250, 100 and 50μM methionine, incubated at the restrictive temperature of 37°C. After four days only the control cells transformed with the *C. elegans* ncc1 gene were able to grow at

37°C. It was not possible to ascertain whether the levels of CRK3 were altered under different methionine concentrations by western blot analysis, possibly because maximal expression is required to generate enough protein to be detectable using the anti-CRK3 antibody.

5.2.2 Co-expression of *T. brucei* cyclins with *L. mexicana CRK3* fails to complement for loss of *S. cerevisiae* CDC28 activity

One explanation for the inability of CRK3 to complement for loss of CDC28 activity is that CRK3 cannot bind to yeast cyclins to form a functional kinase complex. It is known however, that two *Trypanosoma brucei* cyclin homologues, *TbCYC2* and *TbCYC3* are able to complement an *S. cerevisiae CLN1,2* and *3* mutant strain (Neuville and Mottram, unpublished). This suggests that they can bind to CDC28 to form a functional complex that is able to recognise and phosphorylate the correct substrates. Furthermore, a third *T. brucei* cyclin CYC1, is able to complement a *Schizosaccharomyces pombe* $cdc2^{ts}$ mutant (Affranchino *et al.,* 1993). It is likely that *Leishmania* contains homologues of the *T. brucei* cyclin genes, some of which may interact with CRK3.

To test whether CRK3 could bind to the *T. brucei* cyclins to form a functional complex that could complement for loss of CDC28 activity, all three *T. brucei* cyclins were co-expressed with *L. mexicana* CRK3 in the *cdc28-4*^{ts} *S. cerevisiae* mutant. All three *T. brucei* cyclins plus the MET25 promoter and CYC1 terminator regions were cloned into the pRS314 vector which is selectable by growth in tryptophan deficient media. The resultant plasmids CYC1, pGL332 (Fig. 5.5), CYC2, pGL292 (Fig. 5.6) and CYC3, pGL293 (Fig. 5.7) were introduced into the *cdc28-1N*^{ts}, *cdc28-4*^{ts}, and *cdc28-13*^{ts} mutants and transformants were selected. No transformants were recovered for the

cdc28-1N^{ts} and *cdc28-13*^{ts} strains. However transformants were isolated for the *cdc28-4*^{ts} strain and were tested for complementation to verify that expression of the cyclins alone could not suppress the mutant phenotype. Overexpression of genes that interact with a ts mutant protein is known, in some cases to lead to suppression of the mutant phenotype and can be used to clone genes encoding proteins that interact with the mutant protein (Del Priore *et al.,* 1996). As expected none of the transformants were able to grow at the restrictive temperature (Fig. 5.8, panel A), although they grew at the permissive temperature (Panel B). The pGl120 plasmid was then introduced into these cells to generate transformants expressing CRK3 in combination with all three *T. brucei* cyclins. Triplicate clones were selected, plated onto selective medium plates and incubated at 25°C or 37°C. Colonies were formed at 25°C (Fig. 5.9, panel B) but not at 37°C (Panel A), indicating that the temperature sensitive phenotype has not been rescued by co-expression of CRK3 in combination with the three *T. brucei* cyclins. Unfortunately no anti-cyclin antibodies were available at the time of this study to test whether the cyclins were expressed in the CRK3 expressing *cdc28-4*^{ts} strain.

5.3 Discussion

The large degree of evolutionary divergence between the trypanosomatids and other eukaryotes such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* may explain why in some cases trypanosomatid homologues of yeast genes are unable to complement yeast mutants. Both *L. mexicana* CRK1, and *T. brucei* CRK2 failed to complement an *S. pombe cdc2*^{ts} mutant (Mottram *et al.,* 1993; Mottram and Smith, 1995). In the case of CRK3 the degree of divergence with CDC28 in the PSTAIRE box, six amino acid substitutions out of sixteen, may preclude the binding of yeast cyclins

and therefore the activation of the kinase. Homologues of yeast cyclins have been identified in the related trypanosomatid *Trypanosoma brucei*. Two of these genes were cloned during a screen for genes that could restore growth to a conditional G1 cyclin mutant (Neuville and Mottram, unpublished). Both of these cyclins therefore appear to be able to bind to and activate *S. cerevisiae* CDC28, and must be able to recognise the correct substrates. Homologues of *T. brucei* cyclins are likely to exist in *L. mexicana* and may function as cyclin partners of CRK3. All three of the *T. brucei* cyclins were co-expressed in the *S. cerevisiae cdc28-4*^{ts} mutant with *L. mexicana* CRK3, and tested for their ability to restore growth at the restrictive temperature. It was reasoned that one or more of the *T. brucei* cyclins might be able to form a functional kinase complex with *L. mexicana* CRK3, and that such a complex may be able to rescue the temperature-sensitive phenotype at the restrictive temperature. However, again complementation did not occur. Assuming that the cyclins are expressed there are several explanations why complementation failed. It may be that *L. mexicana* CRK3 cannot bind to any of the *T. brucei* cyclins used in this study, possibly because they are not the functional partners of CRK3. It is likely that many more trypanosomatid cyclins remain to be discovered, some of which may be the functional partners of CRK3. An alternative explanation is that the degree of divergence between the *T. brucei* cyclins and presumed *L. mexicana* homologues is too great, and the *T. brucei* cyclins cannot, therefore, bind to *L. mexicana* CRK3. This however is unlikely as *T. brucei* CRK1 (which is 72 % identical to *L. mexicana* CRK1) can function in *L. mexicana* in the absence of native CRK1. Consequently *T. brucei* CRK1 must be able to bind *L. mexicana* cyclins to function. It is likely, therefore, that *L. mexicana* CRK3, which is 78 % identical to *T. brucei* CRK3, is able to bind to *T. brucei* homologues of its cyclin partner. Ideally this experiment should have been performed using the *T. brucei CRK3* gene. This was not done due to the

the failure of attempts to clone the *T. brucei CRK3* gene into the pRS416MET vector, coupled with time constraints.

During the course of this work it became apparent that a separate study on *L. major* CRK3 had resulted in successful complementation of a *Schizosaccharomyces pombe* temperature-sensitive *cdc2* mutant (Wang *et al.,* 1998). However, complementation occurred in only two of 50,000 transformants. Analysis of these two transformants showed that the introduced plasmid had integrated into the genome, and that the expression levels of the CRK3 protein were lower than that of cells in which CRK3 was expressed from a plasmid. The level of expression of CRK3 appears therefore to be critical in determining whether a complementation test will succeed or fail. Overexpression of CRK3 may have a dominant negative effect that is only apparent in the absence of Cdc2 function at the restrictive temperature, as no visible phenotype was observed at the permissive temperature in strains expressing CRK3. Alteration of the expression level of *L. mexicana* CRK3 in the *cdc28-1N*^{ts}, *cdc28-4*^{ts} and *cdc28-13*^{ts} mutants, by growing the transformants in the presence of methionine, did not result in complementation. It was not possible, using the available anti-CRK3 antibody, to determine whether CRK3 levels were indeed affected as predicted. The anti-CRK3 antibody would only detect the protein at the limits of detection when CRK3 was fully expressed. It may be that expression levels were repressed in the presence of methionine, but that the repression was not significant enough to reduce CRK3 levels to the required level. It is clear, however, that although complementation of conditional yeast mutants provides a powerful method for analysing gene function, unforeseen epigenetic effects may hamper such analysis. For some genes it is clearly not enough to express them in a particular mutant, but the level of expression must be tightly controlled to provide a true test of the ability to complement.

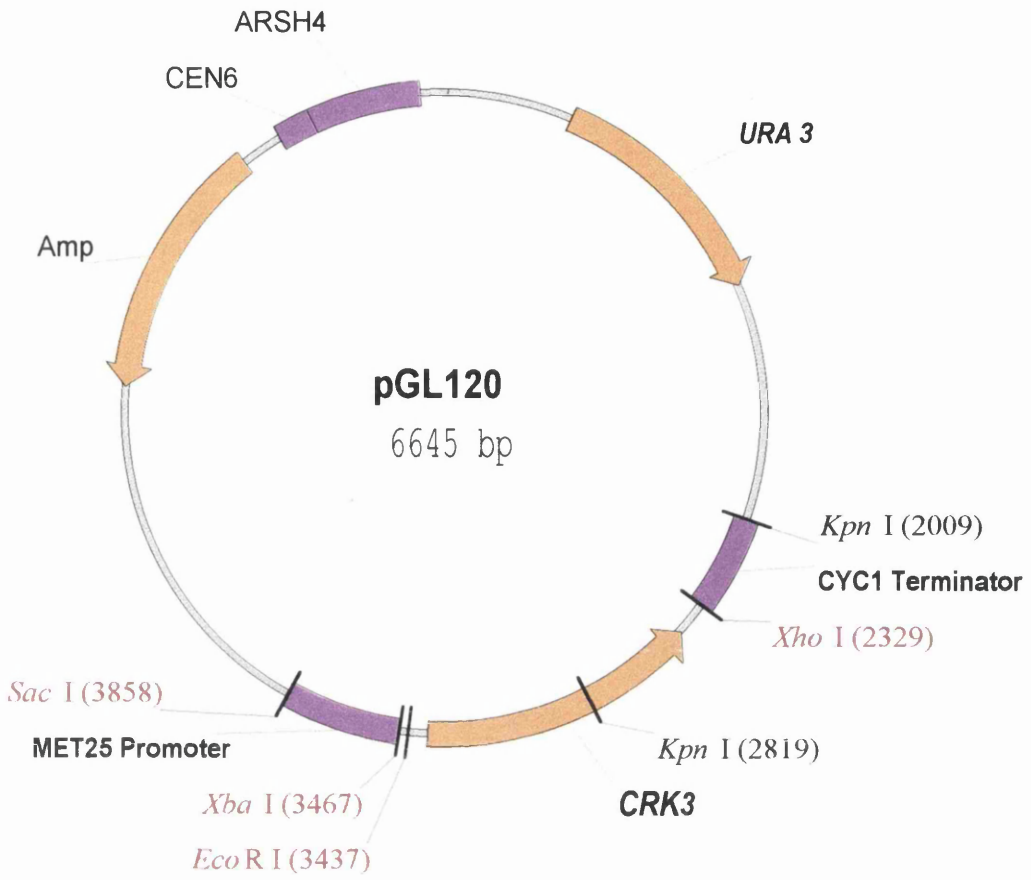
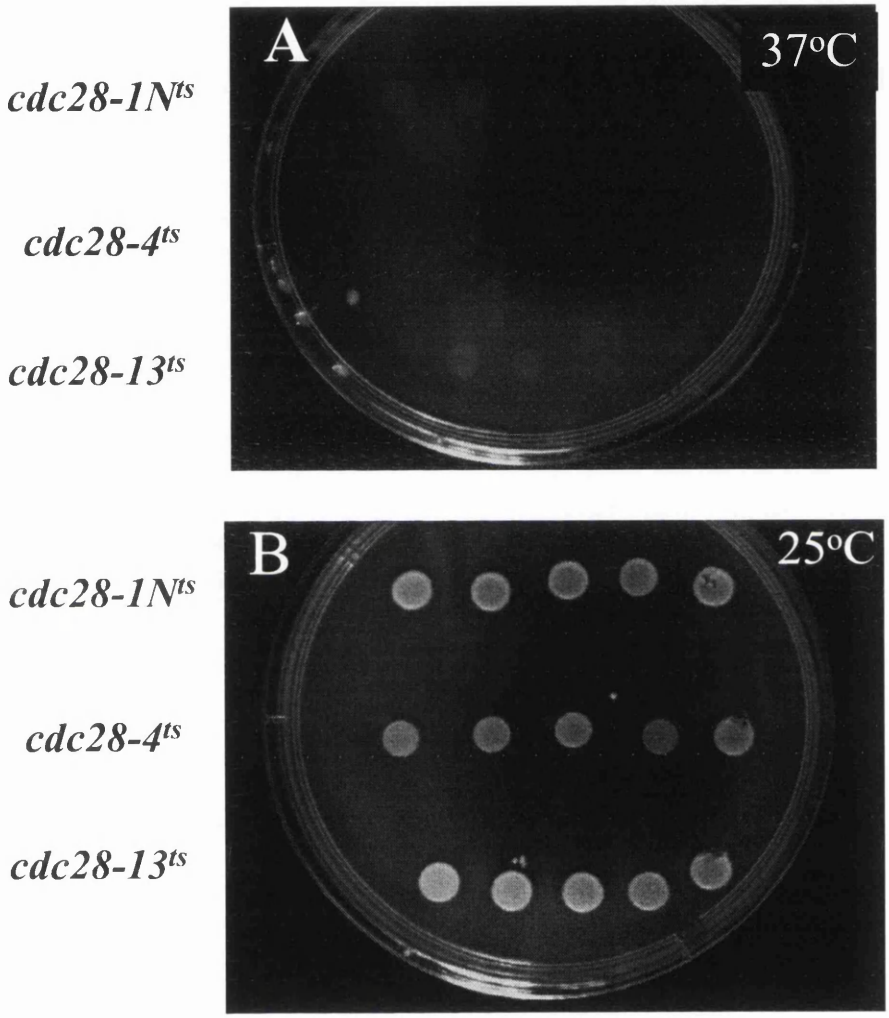


Fig. 5.1 Map of the pGL120 construct

The pGL120 construct allows the expression of *L. mexicana CRK3* in *S. cerevisiae*. The *MET25* promoter and *CYC1* terminator provide the necessary transcriptional control regions. The *URA3* gene allows for selection of clones bearing the plasmid on medium lacking uracil.

Fig. 5.2 Complementation test of *S. cerevisiae cdc28^{ts}* mutant strains expressing the *L. mexicana CRK3* gene

The *cdc28-1N^{ts}*, *cdc28-4^{ts}* and *cdc28-13^{ts}* mutant *S.cerevisiae* strains were transformed with the pGL120 plasmid to allow expression of *L. mexicana CRK3* in *S. cerevisiae*. Transformants were selected on YNB-agar medium lacking uracil. Five colonies derived from each transformation were picked from the plate, resuspended in 50 µl distilled water and 5 µl of this cell suspension was transferred to duplicate plates. One plate (Panel A) was incubated at the restrictive temperature of 37°C and the other at the permissive temperature of 25°C (Panel B).


A
37°C
cdc28-1N^ts
cdc28-4^ts
cdc28-13^ts
B
25°C
cdc28-1N^ts
cdc28-4^ts
cdc28-13^ts

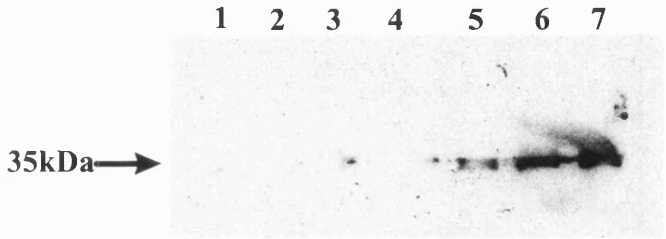


Fig. 5.3 Western blot analysis of transformed *S. cerevisiae* cell lysates

Cell lysates were prepared from the *cdc28-1N*^{ts}, *cdc28-4*^{ts} and *cdc28-13*^{ts} mutant strains transformed with the pGL120 plasmid. Lysates were separated by SDS-PAGE, transferred to PVDF membrane and probed with an antibody against the last ten residues of the C-terminus of CRK3 (Grant *et al.*, 1998).

C. Control lysate, untransformed *cdc28-13*^{ts}.

Lane 1. *cdc28-1N*^{ts}, pGL120, lysate plus 2 μg ml^{-1} competing peptide

Lane 2. *cdc28-4*^{ts}, pGL120, lysate plus 2 μg ml^{-1} competing peptide

Lane 3. *cdc28-13*^{ts}, pGL120, lysate plus 2 μg ml^{-1} competing peptide

Lane 4. *cdc28-13*^{ts}, no plasmid

Lane 5. *cdc28-1N*^{ts}, pGL120, lysate

Lane 6. *cdc28-4*^{ts}, pGL120, lysate

Lane 7. *cdc28-13*^{ts}, pGL120, lysate

Fig. 5.4 Complementation test in the presence of methionine

The *cdc28-1N*^{ts}, *cdc28-4*^{ts}, and *cdc28-13*^{ts} *S. cerevisiae* mutants expressing *L. mexicana CRK3*, and a *cdc28-13*^{ts} strain expressing the *C. elegans ncc1* gene, were transferred to plates containing 1.0 mM or, 500, 250, 100 or 50 μM methionine, to alter the levels of expression of CRK3. The control plate (Panel A) was incubated at the permissive temperature (25°C). All other plates were incubated at the restrictive temperature (37°C).

Panel A. Control plate 30°C.

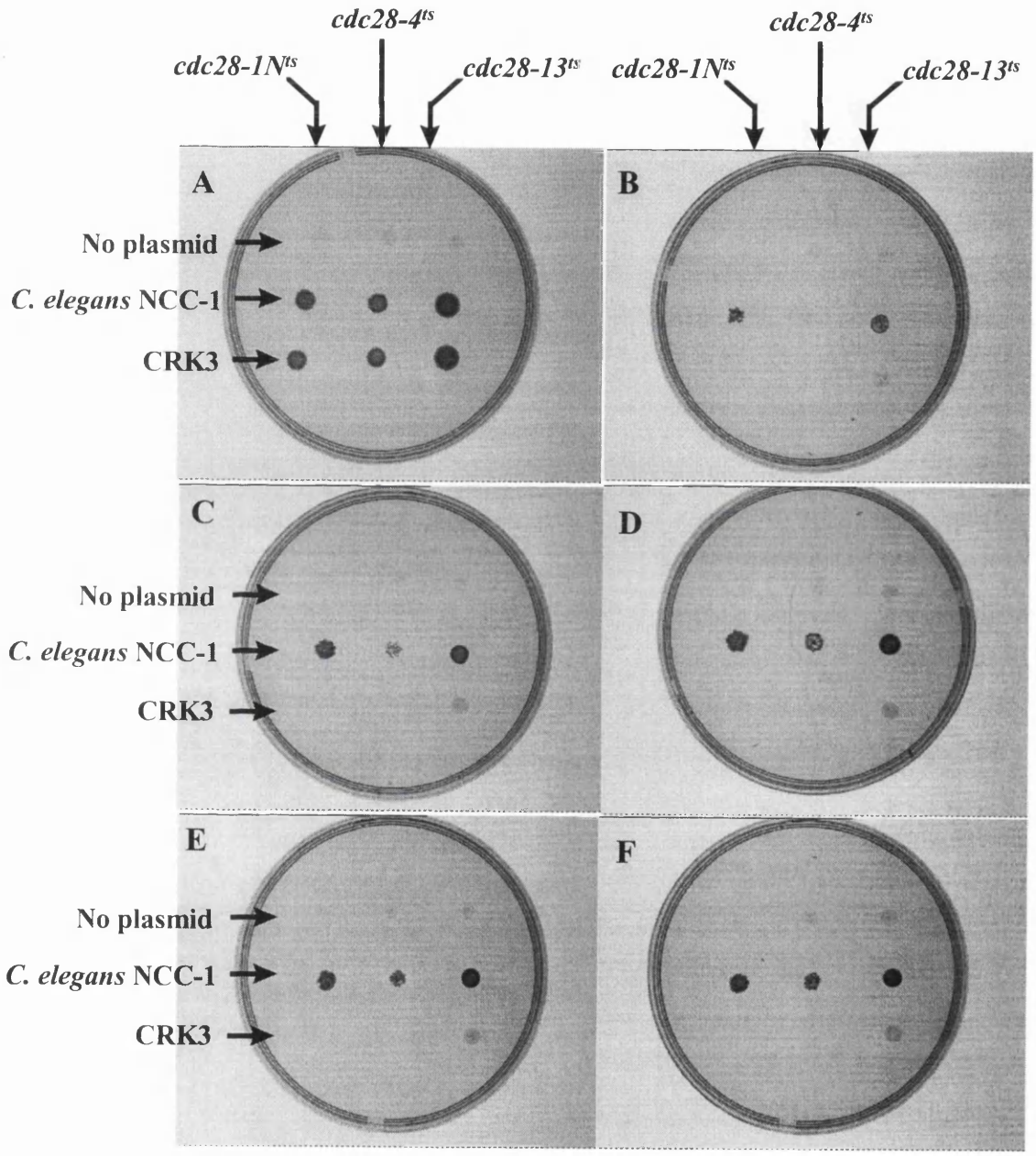
Panel B. 1.0 mM methionine

Panel C. 500 μM methionine

Panel D. 250 μM methionine

Panel E. 100 μM methionine

Panel F. 50 μM methionine


cdc28-4ts
cdc28-1Nts
cdc28-13ts
A
No plasmid
C. elegans NCC-1
CRK3
B
C
No plasmid
C. elegans NCC-1
CRK3
D
E
No plasmid
C. elegans NCC-1
CRK3
F

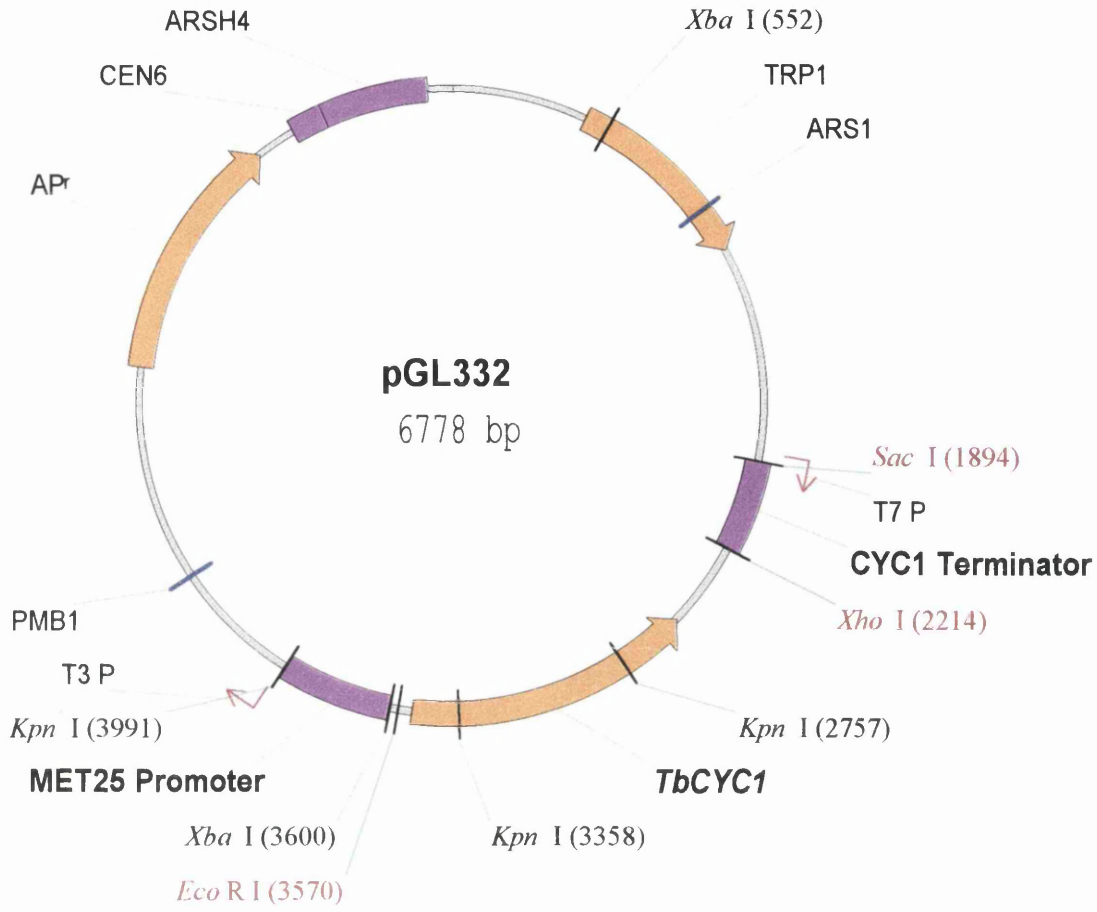


Fig. 5.5 Map of the pGL332 construct

The pGL332 construct allows the expression of *T. brucei CYC1* in *S. cerevisiae*. The *MET25* promoter and *CYC1* terminator provide the necessary transcriptional control regions. The *TRP* gene allows for selection of clones bearing the plasmid on medium lacking tryptophan.

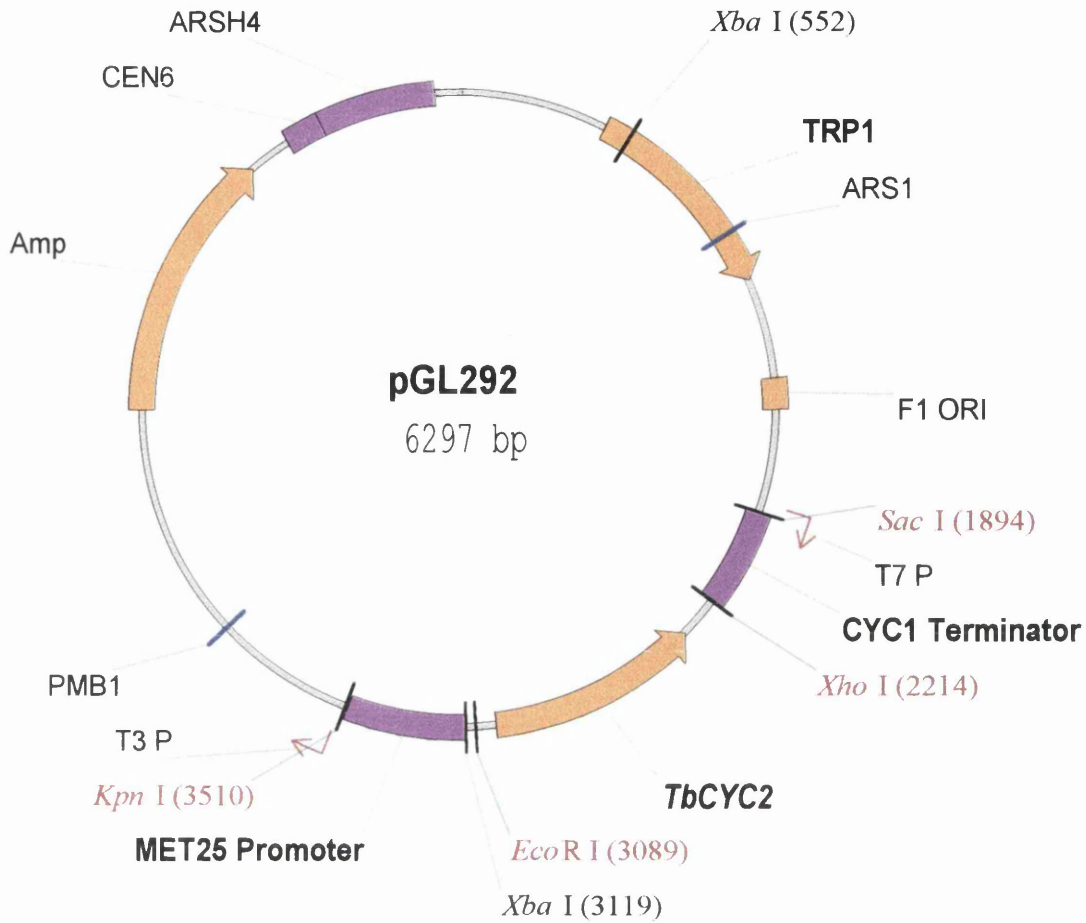


Fig. 5.6 Map of the pGL292 construct

The pGL292 construct allows the expression of *T. brucei CYC2* in *S. cerevisiae*. The *MET25* promoter and *CYC1* terminator provide the necessary transcriptional control regions. The *TRP* gene allows for selection of clones bearing the plasmid on medium lacking tryptophan.

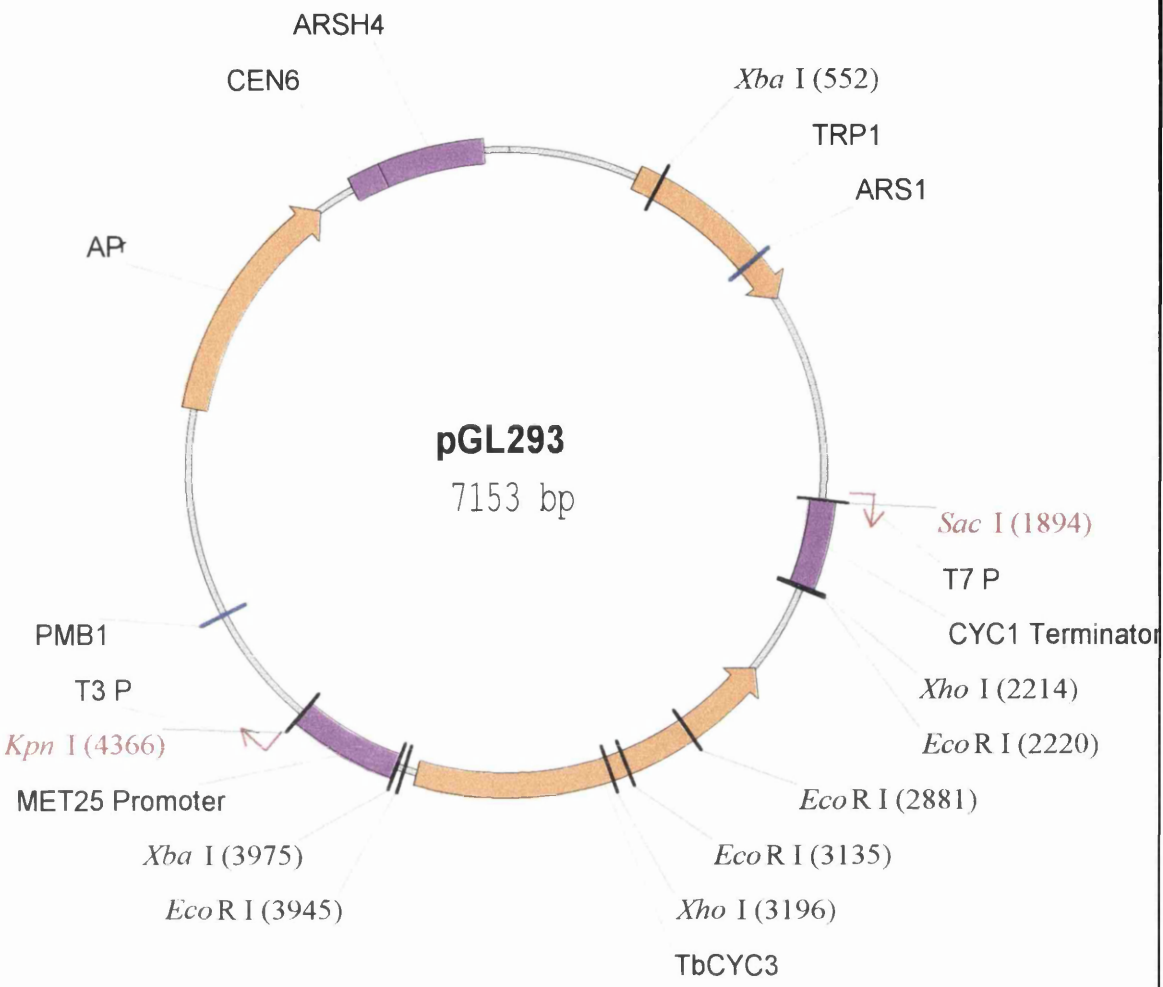


Fig. 5.7 Map of the pGL293 construct

The pGL293 construct allows the expression of *T. brucei CYC3* in *S. cerevisiae*. The *MET25* promoter and *CYC1* terminator provide the necessary transcriptional control regions. The *TRP* gene allows for selection of clones bearing the plasmid on medium lacking tryptophan.

Fig. 5.8 Complementation test of *cdc28-4^{ts}* mutant expressing *T. brucei CYC1*, *CYC2* or *CYC3*

The *cdc28-4^{ts}* mutant *S. cerevisiae* strains was transformed with either the pGL332, pGL292 or pGL293 plasmids, that allow expression of *T. brucei* CYC1, CYC2, and CYC3 in *S. cerevisiae*. Transformants were selected on YNB-agar medium lacking tryptophan. Triplicate colonies derived from each transformation were picked from the plate, resuspended in 50 µl distilled water and 5 µl of this cell suspension was transferred to duplicate plates. One plate (Panel A) was incubated at the restrictive temperature of 37°C and the other at the permissive temperature of 25°C (Panel B).

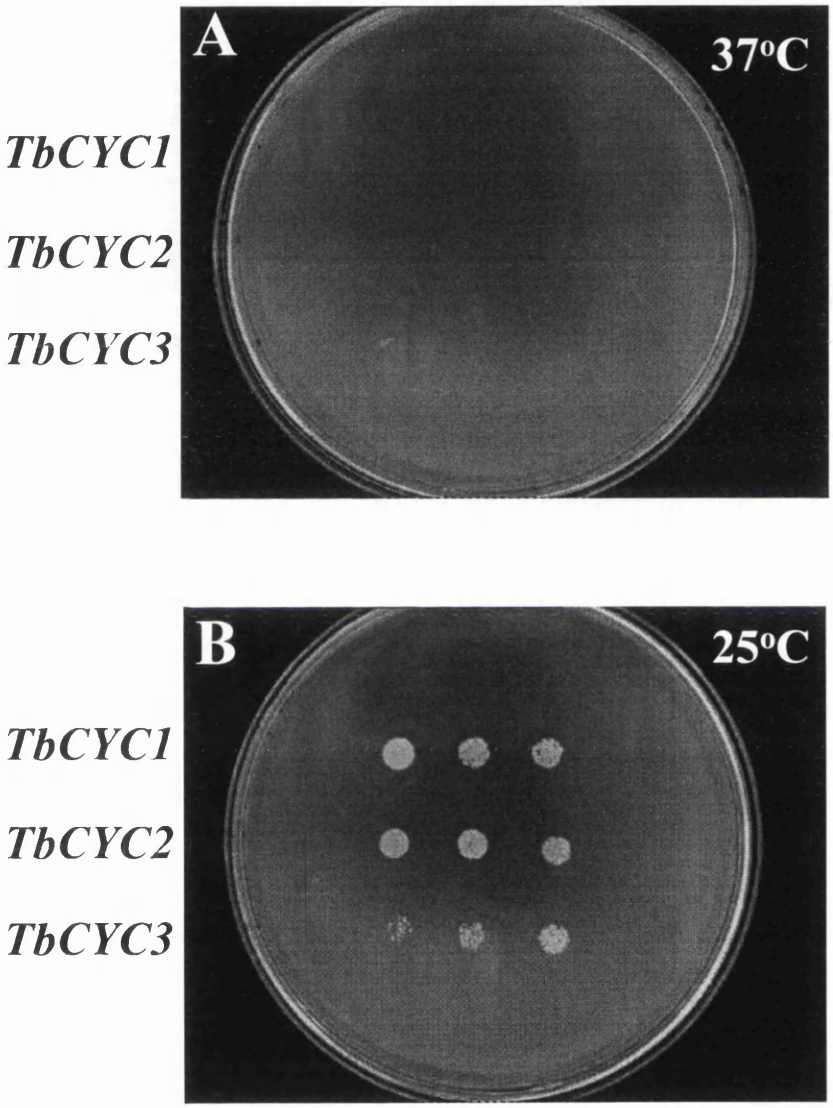
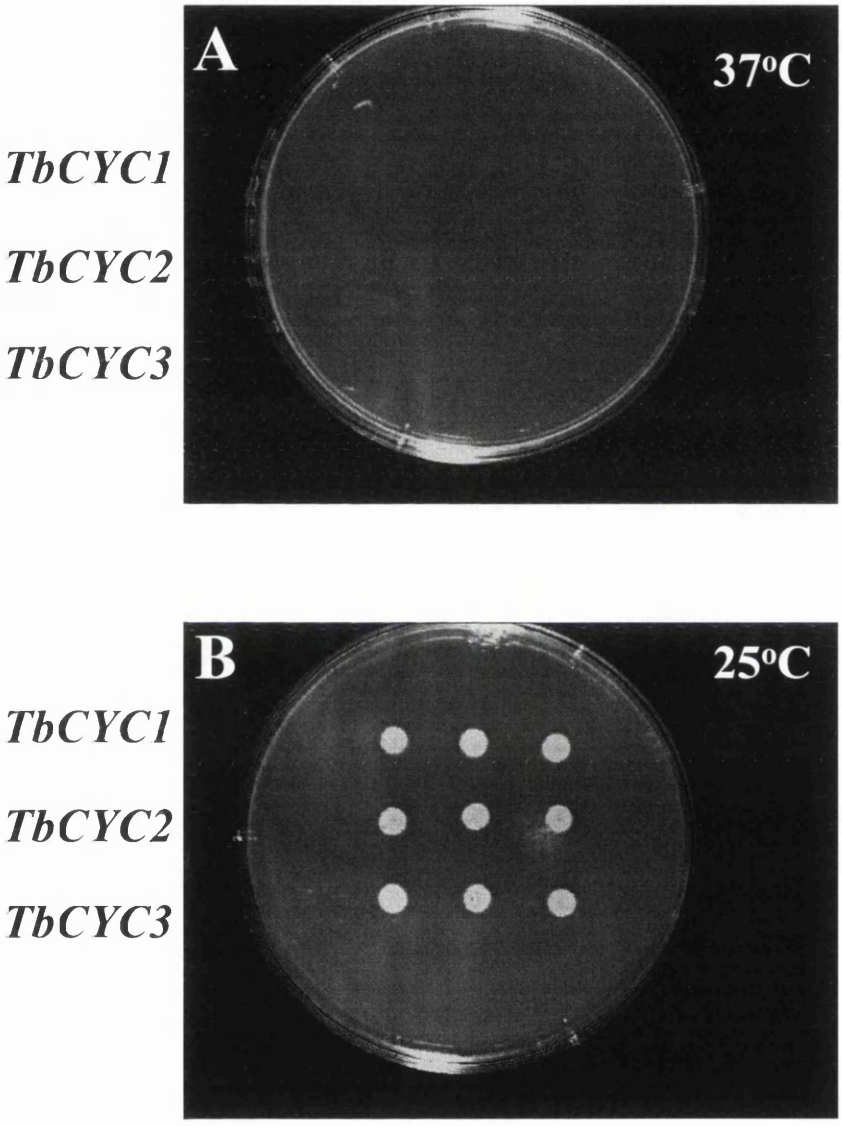

A
37°C
TbCYC1
TbCYC2
TbCYC3
B
25°C
TbCYC1
TbCYC2
TbCYC3

Fig. 5.9 Complementation test of *cdc28-4^{ts}* mutant co-expressing *L. mexicana* CRK3 and *T. brucei CYC1*, *CYC2* and *CYC3*

The *cdc28-4^{ts}* mutant *S. cerevisiae* strains expressing CRK3 from plasmid pGL120 was transformed with the pGL332, pGL292 and pGL293 plasmids that allow expression of *T. brucei CYC1*, *CYC2*, and *CYC3* in yeast. Transformants were selected on YNB-agar medium lacking uracil and tryptophan. Triplicate colonies derived from each transformation were picked from the plate, resuspended in 50 µl distilled water and 5 µl of this cell suspension was transferred to duplicate plates. One plate (Panel A) was incubated at the restrictive temperature of 37°C and the other at the permissive temperature of 25°C (Panel B).


A
37°C
TbCYC1
TbCYC2
TbCYC3
B
25°C
TbCYC1
TbCYC2
TbCYC3

CHAPTER 6

General Discussion

6.1 Gene Disruption of *CRK3*

The aim of this thesis has been to describe the methods used to analyse the function of the *CRK3* gene of *Leishmania mexicana*, and to explain the findings of these analyses. The kinase encoded by the *CRK3* gene of *L. mexicana* is a member of a group of trypanosomatid kinases that show a high degree of homology to members of the cyclin-dependent kinase family of Serine/Threonine protein kinases (Mottram *et al.*, 1993; Mottram and Smith, 1995; Grant *et al.*, 1998). In other organisms members of the cdk family play important roles in initiating transitions between phases of the cell cycle (Piggott *et al.*, 1982), controlling the transcriptional apparatus of the cell (Primig *et al.*, 1992), and integrating extracellular signalling events with cell cycle progression, transcriptional regulation and differentiation (Kato *et al.*, 1993; Polyak *et al.*, 1994). Sequence analysis alone is not sufficient to determine which of the trypanosomatid kinases is the functional homologue of a particular yeast, or vertebrate cdk. The level of similarity between any member of the trypanosomatid CRKs and a member of the vertebrate or yeast cdks varies between 43% and 59% (Mottram and Smith, 1995). We tested whether *CRK3* was essential by attempting to generate *CRK3* null mutant cell lines in which both copies of *CRK3* were disrupted by the site specific integration of a gene targeting construct. Such targeting constructs contained a drug resistance marker gene, conferring resistance to an appropriate antibiotic compound. One *CRK3* allele could be reproducibly deleted by homologous integration of gene disruption constructs (section 3.2.1). The *CRK3::HYG* and *CRK3::BLE* constructs were equally effective. However attempts to integrate the second targeting construct, in order to generate a null

mutant, resulted in ploidy changes in the resulting transfectant cell lines (section 3.2.1). The exact mechanism by which these ploidy changes occur is unknown. But, it is believed that these changes are due to the requirement for the parasite to retain a copy of the gene being targeted (Cruz *et al.,* 1993). If the gene being targeted is essential, then it is not possible to obtain double resistant mutants unless ploidy changes, or a genomic rearrangement, has allowed the double resistant cells to retain at least one copy of the targeted gene (Mottram *et al.,* 1996; Tovar *et al.,* 1998). It is possible that under the extreme conditions of electroporation, when the parasite membrane is temporarily permeabilised to allow entry of the transfected DNA, parasites may fuse together. This would result in a cell containing two wild type and two disrupted *CRK3* alleles. Subsequent incorporation of the transfected DNA molecule at one of the wild type *CRK3* alleles, allows expression of the second drug resistance marker gene, whilst still retaining an intact *CRK3* allele. Another possibility is that aneuploid parasites can arise during serial passage of *Leishmania* promastigotes in liquid culture. Under normal conditions such parasites would have a growth disadvantage and would quickly become outnumbered by more rapidly growing diploid cells. Under the extreme selection conditions that exist during the second round of disruption of an essential gene, then aneuploid parasites would have a distinct selective advantage. They could integrate the second targeting fragment whilst still retaining an intact wild type *CRK3* allele. Under double drug selection such cells would be able to grow. It is also possible that cells in late S-phase, with a greater than 2N DNA content, at the time of electroporation, integrate the targeting construct but fail to undergo cell division. Such cells again have a selective advantage as they contain both drug resistance genes whilst retaining a functional *CRK3* allele.

The sort of changes in ploidy mentioned above do not occur if an extra copy of *CRK3* is introduced into a heterozygote mutant prior to disruption of the second native *CRK3* allele (see section 3.2.4). However this was only possible when the drug selection protocol was manipulated so that the selection of the episome was not coincident with selection for the second integration event. Because *CRK3* is an essential gene then there would be no need to select for the episome in this instance, as only those cells which have retained the episome and integrated the second targeting fragment will be able to grow. It is not clear why selection with all three antibiotics (hygromycin, phleomycin and Geneticin) was lethal, because transfected parasites in which both wild type *CRK3* alleles had been disrupted, and which were expressing *CRK3* from the pTEX episome, were able to grow in liquid culture in the presence of all three antibiotics. For some reason the initial triple drug selection on solid medium is not feasible. It is possible that some subtle, synergistic effect of the triple drug selection becomes apparent only under the extreme conditions of selection of transfectants on solid medium; when a single transfected cell is expected to grow and divide to form a colony of cells.

Attempts to produce a cell line in which both native *CRK3* alleles were disrupted and a modified, hexa-histidine tagged version of the kinase was expressed from an episome, failed at each attempt. Invariably the second targeting construct integrated into the episome (see section 3.2.6). This failure may be due to an effect of the presence of the tag, which is located at the C-terminus of the molecule, on some aspect of the function of the kinase. It is known however, that the tagged kinase can be expressed and purified from *Leishmania*, and has kinase activity (Grant *et al.,* 1998); indicating that regulatory events such as cyclin binding and phosphorylation/dephosphorylation of critical residues are unaffected. It is possible that the presence of the tag interferes with substrate binding. This however is unlikely, as substrate specificity is likely to depend

upon the cyclin subunit, rather than the catalytic subunit. Another possibility is that the tag interferes with the sub-cellular localisation of the kinase. It is known that cdk complexes move between different subcellular compartments at different points in the cell cycle (Hagting *et al.,* 1998). Another explanation is that the approach taken to generate the required cell line was flawed. In this case, unlike the previous instance where a pTEX based episome was used to complement a null background, a pX based episome was used to express the modified CRK3. The pX vector contains *L. major* derived *DHFR-TS* intergenic sequence, which provides the signals for trans-splicing and polyadenylation of the required mRNA. This *DHFR-TS* derived sequence is also present in the gene targeting construct, to provide the signals for trans-splicing and polyadenylation of the mRNA derived from transcription of the drug resistance gene. This situation means that any degradation of the introduced targeting fragment, by exonucleases for example, may expose the *DHFR-TS* sequence, allowing homologous recombination with other *DHFR-TS* sequence, such as is present in the pX episome. Because the pX episome is present in a high copy number in comparison to the target locus, then integration into the episome is highly favourable. It is clearly important to give careful consideration to the design of constructs for gene disruption experiments. Such constructs can be designed using flanking regions of the gene being targeted to provide the RNA processing signals for the drug resistance gene. However such flanking regions may contain sequence motifs conferring cell cycle regulated periodicity on the gene transcript; this occurs in the case of a number of genes involved in kinetoplast DNA replication (Hines and Ray, 1997; Brown and Ray, 1997), and may possibly occur in the case of genes involved in cell cycle control, such as cyclins or crks. In *Saccharomyces cerevisiae* it is known that cyclin transcripts are cell cycle regulated (Wittenberg *et al.,* 1990), however this is not the case for *CDC28* transcript

levels, that remain constant throughout the cell cycle (Lorincz and Reed, 1984). If flanking regions of a constitutively expressed gene are required to ensure constitutive expression of the drug resistance marker gene, then it may be prudent to ensure that such sequence bears no homology to sequence present in any episome that may also be introduced into the parasite. If this is unavoidable then it is important to use a large amount of flanking DNA derived from the target locus. This would minimise any problems associated with the degradation of DNA at either end of the linear targeting fragment.

Expression of hexahistidine tagged CRK3 from a pTEX based episome, followed by disruption of both native *CRK3* alleles following the procedure used in section 3.2.4 should allow expression of the modified kinase in a null background.

The gene disruption evidence presented in this thesis, suggests that CRK3 is essential to *L. mexicana* promastigotes and has a non-redundant function. If the same is true of CRK3 in amastigotes then CRK3 can be considered a potential drug target.

6.2 Indirect inhibition of CRK3

Incubation of *Leishmania* promastigotes with the protein tyrosine phosphatase inhibitor bpV(phen), resulted in growth arrest of the parasites. A parallel study has suggested that this growth arrest is due to indirect inhibition of the *Leishmania* homologue of the cdk1 gene, which is involved in controlling the transition from G2 to M phase in vertebrate cells (Draetta and Beach, 1988). It is thought that this inhibition occurs by inhibition of the *Leishmania* homologue of the vertebrate cdc25 tyrosine phosphatase (Olivier *et al.*, 1998). The cdc25 phosphatase regulates cdk1 activity by removing the inhibitory phosphate at the conserved Tyr 15 residue (Millar *et al.*, 1991). Inhibition of cdc25

therefore results in inhibition of cdk1 activity, as the Tyr 15 residue cannot be dephosphorylated (Faure *et al.,* 1995). Incubation of *L. mexicana* promastigotes with bpV(phen) resulted in a cell cycle arrest in the G1 and S-phases of the cell cycle (section 4.2.1). Analysis of the $p13^{suc1}$ binding kinase activity of *L. mexicana*, due predominantly to CRK3 (Grant *et al.,* 1998), showed that a 24 hour incubation with bpV(phen) drastically reduces the kinase activity (Fig.4.2). Presumably this reduction in kinase activity is due to inhibition of an as yet unidentified *Leishmania* homologue of Cdc25. The inhibition of kinase activity is reversible. Cells which have been arrested by a 24 hour incubation with bpV(phen) can be released from the arrest by washing the cells in PBS before resuspending them in fresh medium. Cells will then re-enter the cell cycle, progressing through S-phase, G2, and M-phase. This re-entry into a normal pattern of cell cycle progression is coincident with a restoration of the $p13^{suc1}$ binding kinase activity. These results may therefore point to a role for CRK3 in control of the G1/S-phase transition in *Leishmania*. A role for other CRKs in controlling this transition cannot be ruled out as it is not yet clear whether other CRKs can bind to $p13^{suc1}$ and make a contribution to the overall $p13^{suc1}$ binding kinase activity. The study mentioned previously in which *L. donovani* promastigotes were incubated with bpV(phen), resulted in a G2/M-phase cell cycle block. It is possible that differences in the experimental conditions have resulted in this apparent difference between the two species of *Leishmania*. If dephosphorylation of CRKs by a *Leishmania* cdc25 homologue is required at both the G1/S and G2/M transition then subtle experimental differences may result in arrest at one or other transition point. It is also possible that there are fundamental differences in the control of cell cycle progression in both these *Leishmania* species with two different cdc25 activities acting at either transition, one which is resistant to bpV(phen) inhibition and the other not. It may be that the

susceptible cdc25 homologue acts at the G1/S transition in *L. mexicana* and acts at the G2/M transition in *L. donovani*. This situation is unlikely, given the close relatedness of both parasite species in evolutionary terms (Croan *et al.,* 1997). An alternative explanation is that CRK3 plays no role in control of the G1/S transition and is not active at this stage of the cell cycle. Inhibition of cell cycle progression by bpV(phen) may be due to another CRK complex that governs the G1/S-phase transition. This may explain why CRK3 activity is low in cells arrested at G1/S but rises as cells are released from the block. A third possibility is that bpV(phen) affects other protein tyrosine phosphatase possibly involved in signal transduction. Differences in tyrosine phosphorylation by as yet unidentified kinases are known to occur during the trypanosome life cycle (Parsons *et al.,* 1990). It is probable that tyrosine phosphorylation/dephosphorylation events play an indirect role in cell cycle progression. However, given that no cdc25 homologues have yet been discovered in any trypanosomatid species and the true nature of bpV(phen) action in *Leishmania* is not fully understood, then it is difficult to distinguish between these possibilities.

6.3 Direct inhibition of CRK3 by the cdk inhibitor flavopiridol

Incubation of *Leishmania* promastigotes in the presence of the cdk inhibitor flavopiridol, resulted in a dose dependent reduction in the growth rate of the parasites. Analysis of the DNA content of growth arrested parasites by FACS, indicated that cells were blocked at the G2/M transition. This block was reversible and cells could be released, and would continue progression through mitosis and into a subsequent cell cycle. Release from the cell cycle block was partially synchronous for the duration of the subsequent cell cycle, after which the synchrony broke down. Purified hexahistidine

tagged CRK3 kinase is inhibited by flavopiridol in a dose dependent manner. 50% inhibition of the purified kinase activity is achieved at a flavopiridol concentration of 100 nM. This figure is broadly comparable with the concentration of 250 nM which is required to give 50% inhibition of *Leishmania* growth. The discrepency between the two figures is probably due to the fact that to inhibit the kinase *in vivo*, flavopiridol must cross the plasma membrane to exert its effect on the kinase. In the *in vitro* situation when purified kinase is assayed for activity, this is not the case. The fact that the two values vary within a single order of magnitude provides good evidence that the effect of flavopiridol on cell cycle progression is due to it's inhibitory effect on CRK3. The CRK3 kinase is one member of a family of CRKs in *Leishmania* and trypanosomes, many of which may have a role in cell cycle progression. Given that CRKs are likely to function in a similar manner and that the site of flavopiridol action, ie. the ATP binding pocket, that is likely to be structurally similar in all CRKs, then it is possible that flavopiridol can inhibit other CRKs with similar kinetics to CRK3. It is known that flavopiridol can inhibit a number of mammalian cdks, but is most effective against cdk1, (Losiewicz *et al.,* 1994), cdk2 and cdk4 (Carlson *et al.,* 1996), the kinases with the most direct affect on cell cycle control. What can be concluded is that as flavopiridol is a highly specific inhibitor of CRK3, and that it causes a block in cell cycle progression, then CRK3 is likely to play a role in signalling cell cycle progression in *Leishmania*. The $p13^{suc1}$ binding properties of CRK3 and the link between bpV(phen) induced perturbation of cell cycle progression and CRK3 kinase activity, provide other independent lines of evidence that CRK3 has a role in cell cycle control. It is not possible based on the available evidence to say whether cell cycle control in *Leishmania* is similar to the situation in yeast, where a single cdk is responsible for controlling both G1/S and G2/M progression, or whether different CRKs act to promote either transition,

as occurs in vertebrate cells. What is clear is that CRK3 plays a role in signalling entry into mitosis. Inhibition of CRK3 activity by the cdk inhibitor flavopiridol blocks entry into mitosis. It remains to be seen whether other CRKs, such as CRK1 are also inhibited by flavopiridol.

In *Trypanosoma brucei*, incubation of procyclic or bloodstream forms with flavopiridol resulted in growth arrest at both the G1/S and G2/M transition (Fig.4.16), suggesting that CRK3 acts at both transition points. Why the same effect is not observed in *Leishmania* is not clear. It is possible that CRK3 interacts with different cyclin partners at each transition and that in *Leishmania*, the G1/S CRK3 complex is more resistant to inhibition by flavopiridol due to structural changes in the ATP binding pocket induced by cyclin binding. Alternatively, the *T. brucei* G1/S kinase may be more sensitive to falvopiridol than its *Leishmania* homologue. Clearly a lot has still to be learned about the mechanisms governing cell cycle control in both *Leishmania* and trypanosomes.

The use of flavopiridol to synchronise *Leishmania* may prove a valuable tool to aid the biochemical analyses of cell cycle control. Synchronised parasites in a given phase of the cell cycle may be used as the starting material for the isolation of CRK/cyclin complexes or for identification of activities that are cell cycle regulated.

6.4 *CRK3* fails to complement *Saccharomyces cerevisiae cdc28^{ts}* mutants

The fundamental components of cell cycle control have been conserved throughout evolution to such a degree that homologues of the *Schizosaccharomyces pombe cdc2* and *Saccharomyces cerevisiae CDC28* genes from distantly related species can complement for loss of function of these genes (Hirt *et al.*, 1991; Miao *et al.*, 1993; Setiady *et al.*, 1996; Mori *et al.*, 1994; Lee and Nurse, 1987; Ninomiya-Tsuji *et al.*,

1991). Expression of Cdc2/CDC28 homologues in temperature sensitive mutant strains can rescue the temperature sensitive phenotype. The human homologue of *Schizosaccharomyces pombe* Cdc2 can complement both an *S. pombe cdc2* as well as a *Saccharomyces cerevisiae cdc28* mutant (Lee and Nurse, 1987; Meyerson *et al.,* 1992). In addition the human cdk2 gene can complement an *S. cerevisiae cdc28* mutant. The use of such conditional mutant yeast strains provides a useful and sensitive assay of potential Cdc2/CDC28 function, and has been used to clone *cdc2/CDC28* homologues from plant species (Hirt *et al.,* 1991; Miao *et al.,* 1993; Setiady *et al.,* 1996), *Caenorhabditis elegans* (Mori *et al.,* 1994) and mammals (Lee and Nurse, 1987; Ninomiya-Tsuji *et al.,* 1991). Given that the CRK3 kinase of *Leishmania mexicana* has features that make it a good candidate to be the functional homologue of Cdc2 (it binds with high affinity to $p13^{suc1}$ and is active only in the proliferative life cycle stages) it was thought to be important to test the *CRK3* gene in such a complementation assay. Sequence analysis of the known CRKs provides no real clue to their function. None of the CRKs obviously resemble any known member of the cdk family to such a degree that the function can be presumed. The *CRK1* gene of *L. mexicana* had previously been tested for ability to complement a fission yeast *cdc2* mutant strain, and failed to do so (Mottram *et al.,* 1993). Attempts to clone a *cdc2* homologue by complementation of a $cdc2^{ts}$ mutant with an *L. mexicana* cDNA library also failed (J.C. Mottram, personal communication). A possible reason for such failure may be that the fission yeast cell cycle is peculiar in that the main point of control occurs at the G2/M transition, whereas the G1/S phase transition seems to be more important in most diploid eukaryotes. This apparent peculiarity of the fission yeast cell cycle may preclude the cloning of *cdc2* homologues from many species. The mammalian cdk2 gene was cloned due to its ability to complement an *S. cerevisiae cdc28* mutant, but was never cloned in a screen

for genes that complement fission yeast *cdc2* mutants. For this reason a number of budding yeast *cdc28* mutants were used in a complementation test of the *CRK3* gene (section 5.1). The particular *cdc28* mutants arrest with different phenotypes and at slightly different points in the cell cycle, *cdc28-1N*^{ts} and *cdc28-13*^{ts} arrest in G1 whilst *cdc28-4*^{ts} arrests in G2 (Lorincz and Reed, 1986). The positions of the mutations responsible for the mutant phenotypes did not correlate with the observed biological characteristics of the mutant alleles. They appeared, however, to occur at regions of structural transition (Lorincz and Reed, 1986). *L. mexicana* CRK3 was expressed from a centromeric plasmid under the control of the *S. cerevisiae MET25* promoter. This promoter is active in the absence of methionine and is repressed in high methionine concentrations (Mumberg *et al.,* 1994). The *L. mexicana CRK3* gene failed to complement any of the three *cdc28* mutant strains tested (Fig.5.2). Regulation of the levels of CRK3 expression by the inclusion of a range of concentrations of methionine in the medium did not affect this result. The CRK3 kinase could be detected by western blotting in all three strains (Fig.5.3), but it is not known whether the levels of the kinase could be regulated by repression in the presence of methionine. Overexpression of CRK3 could conceivably result in an imbalance between essential cell cycle components causing a toxic effect on the cells. Another possibility is that CRK3 is unable to bind to yeast cyclins to form a functional kinase, or cannot phosphorylate the proper substrates. As two trypanosome cyclins were cloned by complementation of a budding yeast *cln1,2* and *3* mutant, *TbCYC2* and *TbCYC3* (Neuville and Mottram, unpublished), and are therefore functional in *S. cerevisiae*; they, along with the *TbCYC1* gene, were expressed in the *cdc28-4*^{ts} mutant, along with the *Leishmania* CRK3 kinase, in the hope that they would be able to bind to CRK3 to form a functional kinase complex. This would help to ascertain which, if any, of the known trypanosome cyclins

is a partner for CRK3. Ideally this experiment would have been performed using the *T. brucei* CRK3 for the co-expression and complementation test, however, due to repeated failure of attempts to clone *T. brucei CRK3* into the pRS416MET vector, this was not possible. Co-expression of all three trypanosome cyclins in combination with *L. mexicana* CRK3 failed to complement for loss of CDC28 activity. It is not known whether *Leishmania* CRK3 can bind to trypanosome cyclins, however the trypanosome CRK1 is able to function in *Leishmania*, suggesting a close functional conservation between the trypanosomatid species (Mottram *et al.,* 1996). It is therefore likely that *L. mexicana* CRK3 is able to bind to the *T. brucei* homologue of it's cyclin partner. However, only three cyclins were tested in these experiments as these are the only known cyclins in trypanosomatids. It is not unreasonable to assume that there are other (as yet unidentified) cyclins in the trypanosomatids, that could be the partners of CRK3. Recently it has been shown by the yeast two-hybrid assay, and by co-immunoprecipitation experiments that *T. brucei* CRK3 interacts with TbCYC2, though it is not yet known at what stage of the cell cycle this complex is active (Van Hellemond and Mottram, unpublished).

A subsequent study in which the *L. major CRK3* gene was expressed in a *Schizosaccharomyces pombe cdc2* mutant, has shown that CRK3 can complement for loss of Cdc2 function (Wang *et al.,* 1998). In this instance, however, complementation only occurred when the *CRK3* gene had been integrated into the *S. pombe* genome. The reason for this is not yet known, but it is possible that a gene dosage effect, or a requirement for a particular genomic location which may affect gene regulation, is needed to achieve the correct level of expression of CRK3 in such an assay. As the experiments described in this study involved expression of *CRK3* from an episome, this may explain the failure to complement the mutant strains used. Complementation

experiments involving species, separated by such a large evolutionary distance may not be widely applicable and are probably only of use to study genes that are very highly conserved. It is also questionable how relevant such complementation tests are to the function of a given gene. Some cyclins that are able to complement a *cln1, 2, 3* mutant have no functional similarity (Lew *et al.,* 1991).

6.5 Future Directions

To gain a more complete understanding of cell cycle control mechanisms in *Leishmania* and trypanosomes, interacting proteins will need to be unambiguously identified and their activities characterised. Given that the trypanosomatid CRKs contain many conserved residues associated with function and regulation of cdks; eg. Tyr 15, involved in regulation of activity (Gould and Nurse, 1989); Thr 161/167, involved in regulation of cyclin binding (Gould *et al.,* 1991); and the PSTAIR box, a region involved in binding to the regulatory cyclin partner protein (Jeffrey *et al.,* 1995); then it is highly likely that proteins involved in phosphorylation/dephosphorylation or binding to such residues/motifs will also be found in trypanosomatids through biochemical purification of CRKs, and through yeast two-hybrid interaction screens. The pathways governing the activity of CRKs are likely to prove to be excellent targets for chemotherapeutic intervention due to the essential role of CRKs in cell growth and their possible role in division and differentiation between infective and non-infective life cycle forms. In pleiomorphic strains of *T. brucei* grown in semi-solid liquid culture, an as yet unidentified factor (stumpy induction factor, or SIF) accumulates and induces differentiation from the long slender proliferative to the short stumpy non-proliferative form (Vassella *et al.,* 1997). The short stumpy form is arrested in the G1 phase of the

cell cycle and the effect of stumpy induction factor (SIF) can be mimicked by analogues of cyclic AMP (Vassella *et al.,* 1997). The links between such signalling pathways and the control of cell cycle progression have yet to be uncovered. However it is likely that regulation of CRK activity by the upregulation of an inhibitory phosphorylation event or an inhibitory protein is a key factor in such a cell cycle arrest. The work described in this thesis has shown a link between the activity of the CRK3 kinase of *Leishmania mexicana* and cell cycle progression of the promastigote form of the parasite. Given that CRK3 is active in promastigotes and amastigotes, and appears to play a central role in control of cell cycle progression in promastigotes, it is likely to play a similar role in amastigotes and can therefore be considered as a good anti-leishmanial chemotherapeutic target. A large number of structurally unrelated inhibitors of cdks now exist (Meijer, 1995; Meijer, 1996). Flavopiridol is one of the most potent and selective of the known chemical inhibitors of cdk activity (Sedlacek *et al.,* 1996). This study has shown that flavopiridol has a potent inhibitory effect on *L. mexicana* CRK3. Flavopiridol inhibits cdk activity by binding to the ATP binding pocket. However the flavopiridol molecule makes contacts with residues that are not involved in ATP binding (De Azevedo *et al.,* 1996). These particular residues, which have no role in ATP binding, are unlikely to be as highly conserved as other residues in the pocket region which are involved in ATP binding. For this reason it is possible that some chemically modified derivatives of flavopiridol may bind more strongly by the parasite enzyme than the host enzyme, thereby limiting toxicity to the host. Flavopiridol itself is highly toxic and the range of concentrations required to kill *Leishmania* in an infected host may exceed thresholds of toxicity (Parker *et al.,* 1998; Arguello *et al.,* 1998).

The inhibition experiments with flavopiridol were all carried out *in vitro* on the promastigote, the insect form of the parasite, or on purified kinase from promastigotes.

It is not known whether *CRK3* is also essential in amastigotes, the parasite life cycle stage found in the host bloodstream or infected macrophages. *In vitro* assay of the effects of flavopiridol on peritoneal exudate cells, from Balb/C mice, infected with stationary phase promastigotes is currently in progress. The results of this work will determine whether flavopiridol should be tested for ability to cure Balb/C mice of an infection with *L. mexicana*. Another possible method of interfering with CRK3 function is by disrupting the interaction with regulatory cyclin partner proteins. The binding of cyclins is necessary for the function and occurs via the PSTAIR box, a sixteen amino acid region found in all cdks (Jeffrey *et al.,* 1995). The PSTAIR box of *L. mexicana* and *T. brucei* CRK3 contains a total of six substitutions (Grant *et al.,* 1998; Mottram and Smith, 1995). This suggests that the structure of the CRK3-binding cyclin/s is/are significantly different from their mammalian counterparts, and may mean that the interaction between the parasite proteins could be disrupted by a specifically designed drug, without interfering with the analogous interaction of the mammalian proteins.

Currently there is no direct proof that CRK3 is the kinase involved in cell cycle progression. Such proof can only come from the analysis of the phenotypes of parasites expressing mutant versions of the protein. As the gene is essential, and no inducible expression system exists for *Leishmania*, this is a difficult task. An inducible system has, however, been developed for use in *Trypanosoma brucei* (Wirtz and Clayton, 1995). This system allows a degree of control of expression of a gene of interest. This technique could be used to express dominant negative mutant forms of the *T. brucei* CRK3, which could be analysed for mutant phenotypes associated with disruption of the cell cycle. For example, expression of a mutant form of CRK3 in which the Tyr 34 residue has been mutated to phenylalinine, may result in premature entry into mitosis upon induction of expression. Such a phenotype has been observed for a fission yeast

mutant in which the corresponding residue had been mutated in the same way (Gould and Nurse, 1989). Alternatively the Thr 161/167 residue, which in *Schizosaccharomyces pombe* is phosphorylated to activate the Cdc2 kinase (Gould *et al.,* 1991), could be mutated to a non-phosphorylatable amino acid such as alanine. This should result in an inactive kinase that is still able to bind a cyclin partner protein. Overexpression of such a mutant protein would be expected to result in a dominant negative phenotype whereby cyclin regulatory sub-units would be sequestered in an inactive complex. This would result in delayed entry into mitosis, a phenotype which is observed when such an experiment is performed in *S. pombe* (Gould *et al.,* 1991).

Another alternative is to mutate the Asp 165 residue to Asparagine. This has been done previously for the Asp 145 and Asp 146 residues of human cdk1 and cdk2 (Van den Heuvel and Harlow, 1993). The analogous residue of human cAMP dependent protein kinase A is involved in the phosphotransfer reaction (Taylor, 1993), and the equivalent residue in *Saccharomyces cerevisiae CDC28* (Asp 154) is mutated in a dominant negative *cdc28* mutant (Mendenhall *et al.,* 1988).

Transcriptional feedback loops are important aspects of cell cycle control in eukaryotes (Reed, 1996). Expression of gene products required for progression through a particular phase of the cell cycle is controlled by the activity of transcription factors (Ohtani *et al.,* 1995; DeGregori *et al.,* 1995), the activity of which may be influenced by members of the cdk family (Serizawa *et al.,* 1995). Because gene regulation in trypanosomatids is not normally regulated at the level of transcription, but is regulated at the level of translation or mRNA stability (Vanhamme and Pays, 1995), it is likely that the expression of specific cell cycle regulated genes involves an interaction between the activity of CRKs and proteins involved in translational control or mRNA stability. A number of cell cycle regulated genes have been identified in *Crithidia fasciculata* that

are upregulated in G1 phase, reaching a peak level immediately prior to kinetoplast replication (Pasion *et al.,* 1994; Hines and Ray, 1997; Brown and Ray, 1997). The mRNA of the *top2* gene was shown to be regulated at the level of mRNA stability. Sequences in the 5' UTR were identified as being involved in this cell cycle regulated stability (Hines and Ray, 1997). It has recently been shown that a number of as yet unidentified proteins bind to this region and are probably involved in stabilising the message (Mahmood and Ray, 1998). This is thought to lead to an increase in the levels of the TOP2 protein, which is required for kinetoplast replication. The protein factors responsible for mRNA stabilisation are themselves probably cell cycle regulated and are possibly regulated by the activity of CRKs involved in cell cycle progression. Such a mechanism would enable the regulated expression of genes required at a particular phase of the cell cycle in the absence of transcriptional control. This prospect raises the possibility of a new set of potential drug targets in trypanosomatids, as cell cycle regulated genes in the mammalian host are more likely to be controlled at the transcriptional level and not by the action of mRNA stabilising proteins. The role played by such trypanosomatid proteins in cell cycle regulated gene expression, and the link with the cell cycle control machinery will surely prove to be an intriguing area of research and may even help to uncover novel molecular mechanisms not present in "model" eukaryotic organisms.

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