Identification, isolation and characterisation of a *CDC37* homologue in *Schizosaccharomyces pombe*

by

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Declaration of Originality

I declare that, unless otherwise stated, this thesis represents my own work and was composed by me.

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Abbreviations

A	absorbance
A	adenosine
~	approximately
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
Ca	calcium ion
cdc	cell division cycle
CDK	cyclin dependent kinase
cDNA	complementary DNA
CIAP	calf intestinal alkaline phosphatase
Cl	chloride ion
cm	centimetre(s)
°C	degrees Celsius
DAPI	4,6,diamidino-2-phenylindole
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	ethylenediaminetetraacetic acid
EMM	Edinburgh minimal medium
FACS	fluoresence acitivated cell sorting
G	guanine
g	gram(s)
H	hydrogen ion
hr	hour(s)
His	histidine
IPTG	isopropyl-β-thiogalactopyranoside
K	potassium ion
kb	kilobase pair(s)
l	litre(s)
LB	Luria-Bertani medium
M	molar
ME	malt extract medium
Mg	magnesium ion
Mn	manganese ion
MOPS	4-morpholinepropane sulphonic acid
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
µg	microgram(s)
µl	microlitre(s)

μM	micromolar
Na	sodium ion
ng	nanogram(s)
nm	nanometre(s)
nmol	nanomole(s)
OAc	acetate
OD	optical density
%	per cent
PCR	polymerase chain reaction
PIPES	Piperazine-N,N'-bis[2-ethanesulfonic acid]
pmol	picomoles
PMSF	phenylmethylsulfonyl fluoride
Rb	rubidium ion
RNA	ribonucleic acid
RNase	ribonuclease
sec	second(s)
SD	synthetic dropout medium
SDS	sodium dodecyl sulphate
SO_4	sulphate ion
SSC	standard-saline-citrate buffer
T	thymidine
TBE	tris-borate-EDTA buffer
TE	tris-EDTA buffer
T_m	melting temperature
Tris	tris[hygroxymethyl]aminomethane
ts	temperature-sensitive
UTR	untranslated region
v/v	volume per volume
vol	volume(s)
w/v	weight per volume
x	times
X-Gal	5-bromo-4-chloro-3-inodyl-β-galactopyranoside
YE	yeast extract medium

Abstract

This work describes the identification of a *Schizosaccharomyces pombe* homologue of the *Saccharomyces cerevisiae CDC37* gene. Homologues have been previously identified in a variety of organisms, including *Drosophila*, chicken, mouse, rat and human. The *S.pombe* gene has been named *cdc37*$^{+}$ and encodes a protein with a predicted molecular weight of 52 kDa. The protein shows extremely high sequence similarity with Cdc37p from *S.cerevisiae* for the first 40 amino acids at the N-terminus but, as with the homologues from other species, this similarity is considerably less over the remainder of the protein.

Deletion of *cdc37*$^{+}$ results in a lethal phenotype, establishing that the gene is essential for viability. The null mutant could survive when *cdc37*$^{+}$ was expressed from the thiamine-regulatable low strength expression vector pREP81; when expression was repressed, the cells showed a variety of phenotypes, becoming swollen, misshapen and/or elongated. The diversity of phenotypes observed suggests that Cdc37 has several biological roles. Elongation of *S.pombe* cells indicates a cell cycle defect and measurements of cell length during Cdc37 depletion confirmed significant elongation in *Δcdc37* cells when *cdc37*$^{+}$ expression from the plasmid pREP81 was repressed. FACS and cytological analysis indicated that cell cycle defects occurred in both the G2 and mitotic phase of the cell cycle.

Genetic analysis carried out to investigate the mechanism of cell cycle arrest suggests that Cdc37 may be involved with the Cdc2/Cdc13 complex, which controls entry into mitosis from the G2 phase. *cdc37*$^{+}$ was strongly overexpressed in wild-type cells from the high strength expression vector pREP1 with no effect. However, in strains carrying either of two *cdc13*^{ts} alleles, strong *cdc37*$^{+}$ overexpression severely reduced the restrictive temperature. A similar but more modest effect was seen in strains carrying various *cdc2*^{ts} alleles. Preliminary biochemical studies were also carried out, which showed that the Cdc2 protein level was lower than normal in cells depleted for Cdc37.

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Chapter 1

Introduction

1.1 The eukaryotic cell cycle

1.1.1 Introduction

The cell cycle is the process by which a cell reproduces and divides into two daughter cells. It comprises two critical phases; S phase, during which chromosomal DNA is replicated and mitosis (M phase) where the replicated genome is distributed equally between the two daughter cells. Separating these two phases are G1 (after M phase and before S phase) and G2 (after S phase and before M phase). The order and integrity of cell cycle events must be maintained to ensure the production of viable progeny.

Cells can exit from the cell cycle from either G1 or G2. In multicellular eukaryotic organisms, most of the cells that are no longer growing and proliferating are arrested at a stationary phase known as G0. Such cells have left the cycle after division at a certain point in G1, known as the restriction point (Pardee, 1974). Cells that do not leave the mitotic cell cycle at this point are committed to progress through the remainder of the cell cycle.

In yeast, the restriction point is broadly equivalent to a transition point in G1, known as Start (Hartwell, 1974). At Start, cells commit to one of three fates: progression through the mitotic cell cycle, entry into a sexual cycle or arrest in stationary phase in which viability can be maintained for a long period.

1.1.2 Regulation of the cell cycle

The cell cycle is a highly ordered and regulated biological process, coordinating cell growth and cell division. Most types of cell grow between each cell division (an

exception being early embryonic cells) and lack of coordination of growth and division would lead to cells becoming progressively smaller or larger (Murray and Kirschner, 1991). In yeasts, this control is achieved by having a critical size requirement for passing through certain cell cycle stages. In the budding yeast, *Saccharomyces cerevisiae*, small daughter cells must grow for a longer time before they pass Start (Hartwell and Unger, 1977). The fission yeast, *Schizosaccharomyces pombe*, also has a similar size requirement but this control is active in G2 so that cells must achieve a certain size before progressing through mitosis and cell division (Nurse, 1975; Nurse and Fantes, 1981; Fantes, 1989).

Regulation of the cell cycle also includes coordination of various cell cycle events. For example, cells must ensure that mitosis does not begin until DNA replication is completed. If these two events are not coordinated, the result could be aneuploid or polyploid daughter cells. A number of cell cycle checkpoints have now been identified and are the subject of much research. These include the S-M replication, G2-M DNA damage and spindle assembly checkpoints (Sheldrick and Carr, 1993). These checkpoints are often signal transduction cascades which feed into the normal cell cycle progression machinery leading to arrest so that the problem can be addressed. An example of this is the response to DNA damage: if DNA damage is detected, cells arrest in G2 until the damage is repaired at which point they re-enter the cell cycle.

1.1.3 Cell cycle control by cyclin dependent kinases (CDKs)

The machinery that underlies the cell cycle regulatory processes includes a highly conserved family of proteins known as the cyclin-dependent kinases (CDKs). By definition, the activity of CDKs at specific stages of the cell cycle is absolutely dependent on their association with cyclin regulatory subunits. However, CDKs are

also regulated both positively and negatively by phosphorylation of key residues and negatively controlled by the binding of CDK inhibitors. Although this section contains a general description of CDKs and their regulation, it mainly focuses on their role in the G2/M transition which is most relevant to this project (Nigg, 1995).

The founding members of the CDK family are the homologous 34 kDa protein kinases Cdc28 in *S.cerevisiae* (Hartwell, 1974) and Cdc2 in *S.pombe* (Nurse *et al.*, 1976). Each has been shown, through genetic analysis, to have key roles in regulating cell cycle progress at both the G1/S and G2/M transitions. Homologues of this kinase, called the $p34^{cdc2}$ family, were then identified in other species and it has since become clear that all eukaryotic cells contain a form of this kinase, which regulates various checkpoints in the cell cycle.

Unlike in the yeasts, where there is only one $p34^{cdc2}$ kinase predominantly regulating all transitions, there are multiple mammalian homologues of $p34^{cdc2}$, all thought to play distinct roles in regulation of the cell cycle. The gene encoding the first human homologue of $p34^{cdc2}$ discovered, Cdc2 (also known as Cdk1), regulates the G2/M transition (Th'ng *et al.*, 1990; Hamaguchi *et al.*, 1992) and was cloned by complementation using a fission yeast $cdc2^{ts}$ strain (Lee and Nurse, 1987). The need for function of Cdk1 at the G1/S transition as with *S.cerevisiae* Cdc28 and *S.pombe* Cdc2 is not conserved in the mammalian cell cycle. Another 6 CDKs have been identified in mammalian cells, named Cdk2-7, and with the exception of Cdk5, all are involved in regulating various transitions within the cell cycle (Nigg, 1995). For the rest of this section, the name Cdc2 will be used to describe the homologues *S.cerevisiae* Cdc28, *S.pombe* Cdc2 and mammalian Cdk1 in the role they play in the G2/M transition.

As previously mentioned, the activity of CDKs is absolutely dependent on association with their cyclin partners (Morgan, 1995). Cyclins were first

characterised as proteins that accumulate periodically during the rapid synchronous early divisions of sea urchin embryos (Evans *et al.*, 1983). Most cyclins can be categorised depending on their structure and on the stage of the cell cycle at which they are required. The five major classes in higher cells are known as cyclins A, B, C, D, H. Some of these cyclins and CDKs undergo combinational interactions, in that a given cyclin may associate with multiple CDK partners and *vice versa*. Due to the universal function of Cdc2 in *S.cerevisiae* and *S.pombe*, it is the specific cyclin bound that determines at which stage of the cell cycle the CDK is required. In all eukaryotes, cyclin B binds to Cdc2 to form a complex that is required for initiation of M phase.

Reversible phosphorylation of specific residues on Cdc2 also contribute to the regulation of Cdc2/Cyclin B kinase activity. These modifications are executed in a cell cycle dependent manner. Cdc2 is activated via phosphorylation of a specific Thr residue in the activation loop of the CDK catalytic domain by CDK-activating kinases (CAKs). This residue is Thr167 in *S.pombe*, which is equivalent to Thr161 in human (Gould and Nurse, 1989; Krek and Nigg, 1991). This phosphorylation event is required for Cdc2 kinase activity (Gould *et al.*, 1991). In contrast, phosphorylation of Tyr15 negatively regulates Cdc2 kinase activity in *S.pombe*. Tyr15 phosphorylation maintains Cdc2/Cyclin B in an inactive form throughout the S and G2 phases and removal of this inhibitory phosphate is absolutely required for activation of Cdc2 kinase activity and entry into mitosis (Gould and Nurse, 1989). Inhibitory phosphorylation of Tyr15 is conserved in vertebrates, however, there is an additional inhibitory phosphorylation on the adjacent residue Thr14 (Krek and Nigg, 1991; Norbury *et al.*, 1991).

1.2 *S.pombe* as a model organism

The fission yeast, *S.pombe*, is a unicellular ascomycete fungus. *S.pombe* cells are rod shaped, 3.5 μm in diameter and between 7 μm and 14 μm in length depending on cell cycle position. The cells grow by apical extension and divide by medial fission. Following the birth of a new cell, growth occurs only at the old end of the cell, which existed in the previous cycle; the new end starts to grow at a defined stage termed "New End Take Off' (NETO, Mitchison and Nurse, 1985). *S.pombe* cell division by medial fission is more typical of higher eukaryotes than the budding of *S.cerevisiae*.

Two features make *S.pombe* an excellent model for studying the eukaryotic cell cycle. Firstly, the cells grow only by length extension, making it possible to determine at which stage a cell was in the cell cycle simply by measuring its length (Mitchison, 1957; Mitchison, 1990). Secondly, *S.pombe* is amenable to both classical and molecular genetic analysis (Gutz *et al.*, 1974; Moreno *et al.*, 1991; Alfa *et al.*, 1993).

S.pombe cells grow under relatively simple conditions. For laboratory purposes, *S.pombe* are grown in either complex or minimal medium at 20°C to 36°C (Moreno *et al.*, 1991). The generation time varies with strain, medium and temperature. Under favourable conditions, *S.pombe* cells reproduce asexually by means of the mitotic cell cycle, whereas starvation induces one of several alternative developmental fates (Fig 1.1). Essentially, haploid cells are of two mating types, known as h^+ and h^-, and if cells of a single mating type are present, then upon starvation they will exit the mitotic cell cycle at G1 or G2 and accumulate in stationary phase. If cells of both mating types are present, they will transiently arrest in G1 and then conjugate to form a diploid zygote. Under continued nutrient limitation, such zygotes usually undergo meiosis directly, forming an ascus containing four haploid spores that lie dormant until nutrient conditions improve. However, if newly formed diploid

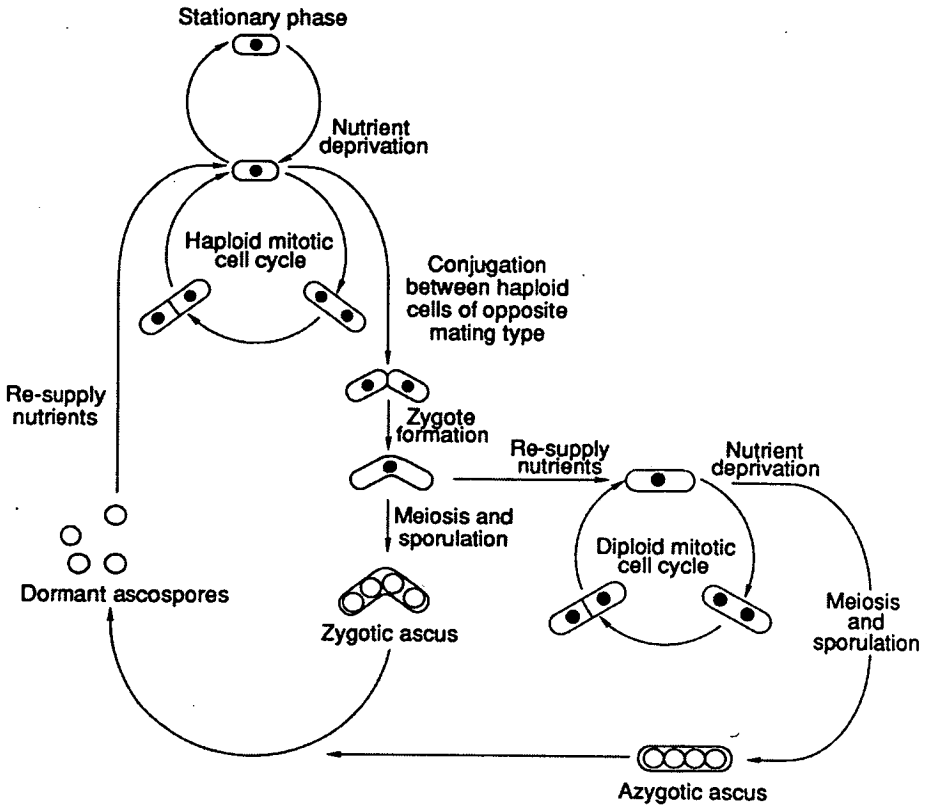

Fig 1.1 The life cycle of *Schizosaccharomyces pombe*

Shown is a schematic diagram representing the life cycle of *S.pombe*. Normally, cells reproduce by means of the haploid mitotic cell cycle (top left). However, if cells are deprived of nutrients they will exit the cell cycle and enter stationary phase (top) or undergo conjugation to form diploid zygotes (upper centre). If starvation continues, the zygotes will undergo meiosis and sporulation, forming an ascus containing four haploid spores which lie dormant until nutrient conditions improve (lower centre and left). However, if newly formed diploid zygotes are resupplied with nutrients, they will resume the mitotic cell cycle as a diploid (lower right). This figure is reproduced from Macneill and Fantes, 1993.

zygotes are supplied with adequate nutrients, some will resume the mitotic cell cycle and divide vegatively as a diploid (Egel and Egel-Mitani, 1974).

The *S.pombe* genome is ~14 Mb, approximately the same as the budding yeast *S.cerevisiae*. However, unlike *S. cerevisiae*, the genome is contained in just three chromosomes. An international project, coordinated by the Sanger Centre in Cambridge, commenced in 1995 to sequence the entire *S.pombe* genome and is almost complete. The data generated from this work can be easily searched from the Sanger Centre website:

http://www.sanger.ac.uk/Projects/S_pombe/

This resource has proved to be extremely important for this project and allowed the identification and subsequent isolation of the gene described in this work.

1.3 Regulation of the cell cycle in *S.pombe*

1.3.1 The mitotic cell cycle in *S.pombe*

The mitotic cell cycle of *S.pombe* (Fig 1.2) is typically eukaryotic, with discrete G1, S, G2 and M phases (Fantes, 1989). G1 is very short in *S.pombe*; DNA replication has already taken place by the time of cell division and the daughter cells are already in G2 at separation (Nasmyth and Nurse, 1979). Conversely, G2 occupies ~70% of the cell cycle, with most growth taking place during this phase. Entry into mitosis is marked by chromosome condensation and rapid microtubule rearrangement, where the cytoplasmic microtubule array disappears and a short spindle is formed. In contrast to higher eukaryotes, the nuclear envelope remains intact during mitosis, which is also true for *S.cerevisiae* (Nurse, 1985). After mitosis, the cell forms a septum across its centre and separation occurs when the septum is cleaved.

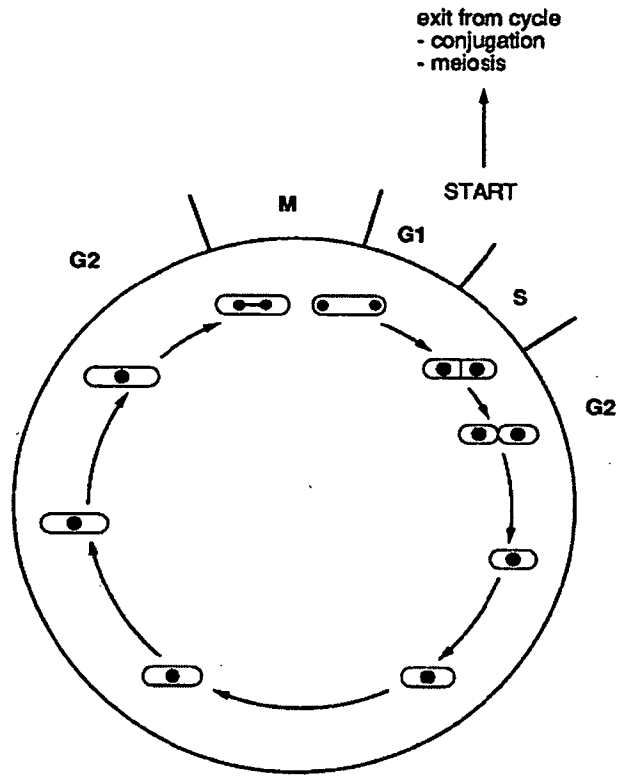

Fig 1.2 The mitotic cell cycle of *S.pombe*

Shown is a schematic representation of the cell cycle in *S.pombe*. It is typically eukaryotic with discrete G1, S, G2 and M phases. G2 is by far the longest phase, occupying about 70% of the cell cycle, whilst the duration of each of the G1, S and M phases is about 10% of the cycle. In *S.pombe*, septation and cytokinesis occur simultaneously with S phase so the newly created daughter cells have already undergone DNA replication.

The G1 and G2 phases of the cell cycle contain major control points concerned with regulating the onset of S phase and mitosis. The G2 phase includes the main growth control, ensuring that mitosis and cell division occur only when the cell has reached a critical size (Nurse, 1990). The transition from G2 to mitosis also requires the prior completion of DNA replication. Cells that are blocked at the DNA replication stage will not enter mitosis (Enoch, 1990; Sheldrick and Carr, 1993).

Cell cycle controls also exist in G1. An important G1 control mediates the requirement for the completion of mitosis before S phase can commence; cells blocked in G2 cannot undergo S phase (Moreno and Nurse, 1994). As mentioned earlier, there is an elaborate system of checkpoints and controls that are the subject of much ongoing work, however, these will not be discussed in great detail in this thesis (Murray, 1992; Woollard and Nurse, 1995; Russell, 1998).

1.3.2 Cell cycle mutants and cell cycle regulatory genes

The study of the cell cycle in *S.pombe* has been largely based on the analysis of mutants, which can be classed into three broad categories which are discussed below: mutants which are blocked at a specific stage of the cell cycle (mainly *cdc* mutants); mutants that are defective in coordination of cell growth and cell division; mutants that are unable to maintain the dependency between cell cycle events. A fourth category of mutants exist which overlaps somewhat with the three previously mentioned, where the mutants are identified due to their genetical interaction with already established cell cycle mutants.

Most *S.pombe* mutants that arrest at specific stages of the cell cycle are known as *cdc* (cell division cycle) mutants and typically display a cell elongation phenotype (Fantes, 1989; Macneill and Fantes, 1994). Since *cdc* phenotypes are lethal,

conditional mutants have been isolated and are usually temperature sensitive (*ts*), where the *cdc* phenotype is only visible at certain 'restrictive' temperatures (usually ≥35°C). The stage of the cell cycle at which the arrest takes place allows *cdc* mutants to be further classified and include genes required for passage through Start, DNA replication, entry into mitosis and septation (Fantes, 1989; Carr *et al.*, 1989).

As mentioned previously (section 1.3.1), the predominant cell cycle control in *S.pombe* exists at the G2 to M transition and controls entry into mitosis. Mutants that alter the timing of entry into mitosis therefore affect the coordination of cell size and cell cycle stage. Advancing the timing of entry into mitosis shortens G2, the phase where most cell growth takes place and results in cells which are shorter than wild-type cells, called the 'wee' phenotype (Nurse, 1975). This can be achieved by mutants of Cdc2 that lose the normal regulation mechanisms (Nurse and Thuriaux, 1980; Fantes, 1981) or by mutations of genes which regulate Cdc2 kinase activity (see section 1.3.3).

Mutations which cause disruption of the normal dependencies of cell cycle events on the completion of earlier events can lead to a variety of lethal phenotypes. These include defects allowing cytokinesis to proceed in the absence of nuclear division, leading to cells with aberrant septation or 'cut' phenotypes (Yanagida, 1998) and cells containing polyploid nuclei which indicate that extra rounds of DNA replication have taken place without an intervening mitosis (Broek *et al.*, 1991, Moreno and Nurse, 1994).

1.3.3 Regulation of entry into mitosis

S.pombe has proved an excellent model organism for investigating the mechanisms controlling entry into mitosis. A mechanistic model has now been firmly established

and homologues have been identified in higher eukaryotes for many of the components of this model (Fig 1.3).

At the G2 to M transition point, Cdc2 is complexed with Cdc13, a B-type cyclin (Booher and Beach, 1987; Booher and Beach, 1988). This complex begins to accumulate during G1 and S phase and is most abundant when required to initiate mitosis. Cdc13 is absolutely required for Cdc2 kinase activity in G2 and for entry into mitosis. Once the Cdc2-Cdc13 complex has formed, it is regulated by phosphorylation of specific residues of Cdc2 (Draetta and Beach, 1988; Gould and Nurse, 1989).

As previously mentioned (section 1.1.3), phosphorylation of Thr167 by a CDK activating kinase (CAK) is required for tight cyclin binding and activation of Cdc2 kinase activity. To date, it appears that *S.pombe* contains two partially redundant CAKs: the Mcs6-Mcs2 complex and Csk1 (Lee *et al.*, 1999). Inactivation of both these CAKs prevents Cdc2 activation and causes cell cycle arrest. In *S.pombe* it is still not certain which protein dephosphorylates Thr167, though the phosphatase Ppa2 has become the most likely candidate. Deletion of $ppa2^{+}$ causes cells to divide at a reduced cell size (semi-wee phenotype), whilst overexpression causes a delay in G2, resulting in elongated cells (Kinoshita *et al.*, 1993). These observations are consistent with Ppa2 being a negative regulator of entry into mitosis. However, at present the genetic interactions of $ppa2^{+}$ with other cell cycle genes are not conclusive and cannot determine which of the mitotic control gene products serves as a substrate for Ppa2 activity.

Two genes, $wee1^{+}$ and $mik1^{+}$, have been identified as negative regulators of Cdc2-Cdc13 kinase activity which act by phosphorylating residue Tyr15 of Cdc2 (Russell and Nurse, 1987a; Lundgren *et al.*, 1991). The $wee1^{+}$ gene product belongs to an unusual class of protein kinase capable of phosphorylating both serine and tyrosine

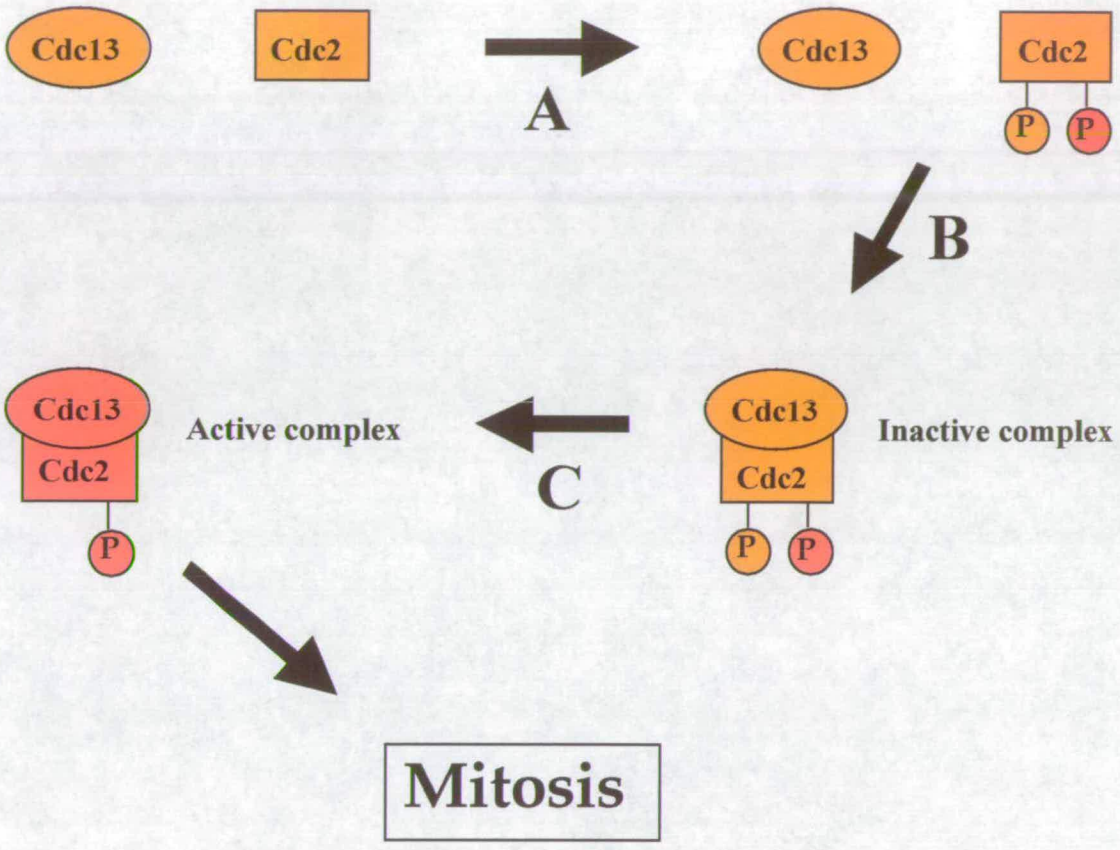

Fig 1.3 The control of entry into mitosis by the Cdc2/Cdc13 complex

The timing of entry into mitosis is controlled by the Cdc2/Cdc13 complex and the pathways which regulate it. **A.** The association of Cdc2 with Cdc13 is absolutely dependent on the activating phosphorylation of Thr167 (red P) of Cdc2 by CDK activating kinases (CAKs). The inhibitory phosphorylation of Tyr15 (orange P) by Wee1 (and Mik1) ensure that Cdc2 remains inactive. **B.** Upon phosphorylation of Thr167, Cdc2 forms a complex with the cyclin, Cdc13. **C.** Removal of the Tyr15 phosphorylation by Cdc25 (and Pyp3) convert the inactive complex into its active state, allowing entry into mitosis.

residues (Featherstone and Russell, 1991, Parker and Piwnica-Worms, 1992). The *mik1*$^+$ gene shares extensive sequence homology with *wee1*$^+$ (Lundgren *et al.*, 1991). The *wee1*$^+$ gene is a dosage dependent inhibitor of mitosis and loss of *wee1*$^+$ function advances mitosis resulting in the short 'wee' phenotype (Russell and Nurse, 1987b). Loss of *mik1*$^+$ function has no effect on cell length in otherwise wild-type cells. Although neither *wee1*$^+$ nor *mik1*$^+$ is essential, loss of both activities leads to rapid dephosphorylation of Tyr15 and to mitotic catastrophe that is lethal (Lundgren *et al.*, 1991). This suggests that Wee1 and Mik1 share an overlapping function, which is required for phosphorylation of the Tyr15 residue of Cdc2 but that Wee1 plays the major role.

The phosphorylation of the Tyr15 residue of Cdc2 by Wee1 and Mik1 must be reversed to activate the Cdc2/Cdc13 complex and this is carried out by Cdc25 and to a lesser extent, Pyp3. The *cdc25*$^+$ gene product has been identified as a dosage dependent activator of mitosis, acting in opposition to Wee1 (Russell and Nurse, 1986). Both *cdc25*$^+$ mRNA and Cdc25 protein levels fluctuate during the mitotic cell cycle, reaching a maximum at the G2 to mitosis transition point (Moreno *et al.*, 1990; Ducommun *et al.*, 1990). *cdc25*$^+$ is essential for cell cycle progression and cells lacking *cdc25*$^+$ function arrest in G2 but only in the presence of functional *wee1*$^+$ (Fantes, 1979; Russell and Nurse, 1986; Russell and Nurse, 1987b). Both genetic (Gould and Nurse, 1989; Gould *et al.*, 1990) and *in vitro* biochemical evidence (Millar *et al.*, 1991) suggest that the *cdc25*$^+$ gene product induces mitosis by dephosphorylating the Tyr15 residue of Cdc2 , thereby activating Cdc2 kinase activity. Pyp3 is another protein tyrosine phosphatase which also contributes to this Tyr15 dephosphorylation (Millar *et al.*, 1992). Pyp3 function is not essential in otherwise wild-type cells but does cause a mitotic delay that is enhanced in cells which are partially defective in Cdc25 function (Millar *et al.*, 1992). However, *pyp3*$^+$ is essential in a *cdc25 wee1* double mutant, suggesting that Cdc25 and Pyp3 also share an overlapping function but that Cdc25 plays the major role.

In order for a cell to enter mitosis and progress through the cell cycle, Cdc25 must be activated, and Wee1 and Mik1 must be inhibited. This is achieved by phosphorylation of these three proteins by multiple kinases, probably including Cdc2 itself as in *S.cerevisiae* (Patra *et al.*, 1999). The *nim1*$^{+}$/*cdr1*$^{+}$ gene (Russell and Nurse, 1987a; Young and Fantes, 1987) encodes a protein kinase that can phosphorylate and inhibit the Wee1 protein *in vitro* (Wu and Russell, 1993; Coleman *et al.*, 1993; Parker *et al.*, 1993). *In vivo*, the levels of Nim1/Cdr1 kinase activity correlate directly with levels of Wee1 phosphorylation (Wu and Russell, 1993).

Cdc25, Wee1 and Mik1 are also targets of checkpoint kinases that prevent mitosis in response to upstream signals. Cds1, a protein kinase involved in the S-M DNA replication checkpoint, is believed to phosphorylate and activate Wee1, thus delaying the onset of mitosis in response to incomplete DNA replication (Boddy *et al.*, 1998). There is also evidence that Cds1 phosphorylates and inhibits Cdc25, another measure towards delaying the onset of mitosis (Furnari *et al.*, 1999). Chk1 is kinase involved in the G2-M DNA damage checkpoint and has been shown to phosphorylate and inhibit Cdc25 *in vitro* and *in vivo*, delaying entry into mitosis in response to DNA damage (Furnari *et al.*, 1999).

1.4 The heat shock response and the role of heat shock proteins as molecular chaperones

When cultured cells or whole organisms are exposed to elevated temperature, they increase the synthesis of several highly conserved proteins known as heat shock proteins (Hsps). As well as heat-shock, these Hsps are also produced in response to a wide variety of other stresses such as exposure to radiation or toxic chemicals (Ritossa, 1996; Lindquist, 1986). This heat shock response is universal; it has been

observed in every organism where it has been investigated and is found in nearly every cell and tissue type of multicellular organisms (Lindquist, 1986).

Heat stress and the other stresses which increase Hsp synthesis result in a much higher than usual intracellular concentration of proteins in denatured conformations, which require 'molecular chaperones' to assist them in refolding properly back into their normal native conformations. Most Hsps are now known to act as molecular chaperones even under normal conditions and their mechanism of action has been subject to intense investigation (Gething and Sambrook, 1992; Morimoto, 1994; Hartl, 1996). They have been shown to play diverse roles in successful folding, assembly, intracellular localisation and regulation of many proteins, even in unstressed cells (Gething, 1997).

The genes encoding Hsps are highly conserved and have been identified in every species in which they have been examined. Most of these genes and their products can be assigned to families based on the basis of sequence similarity and typical molecular weight (Gething, 1997). These families include the Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, Hsp25 and small Hsp families. The Hsps recognise and bind to target proteins that are in non-native conformations, whether due to denaturation induced by stress or because they have not yet been fully synthesised. Binding and/or release of the target proteins is often regulated by association and/or hydrolysis of nucleotides. Typically, Hsps function as complexes with several other chaperones or in association with partners known as cochaperones. The Hsps that have been best studied are Hsp40, Hsp60, Hsp70 and Hsp90. The most interesting Hsp with regard to this project is Hsp90 and is described in the next section. However, brief descriptions of the roles of the other Hsps are included here.

Hsp70 is the most highly conserved of the Hsps and therefore has been investigated most thoroughly. All known Hsp70s have Hsp40 cochaperones and together assist a

large variety of protein folding processes in almost all cellular compartments (Bukau and Horwich, 1998). They play an essential role in the folding of newly translated proteins, targetting proteins and assisting their translocation across organellar membranes, disassembling oligomeric protein structures, facilitating proteolytic degradation of unstable proteins and controlling the biological acivity of folded regulatory proteins (Morimoto *et al.*, 1994; Hartl, 1996). In Hsp70 assisted folding reactions, substrates undergo repeated cycles of binding and release, causing local conformational changes in the substrate, thus preventing aggregation and facilitating folding to its native state (Szabo *et al.*, 1994; Buchberger *et al.*, 1996). Hsp40 is believed to target Hsp70 to its destination and also stimulate ATP hydrolysis.

Hsp60s, or the chaperonins as they are also known, function as a collective of double-ring assemblies that promote folding of proteins into their native state (Bukau and Horwich, 1998). The huge complexes (~1 MDa) are made up of back to back rings of identical or closely related rotationally symmetric subunits of ~60 kDa. Chaperonins play an essential role in all cells, assisting a large variety of newly synthesised and newly translocated proteins to reach their native forms by binding them and facilitating their folding inside a large central channel within each ring (Ellis, 1996; Fenton *et al.*, 1997).

All known stresses, if sufficiently intense, induce Hsp expression (Feder and Hofmann, 1999) and their increase in expression is termed the stress response. A common aspect of these inducing stresses is that they result in proteins having non-native conformations (Somero, 1995), which is consistent with the function of Hsps as molecular chaperones, binding only proteins that are in non-native conformations.

1.5 The Hsp90-based chaperone system

1.5.1 The ubiquitous protein chaperone, Hsp90

Hsp90 is a highly conserved stress protein and is expressed in the cytoplasm of all eukaryotic cells, though very small amounts are present in the nucleus as well (Parsell and Lindquist, 1993). It is highly abundant in unstressed eukaryotic cells, accounting for ~1% of cytosolic protein but in response to stress, Hsp90 protein levels increase up to ten-fold (Welch and Feramisco, 1982). It is essential in both yeast (Borkovich, 1989) and *Drosophila* (Cutforth and Rubin, 1994). Hsp90 purifies as a dimer and dimerisation is required for its function *in vivo* (Minami *et al.*, 1994). *In vitro*, Hsp90 alone functions as a molecular chaperone that facilitates the folding of a number of proteins (Jakob and Buchner, 1994), however, this ability to assist in protein folding is probably only part of its function *in vivo* as a component of the Hsp90-based chaperone system. Several transcription factors and protein kinases involved in signal transduction have been recovered from cells in association with Hsp90. In many cases, the association with this protein chaperone is essential for the proper functioning of the signalling pathway.

1.5.2 The subcomplexes of Hsp90

There are several Hsp90 cochaperones that have been identified and they appear to be organised into discrete subcomplexes (Fig 1.4). To date, four different Hsp90 subcomplexes have been described and are believed to have distinct roles (Caplan, 1999). It is thought that the subcomplex which Hsp90 forms, directly affects the downstream function of Hsp90. Both the 'Hop' complex and the 'p23' complex (Fig 1.4) are associated with maturation of steroid hormone receptors (SHRs), though the exact mechanisms have yet to be determined. The Hsp90/Cdc37 complex is

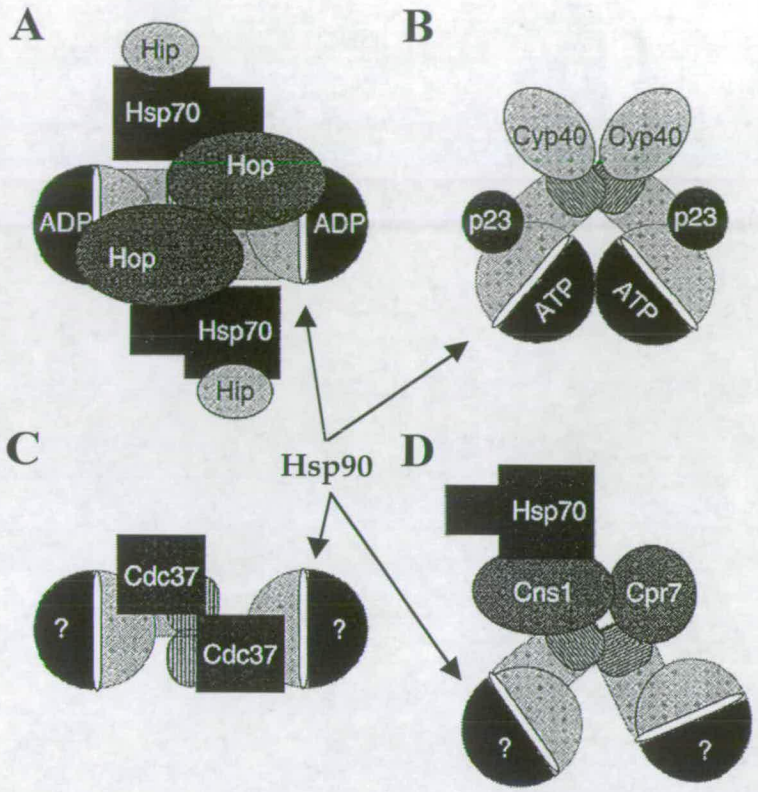

Fig 1.4 The cochaperones and subcomplexes of Hsp90

Shown are the known cochaperones of Hsp90 to date and the subcomplexes they form. Complex A, called the 'Hop' complex (Caplan, 1999) and complex B, called the 'p23' complex (Caplan, 1999), are both involved in the maturation of steroid hormone receptors. Complex C, containing $p50^{cdc37}$, is involved in the folding and activity of various protein kinases, whilst the function of complex D is at present unknown. This figure is reproduced and modified from Caplan, 1999.

thought not be involved with SHRs but with the correct folding of protein kinases. The function of the fourth complex found in *S.cerevisiae* containing Cpr7 and Cns1 is not well understood but Cpr7 has been shown to play a role in the maturation of glucocorticoid receptors (Pauli and Mahowald, 1990).

Hsp90 binds to several of its cochaperones at its C-terminal dimerisation domain (Young *et al.*, 1998; Carrello, 1999). All of the cochaperones that bind in this region, except Cdc37, contain tetratricopeptide repeat sequences (TPR) that mediate their association in a competitive manner with Hsp90 (OwensGrillo *et al.*, 1996). Although Cdc37 does not contain TPR sequences, it also appears to competitively bind Hsp90 at a site adjacent to, although distinct from, the binding site for TPR-containing proteins (Silverstein *et al.*, 1998). This competitivity between cochaperones is unlikely to be the only factor in complex assembly, as this might also depend on the nucleotide state of Hsp90 and the presence of other cochaperones.

1.5.3 Steroid hormone receptors and Hsp90

Steroid hormone receptors (SHRs) are soluble intracellular proteins that shuttle between the cytosol and the nucleus. SHRs are the best studied *in vivo* substrates of Hsp90 (Pratt and Toft, 1997). This interaction occurs only in the absence of steroid hormones; the addition of hormones causes dissociation of the Hsp90/SHR complex and dimerisation of the receptors, allowing them to enter the nucleus and bind specific DNA sequences, thus regulating transcription (Louvion *et al.*, 1996). Genetic experiments in a heterologous system have demonstrated that Hsp90 is required to maintain receptors in an inactive but activatable state in the absence of hormone (Xu and Lindquist, 1993; Bohen and Yamamoto, 1994).

Both the 'Hop' complex and the 'p23' complex have been found to be associated with SHRs but the exact mechanisms are not clearly understood. Sequential passage through at least both of these two Hsp90 multicomponent complexes are necessary to keep SHRs competent for hormone binding and subsequent activation (Caplan, 1999). The unstable 'Hop' complex is believed to bind the SHR initially and then subsequently, the stable mature complex Hsp90/SHR/p23 complex is formed. The intermediate stages of this transition are unclear.

1.5.4 Protein kinases and Hsp90

Hsp90 has been found in complexes with a number of protein kinases including several CDKs (Hunter and Poon, 1997). In most of the complexes formed between protein kinases and Hsp90, a cochaperone, p50, has also been found to be present. It has recently been shown that p50 is actually a mammalian homologue of Cdc37 (Perdew *et al.*, 1997), however, this was unknown at the time most of the work described in this section was carried out.

Several independent lines of evidence link an Hsp90/p50 complex with kinases of unrelated signalling pathways, including two examples which are discussed here. These studies have shown that this complex is found associated with newly synthesised and inactive forms of Src homologues (Brugge *et al.*, 1981; Brugge, 1986) and Raf-1 (Stancato *et al.*, 1993) in fibroblasts. However, active forms of Raf-1 and src homologues found at the cell membrane lack this chaperone complex.

v-Src is a membrane-associated protein-tyrosine kinase encoded by Rous sarcoma virus (RSV) and had been shown previously to associate with a Hsp90/p50 complex in chicken fibroblasts (Brugge *et al.*, 1981; Brugge, 1986; Oppermann *et al.*, 1981). Ectopic expression of v-Src in wild-type *S. cerevisiae* causes rapid cell death with

associated disruption of the cell cycle, due to abnormal protein phosphorylation on tyrosine residues (Boschelli *et al.*, 1993). However, mutations in *CDC37* suppress the lethality resulting from v-Src expression, apparently through kinase destabilisation (Dey *et al.*, 1996). In chicken fibroblasts, newly synthesised v-Src remains complexed with Cdc37 and Hsp90 in the cytoplasm until it is translocated to the cytoplasmic membranes (Courtneidge and Bishop, 1982; Brugge *et al.*, 1983). When bound to Hsp90/Cdc37 complexes, v-Src was shown to be hypophosphorylated and lack protein kinase activity. However, after dissociation from the complex, v-Src was shown to be phosphorylated on tyrosine and serine residues, to have protein kinase activity and to be associated with membrane fractions.

It is interesting, however, that Hsp90/Cdc37 complexes have not been found with the v-Src cellular homologue, c-Src. There are two very different reasons as to why this could be: either because the interaction of Hsp90/Cdc37 complexes with v-Src could represent a nonphysiological relationship or possibly because mutant protein kinases contain a larger fraction of misfolded protein and therefore are better targets for Hsp90/Cdc37 association. The Hsp90/Cdc37 complex has also been detected in association with three other oncogenic protein-tyrosine kinases, v-Yes (Adkins *et al.*, 1982), v-Fps (Lipsich *et al.*, 1982) and v-Fgr (Ziemiecki, 1986).

Raf-1 is another protein kinase that interacts with the Hsp90/Cdc37 complex (Stancato *et al.*, 1993; Wartmann and Davis, 1994). It is a serine/threonine protein kinase which is normally involved in activating the MAP kinase kinase, Mek, thus playing an important role in mitogenic signal transduction. It has also been shown to be directly activated by the potent oncoprotein Ras (Ahn, 1993). Unlike the case of c-Src, both v-Raf and its cellular analogue c-Raf have been demonstrated to interact stably with Hsp90/Cdc37 complexes (Stancato *et al.*, 1993; Wartmann and Davis, 1994). Raf activation by Ras involves the translocation of the Raf/Hsp90/Cdc37

complex to the cell membrane and only membrane-bound Raf exhibits protein kinase activity (Wartmann and Davis, 1994). This work also showed that although it is predominantly cytosolic, c-Raf also undergoes trafficking to the plasma membrane, and only after translocation to membrane sites is its kinase activity stimulated.

1.6 Cdc37 and its role in the cell cycle with CDKs

1.6.1 The identification of Cdc37p in *S.cerevisiae*

CDC37 is an essential gene in *S. cerevisiae* and is required for passage through the G1 phase of the cell cycle (Gerber *et al.*, 1995). It was first identified through a screen which selected for temperature-sensitive mutations that cause cell cycle arrest in G1 at Start (Reed, 1980b), the point at which the cell becomes committed to the cell division cycle (Hereford and Hartwell, 1974; Johnston *et al.*, 1977; Hartwell and Unger, 1977). Cells also arrest in G1 if treated with mating pheromone, or are starved of nitrogen, allowing exit from the mitotic cell cycle. Four genes were identified in which mutations caused this characteristic arrest at Start: *CDC28*, *CDC36*, *CDC37* and *CDC39* (Breter *et al.*, 1983; Ferguson *et al.*, 1986). Mutations in *CDC36* and *CDC39* result in constitutive activation of the mating pheromone pathway which in turn leads to G1 arrest (Neiman *et al.*, 1990). In contrast, *CDC28* is known to be directly involved in the cell cycle (Forsburg and Nurse, 1991; Reed, 1992; Nasmyth, 1993).

The first clue that *S.cerevisiae* Cdc37p was involved in the function of CDKs was the discovery that the G1 arrest in the *cdc37-1*^{ts} mutant is accompanied by a decrease in the kinase activity of Cdc28. This was shown to be due to the lack of association of Cdc28 with the G1 cyclin Cln2 (Gerber *et al.*, 1995), which could account for the defect in progression from G1 to S phase in *cdc37-1*^{ts} cells. Also in these cells, the

kinase activity of Cdc28 associated with the mitotic cyclin Clb2 was also shown to be much lower. These results indicated that Cdc37 might play a role in regulating CDK activity in G1 and G2 by somehow regulating the association between Cdc28 and the cyclins. This evidence was supported by work carried out with the *D.melanogaster* homologue of *CDC37*, in which mutations were shown to dominantly enhance mutations in the *cdc2* gene, the closest homologue of mammalian Cdk1 (Cutforth and Rubin, 1994).

1.6.2 The role of mammalian Cdc37p homologues with CDKs

In mammalian cells, the Cdks 4 and 6 associate with D-type cyclins and are involved in the cell cycle at the G0/G1 transition and probably at the G1/S phase transition (Nigg, 1995). At the same time as the *S.cerevisiae* work described in the previous section (1.6.1) was going on, work on mammalian systems indicated that the assembly of these cyclin D-Cdk4/6 complexes was thought to require additional factors, partly due to the fact that the complexes were much more difficult to produce *in vitro* than other active complexes such as cyclin-Cdk1 or cyclin-Cdk2 complexes. When cells are stimulated with growth factors to enter the cell cycle from quiescence (G0), cyclin D and Cdk4 are synthesised and assemble into cyclin D-Cdk4 complexes in mid-G1 (Matsushime *et al.*, 1994). However, when both cyclin D and Cdk4 are ectopically expressed in quiescent cells, cyclin D-Cdk4 complexes are not formed. It has since been shown that assembly of these complexes does indeed require a mitogen-dependent step that is independent of CAK function (Kato *et al.*, 1994; Matsuoka *et al.*, 1994).

When a mammalian homologue of Cdc37p was found to associate directly *in vitro* with the mammalian kinases Cdk4 and Cdk6 (Stepanova *et al.*, 1996), it began to be implicated as an additional factor for cyclin D-Cdk4/6 assembly. Furthermore, by

immunoprecipitation, Cdc37 was found to interact with Cdk4 *in vivo* and the immunoprecipitation results have been confirmed with the yeast-two hybrid system (Lamphere *et al.*, 1997). Since Cdk4 had also been found to associate with Hsp90 (Stepanova *et al.*, 1996; Dai *et al.*, 1996) and Cdc37 (p50 at the time), and Hsp90 had been shown to colocalise in the cytoplasm (Perdew and Whitelaw, 1991; Whitelaw *et al.*, 1991), it was considered that this was all part of the same complex. This hypothesis was strengthened by the fact that Hsp90 could only associate with Cdk4 when Cdc37 was present (Stepanova *et al.*, 1996), suggesting that Cdc37 is a targeting subunit of Hsp90 which somehow directs it to and mediates its interactions with the protein kinases that require it. Therefore, the association of both Hsp90 and Cdc37 with Cdk4 can be explained by the interaction of Cdk4 with the Hsp90-Cdc37 complex. It is interesting to note, however, that no cyclin D was present in Cdc37 immune complexes. Based on gel-filtration analysis, there were two populations of Cdk4, one containing cyclin D, and the other as part of a high molecular weight complex (~450 kDa) containing Cdc37 and Hsp90 but no cyclin D (Stepanova *et al.*, 1996). Cdk4 has been found in both the nucleus and the cytoplasm, though never in monomeric form, whereas Cdk4/cyclin D complexes are mainly located in the nucleus (Whitelaw *et al.*, 1991). Furthermore, Cdc37 has been shown to enhance the binding of cyclin D to Cdk4 (Dai *et al.*, 1996). Together, these observations suggest that a Cdc37/Hsp90 complex may play a role in the assembly or activation of cyclin D-Cdk4 complexes, possibly by being required for stabilisation of newly-synthesised monomeric Cdk4, 'holding' it in a state in which it is able to bind its cyclin partner.

Although there is a considerable amount of interest in Cdk4/cyclin D1 complexes now that it has been shown that they seem to play a role in tumorigenesis (Hall and Peters, 1996), much less is known about the regulation of Cdk6/cyclin D complexes. Some headway has been made since elevated Cdk6 activity was detected in squamous cell carcinoma cell lines, suggesting that deregulation of Cdk6 activity may play a role

in the onset of some tumours (Timmerman *et al.*, 1997). Gel filtration chromatography has shown that Cdk6 exists in at least three forms in T-cells (Mahony *et al.*, 1998). The most abundant form was as part of a complex (~450 kDa) which was shown to contain both Hsp90 and Cdc37 and was present in cytoplasmic extracts but not in nuclear extracts. The second most abundant form was found to be monomeric Cdk6 and was found in both the nucleus and cytoplasm. The third and perhaps surprisingly least abundant Cdk6 complex was found to contain cyclin D and again, these complexes were found in both the nucleus and the cytoplasm. However, only Cdk6 that was complexed to cyclin D was active, and furthermore, Cdk6 kinase activity was largely confined to the nuclear extracts.

The interactions between Cdk4 and Cdk6 with Hsp90/Cdc37 complexes are strikingly similar. The Cdk/Hsp90/Cdc37 complexes exist predominantly in the cytoplasm where the kinase activity is not detected and are not found in the nucleus where the kinase activity has been shown to exist. From these observations, it seems likely that Hsp90/Cdc37 complexes are required to bind and stabilise newly synthesised Cdk4/6 in monomeric form.

The investigations of Cdc37 with the mammalian cell cycle CDKs, Cdk4 and Cdk6, and also with *S.cerevisiae* Cdc28 indicate that it is crucial in cell cycle regulation. It is interesting to note, however, that whilst physical interactions between Cdc37 and both Cdk4 and Cdk6 have been demonstrated, no evidence of binding between Cdc37 and Cdc28 has yet been shown.

1.7 The genetic interactions of *S.cerevisiae CDC37* with other protein kinases

Cdc37 has been found to interact genetically with a number of genes encoding other kinases with seemingly diverse biological functions, examples of which are described here. The *S.cerevisiae cdc37-1*^{ts} mutant was used to investigate a *S.cerevisiae* homologue of mammalian mitogen-activated protein (MAP) kinase, *FUS3/DAC2* protein kinase (Fujimura, 1994). Since the *FUS3/DAC2* kinase controls the activity of G1 cyclins (Peter *et al.*, 1993; Tyers and Futcher, 1993), it was investigated for interactions with the *CDC28*, *CDC36*, *CDC37*, or *CDC39* gene products in G1 progression or in the pheromone response pathway. *FUS3/DAC2* was shown to be required for the G1 arrest of the cell cycle caused by the *cdc37* mutation but not for the similar G1 arrest caused by the *cdc28* mutation (Fujimura, 1994). These data suggest that either *CDC37* and *FUS3/DAC2* regulate *CDC28* positively and negatively, respectively, or that CDC37 negatively regulates FUS3/DAC2, which controls CDC28 for G1 progression.

Another genetic interaction of *CDC37* in *S.cerevisiae* is that with the essential protein kinase gene *MPS1*, which is required for spindle pole body (SPB) duplication and for the mitotic spindle assembly checkpoint (Schutz *et al.*, 1997). *CDC37* was identified as a multicopy suppressor of the restrictive phenotype of the *mps1-1*^{ts} mutant and synthetic lethal interactions were shown to occur between *mps1* and *cdc37* alleles. The kinase activity of Mps1p was also shown to be diminished in the *cdc37-1*^{ts} mutant.

Finally, a genetic interaction in *S.cerevisiae* has also been described between *CDC37* and *KIN28*, which encodes a putative CDK sharing 38% homology with Cdc28p but does not appear to be involved in the cell cycle (Valay *et al.*, 1995). Instead, Kin28

has since been shown to be involved in the recruitment of mRNA processing machinery to RNA polymerase II (Rodriguez *et al.*, 2000).

1.8 Structure of Cdc37 in relation to its function

Little is known about the structure of Cdc37 and how it relates to its function. It has been shown that the interaction of Cdc37 with Raf-1 occurs via the N-terminal half of Cdc37 (Grammatikakis *et al.*, 1999). This work also suggested that the interaction of Cdc37 with Hsp90 occurs via the C-terminal half of Cdc37. The interpretation of these results propose that the highly conserved N-terminus of Cdc37 is required for interaction with the target protein kinase, whilst the less conserved C-terminus is involved in the association with the Hsp90 dimer.

1.9 The physiological roles of Cdc37

Even though there has been considerable analysis of Cdc37, its precise biochemical function is still largely unknown. There are many questions that remain unanswered and need to be addressed before the function of Cdc37 is elucidated in full. It is widely believed that Cdc37 acts as a targeting subunit for Hsp90 and that together the Hsp90/Cdc37 complex somehow aids in the folding of newly translated protein kinases and/or regulates their activity. These kinases include specific CDKs, which explains the cell cycle role of Cdc37 and the means by which it was first isolated in *S.cerevisiae*. It will be interesting to find out exactly what role it plays in the overall system and how it recognises monomeric kinases, or if indeed its function is limited to protein kinases. Fig.1.5 shows the genetical and physical interactions that have been identified with various protein kinases, Cdc37 and Hsp90.

There is limited evidence that Cdc37 acts as a chaperone in its own right, so that as well as acting as an accessory factor for Hsp90, Cdc37 itself may perform a protein folding function similar to Hsp90 (Kimura *et al.*, 1997). Other investigations have implicated Cdc37 in signal transduction processes other than regulation of protein kinases, such as steroid hormone receptor activation (Fliss *et al.*, 1997). However, it does seem possible that Cdc37 has a targeting function, perhaps signalling transport to the plasma membrane such as in the cases with Raf and v-Src.

1.10 Finding an *S.pombe* homologue of *CDC37*

The principal aims of this project were to identify, clone and characterise any homologues of *CDC37* in *S. pombe*. As described above, *CDC37* homologues have been isolated from a variety of species and, given its essential function in CDK activity in *S. cerevisiae*, it seemed probable that at least one similar homologue would exist in *S. pombe*. Investigating for the presence of this gene or genes is interesting because it seems that Cdc37 in all species plays an important role in regulating CDK activity, so it may be that it is also important for the cell cycle in *S. pombe* via regulation of central kinases, an area which our lab has been interested in for some time. Also, examining Cdc37 in another system, such as *S.pombe*, is important for several reasons: firstly, by virtue of the way *S.pombe* grows and divides, the system is amenable to genetic and cytological techniques that are not possible in other systems. Also, due to fundamental differences in the cell cycle of *S.pombe* compared to that in, for example, *S.cerevisiae*, *Drosophila* and mammalian cells, studies of the effect on the cell cycle in *S.pombe* are extremely interesting. In particular, in *S.cerevisiae*, the major cell cycle control exists in G1, whereas in *S.pombe*, the main control is during G2. Therefore, of immediate interest would be whether Cdc37 function is mainly involved in G1, as in *S.cerevisiae* and mammalian systems, or whether in *S.pombe* the main role of Cdc37 is during G2.

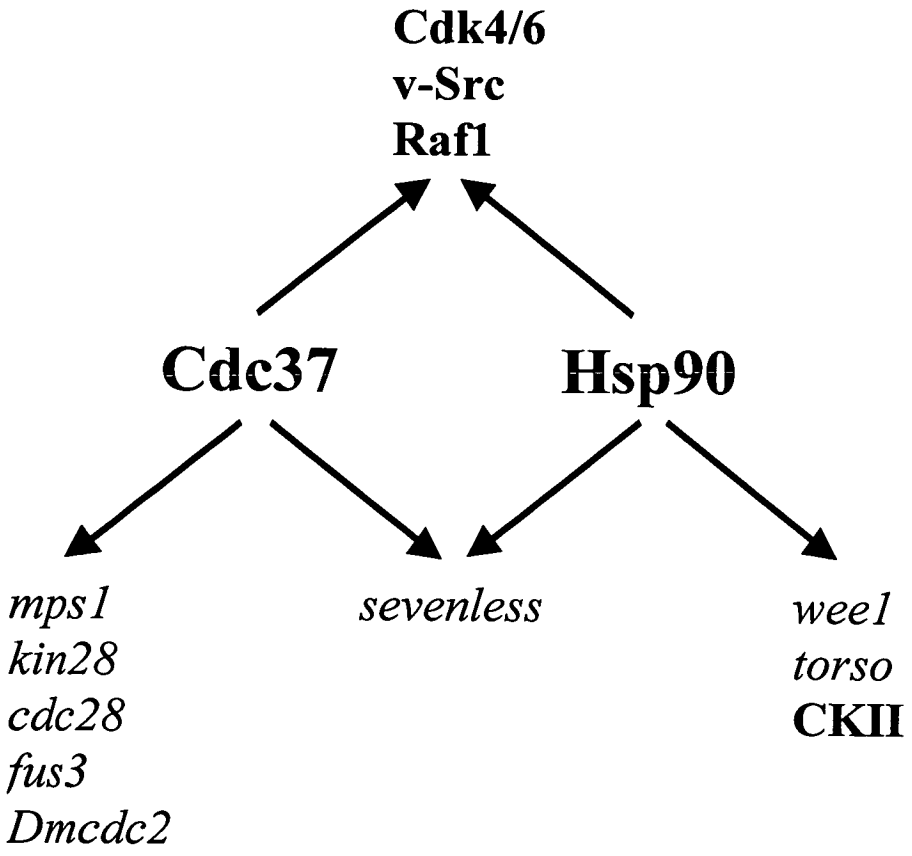

Fig 1.5 Interactions between Cdc37, Hsp90 and various protein kinases

Shown are the physical and genetical interactions found between Cdc37, Hsp90 and various protein kinases. Kinases shown in bold type indicate physical interactions with the chaperones and plain type show genetic interactions. This figure is reproduced and modified from a combination of diagrams from Hunter and Poon, 1997 and Stepanova *et al.*, 1997.

Chapter 2

Materials and Methods

2.1 Materials and Reagents

2.1.1 Chemicals

All chemicals were of molecular biology grade, supplied by the Sigma-Aldrich Company Ltd., Dorset, UK, except where otherwise stated.

2.1.2 Molecular biology reagents

Amersham, Little Chalfont, Bucks, UK

^{32}P-labelled dCTP; Hybond-N nylon hybridisation transfer membrane; ECL™ detection kit; Hyperfilm™ECL™ chemiluminescent film

BioRad Laboratories Ltd., Hertfordshire, UK

Muta-Gene® Phagemid *In Vitro* Mutagenesis Kit (Version 2); PVDF transfer membrane

Boehringer Mannheim, Lewes, Sussex, UK

Expand™ High Fidelity PCR polymerase mix; Klenow DNA polymerase; dNTPs

Clontech Laboratories Inc., La Jolla, CA

Herring testes carrier DNA

Diagnostics Scotland, Edinburgh, UK

HRP sheep anti-mouse IgG; HRP donkey anti-rabbit IgG

GIBCO BRL, Paisley, UK

1 kb DNA ladder; 0.24-9.5 kb RNA ladder

New England Biolabs (UK) Ltd., Hertfordshire, UK

Various restriction endonucleases; Calf Intestinal Alkaline Phosphatase

PE Applied Biosystems, Perkin Elmer, Warrington, UK

dRhodamine Terminator Cycle DNA Sequencing kit

Pharmacia Biotech, St. Albans, UK

NICK™ Sephadex G-50 Columns; Hexadeoxyribonucleotides

Promega Corporation UK, Southampton, UK

Various restriction endonucleases; T4 DNA ligase; *Taq* DNA polymerase; PCR cloning vectors pGEM-T and pGEM-T Easy

Qiagen Ltd., Dorking, Surrey, UK

QIAquick Gel Extraction kit

Sigma-Aldrich Company Ltd., Dorset, UK

Acid-washed glass beads; Salmon sperm DNA

Stratagene, La Jolla, CA

QuikChange™ Site-Directed Mutagenesis kit

2.1.3 Deoxyoligonucleotide synthesis

Synthesis of all oligonucleotides was carried out by Genosys Biotechnologies (Europe) Ltd., Cambridgeshire, UK.

2.2 Nucleic acid manipulation protocols

2.2.1 General techniques

2.2.1a Phenol/chloroform extraction

Proteins were removed from nucleic acid preparations by extraction with phenol. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the preparation, vortexed for 1 min and then the phases were separated by centrifugation at 14,000 rpm. The upper, aqueous layer containing the nucleic acid was removed and recovered by precipitation.

2.2.1b Ethanol precipitation

Nucleic acids were precipitated from solution by adding 0.1 vol of 3 M NaOAc, pH 5.2 and 2.5 vol of absolute ethanol. The solution was mixed by vortexing, incubated for 15 min on ice and the DNA recovered by centrifugation at 14,000 rpm for 10 min. The pellet was washed in 70% ethanol, air-dried and then dissolved in an appropriate volume of distilled water or TE buffer (10 mM Tris-HCl, pH 7.0; 1 mM EDTA).

2.2.1c Quantification

The amount and quality of DNA or RNA was assayed by measuring the absorbance of the preparations at 260 nm and 280 nm. The reading at 260 nm allowed calculation of the concentration of nucleic acid in the sample. An A_{260} of 1 corresponded to a preparation containing approximately 50 μg/ml of double stranded DNA or 40 μg/ml of single stranded DNA or RNA. The ratio between the readings at 260 nm and 280 nm provided an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have ratios of 1.8 and 2.0, respectively. Any ratios less than these values indicate protein and/or phenol contamination of the sample.

2.2.2. Cloning in plasmid DNA vectors

2.2.2a Restriction enzyme digestion

Digestion of DNA with restriction enzymes was carried out according to the manufacturers' instructions. The DNA to be treated was incubated at 37°C (unless otherwise instructed) with the restriction enzyme, in the appropriate buffer and 100 μg/ml BSA (if instructed) for 1-16 hr. Often the DNA was treated with more than

one enzyme. If the conditions were compatible then the two enzymes were added together; where the conditions were incompatible the digests were performed sequentially, with a phenol extraction/ethanol precipitation step in between.

2.2.2b Dephosphorylation of vector 5'-ends

Where the cloning step only required digestion with one restriction enzyme, the vector DNA was phenol extracted/ethanol precipitated and treated with Calf Intestinal Alkaline Phosphatase (New England Biolabs) according to the instructions. The DNA to be treated was incubated with the CIAP in a final volume of 100 µl at 37°C for 30 min. After treatment, the DNA was phenol extracted/ethanol precipitated before ligation.

2.2.2c Ligation

Concentrations of vector and insert DNA were estimated by electrophoresis and comparison with DNA molecular weight markers of known concentration. A vector:insert molar mass ratio of between 1:1 and 1:3 was used in most cases and was found to work well. The molar mass ratio for DNA molecules was estimated using the following formula: [ng vector x insert size (kb)/ vector size (kb)] x molar ratio of insert = ng of vector required. Individual ligation reactions were set up in a final volume of 10 µl and carried out according to the T4 DNA ligase (Promega) manufacturers' protocol. The ligation reactions were incubated for 16 hr at 16°C for cohesive termini and for 16 hr at 25°C for blunt-ended termini.

2.2.3 Agarose gel electrophoresis of DNA

Agarose gels of 0.5%-1.5% were used depending on the size of the DNA that was being analysed. An appropriate amount of agarose (electrophoresis grade, Boehringer Mannheim) was dissolved in 1x TBE buffer (10 mM Tris-HCl, pH 7.0; 90 mM Boric acid; 1mM EDTA) by boiling. The solution was allowed to cool to ~50°C before adding ethidium bromide to a final concentration of 0.5 μg/ml (except where the gel was to be blotted) and pouring. 0.2 vol of 5x DNA loading buffer (33% glycerol; 0.25% bromophenol blue; 0.25% xylene cyanol) was added to each sample and once loaded, gels were run with an applied voltage of 4-8 V/cm in 1x TBE. Following electrophoresis, the DNA was visualised with an ultra-violet transilluminator (Ultra-Violet Products, Inc.) and photographs taken with a UVP DOC-IT™ camera (Ultra-Violet Products, Inc.).

2.2.4 Gel purification of DNA fragments

DNA fragments were isolated using QIAGEN's QIAquick gel extraction kit, following the manufacturers' instructions precisely. The purified DNA was resuspended in an appropriate volume of distilled water or TE buffer (10 mM Tris-HCl, pH 7.0; 1 mM EDTA).

2.2.5 Denaturing agarose gel electrophoresis of RNA

Agarose gels of 1% were used for all RNA gels. An appropriate amount of agarose (electrophoresis grade, Boehringer Mannheim) was dissolved in 1x FRB (formaldehyde running buffer: 20 mM MOPS; 5 mM NaOAc; 1 mM EDTA). The solution was allowed to cool to ~50°C before adding 37% (v/v) formaldehyde to a

final concentration of 2.2 M and pouring. Each sample was incubated at 65°C for 15 min in an equal volume of RNA loading buffer (50% (v/v) formamide; 1x FRB; 2.2 M formaldehyde; 0.01% (w/v) bromophenol blue) and once loaded, gels were run with an applied voltage of 5 V/cm in 1x FRB. Following electrophoresis, the gels were rinsed briefly in DEPC-treated distilled water. They were then stained for 30-45 min in 0.5 μg/ml ethidium bromide, destained in distilled water for 10 min and visualised as previously described.

2.2.6 Filter hybridisation of DNA

2.2.6a Southern blotting

After restriction digestion and separation of the DNA by agarose gel electrophoresis (no ethidium bromide), the gel was stained for 30-45 min in 0.5 μg/ml ethidium bromide, destained in distilled water for 10 min, and visualised as previously described. The DNA was denatured by soaking the gel in 1.5 M NaCl; 0.5 M NaOH for 30 min with gentle agitation. The gel was then rinsed briefly in distilled water and neutralised by soaking it in 0.5 M Tris, pH 7.2; 1.5 M NaCl; 0.001 M EDTA for 30 min with gentle agitation. The transfer of DNA to the nylon filters (Hybond-N, Amersham) was carried out as described in Sambrook *et al.*, 1989. After transfer, the DNA was crosslinked to the filters using UV-light in a Stratalinker (Stratagene), according to the manufacturers' instructions. Finally, the filters were baked at 80°C for 2 hr in a vacuum oven (Townson & Mercer).

2.2.6b Random-primed labelling of DNA probes

10-50 ng of fragment DNA to be used as probe was diluted in distilled water to 11 µl. The DNA was incubated at 100°C for 5 min, then cooled rapidly on ice. 5 µl of Rxn-C (250 mM Tris HCl, pH 8.0; 25 mM $MgCl_2$; 50 mM β-mercaptoethanol; 100 µM each dATP, dGTP, dTTP; 1 M HEPES, pH 6.6; 2.7 OD units hexadeoxyribonucleotides (Pharmacia Biotech) in TE buffer (10 mM Tris-HCl, pH 7.0; 1 mM EDTA)) was added, along with 1 µl of Klenow enzyme (NEB) and 3 µl (30 mCi) of [α-^{32}P] dCTP (Amersham). The contents were mixed thoroughly and incubated at 37°C for 30 min. NICK™ Sephadex G-50 columns (Pharmacia Biotech) were used to remove unincorporated ^{32}P, following the manufacturers' instructions precisely. The labelled probe was eluted in 400 µl of TE buffer (10 mM Tris-HCl, pH 7.0; 1 mM EDTA).

2.2.6c Hybridisation of filters

The filter was pre-hybridised for 1 hr at 65°C in hybridisation buffer (7.5 ml 20x SSC (3 M NaCl; 300 mM NaOAc); 2.5 ml 50x Denhardt's solution (5% (w/v) Ficoll, 5% (w/v) polyvinylpyrollidine, 5% (w/v) BSA); 625 µl 20% (w/v) SDS; 1 ml single-stranded salmon sperm DNA (10 mg/ml, Sigma); 10.25 ml distilled water) in a rolling bottle hybridisation oven (Techne). The labelled probe was then added and allowed to hybridise for 16 hr at 65°C. Washing conditions were as follows:

2x SSC; 0.1% SDS; 65°C; 2x 15 min	**low stringency**
2x SSC; 0.1% SDS; 65°C; 1x 30 min	**moderate stringency**
0.2x SSC; 0.1% SDS; 65°C; 1x 30min	**high stringency**

After the final wash, the filters were wrapped in SaranWrap and autoradiographed.

2.2.6d Autoradiography

The wrapped filters were placed in an X-ray film cassette in contact with Kodak MS film and with an intensifying screen. The cassettes were incubated at -70°C for a sufficient exposure time (between 1 hr and 16 hr) and then the films were developed in a Konica SRX-101A automatic developer for analysis.

2.2.7 Filter hybridisation of RNA

2.2.7a Northern blotting

After denaturation and separation of the RNA by agarose gel, the gel was rinsed briefly in DEPC-treated distilled water for 5 min with shaking to remove excess formaldehyde and agarose. The transfer of denatured RNA to the nylon filters (Hybond-N, Amersham) was carried out as described in Sambrook *et al.*, 1989. After transfer, the RNA was crosslinked to the filters using UV-light in a Stratalinker (Stratagene), according to the manufacturers' instructions. Finally, the filters were baked at 80°C for 2 hr in a vacuum oven (Townson & Mercer).

2.2.7b Random-primed labelling of DNA probes

DNA probes were used and were labelled as described for Southern blotting.

2.2.7c Hybridisation of filters

Labelled DNA probes were hybridised to filters as described for Southern blotting.

2.2.7d Autoradiography

Autoradiography was carried out as described for Southern blotting.

2.2.8 Polymerase Chain Reaction (PCR)

PCR (Saiki *et al.*, 1985; Mullis *et al.*, 1986) was used routinely in this project for amplification of specific regions of DNA for cloning. It was also used as a convenient tool for analysing transformants after cloning experiments (Gussow and Clackson, 1989), and for creating suitable restriction endonuclease sites to aid the cloning of specific DNA regions (Clackson *et al.*, 1994).

Every PCR experiment is unique and is differentiated by differences in thermal cycle conditions. The thermal cycle can be divided into three distinct steps:

(i) Denaturing step
The reaction mixture was incubated at 95°C to denature the double stranded template.

(ii) Annealing step
The annealing temperature varies with each reaction depending on the melting point values of the primers in a specific reaction. Generally, the annealing temperature used was 5°C below the lowest melting point of the two primers. The melting point

of each primer was calculated using the following formula (Bolton and McCarthy, 1962):

$$T_m = 81.5 + 16.6(\log_{10}[Na^+] + 41.0 \text{ (fraction C + G)} - (600/N)$$

where N = oligonucleotide length in bp

(iii) Extension step

The reaction mixture was incubated at the optimum temperature for the thermostable polymerase being used in the reaction. For this project two different PCR enzymes were used: *Taq* polymerase (Promega) where the fidelity of the product was not important, or Expand™ High Fidelity (Boehringer Mannheim), which is a mixture of *Taq* and *Pwo* DNA polymerases, where the accuracy of the product sequence was important.

PCR amplification was carried out using 25-30 thermal cycles on a Hybaid 'Touchdown' PCR thermal cycler. The reactions were set up as follows:

	Taq DNA polymerase	Expand™ High Fidelity
Template DNA	0.1 - 0.75 µg	0.1 - 0.75 µg
$MgCl_2$	1.5 mM	1.5 mM (in buffer)
10x buffer	10µl (Promega)	10µl (Boehringer)
dNTPs	200 µM	200 µM
Forward primer	200 nmol	200 nmol
Reverse primer	200 nmol	200 nmol
Enzyme	3 units	1.5 units
Distilled water	up to 50 µl	up to 50 µl

In every PCR amplification the addition of the thermostable polymerase was withheld until the reaction had been incubated at 94°C for 1 min. After that, the incubation times at each step were then generally as follows:

Denaturing (94°C)	1 min
Annealing (40°C - 60°C)	1 min
Extension (68°C - 72°C)	1 min per kb to be amplified.

The oligonucleotides that were used as primers for PCR in this project are shown in Table 2.1. Their use is described in detail within the relevant sections.

2.2.9 *In vitro* site-directed mutagenesis

2.2.9a Single stranded mutagenesis

Mutagenesis was carried out on single stranded phagemid DNA using the Muta-Gene® Phagemid *In Vitro* Mutagenesis Version 2 kit (BioRad), following the manufacturers' instructions precisely. Single stranded phagemid DNA was prepared as described elsewhere in this section (Section 2.4.5). The oligonucleotides that were used as primers for mutagenesis in this project are shown in Table 2.1.

2.2.9b Double stranded mutagenesis

Mutagenesis was carried out on double stranded plasmid DNA using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene), following the manufacturers' instructions precisely. The oligonucleotides that were used as primers for mutagenesis in this project are shown in Table 2.1.

Table 2.1 Details of the oligonucleotides used as primers used for PCR and site-directed mutagenesis

PCR

Oligo	Sequence (5'→3')	Length	Direction	T_m	Position (Fig 3.1)
PW3	AACATAAGGATCCGAAGG AGA	21 bp	Reverse	61.4°C	680→660
PW4	CGATGAATCGTAGGCTCT TGT	21 bp	Forward	63.0°C	601→621
PW5	**CCGAATTCAT**ATGGCAAT TGATTACAGC	28 bp	Forward	70.6°C	1→18
PW6	**GCAGCCCGGG**TTAAGACA ATTGAGGAAT	28 bp	Reverse	75.1°C	1832→1815
PW7	CGTACCAGAAGGAATTCC CATG	22 bp	Forward	66.3°C	-956→-935
PW8	**GATCAAGCTT**GCACCTAC CTTATGCGAA	28 bp	Reverse	72.4°C	-149→-132
PW9	**GATCAAGCTT**CCATACCT TGTGTGTACC	28 bp	Forward	68.9°C	1943→1959
PW10	GATGAC**T**CGAGCAGAGGG TTGTTC	24 bp	Reverse	69.1°C	2723→2746
PW13	GTGCTCGAAGACGACTGA	18 bp	Forward	60.3°C	-1065→-1048
RB1	GCATACATATAGCCAGTG	18 bp	Reverse	50.9°C	-
TG1	GCTATACCAAGCATACAA TCAACTCC	26 bp	Forward	64.4°C	-
TG2	CGTATCTACCAACGATTT GACCC	23 bp	Reverse	65.1°C	-

Mutagenesis

Oligo	Sequence (5'→3')	Length	Direction	T_m	Position (Fig 3.1)
PW19	CATAAGCAT**G**TGATGATA	18 bp	Reverse	49.2°C	750→767
PW20	TCGGACATA**A**GTATTATG	18 bp	Reverse	47.6°C	1461→1478
PW27	CTATTCCTCAATTGTCTT **GCGGCCGC***TACCGAGCTC GAATTCCC*	44 bp	Forward	86.5°C	1813→1829
PW28	*GGGAATTCGAGCTCGGTA* **GCGGCCGC**AAGACAATTG AGGAATAG	44 bp	Reverse	86.5°C	1829→1813

The position of the oligonucleotides designed using the *S.pombe cdc37*$^+$ sequence can be located using the sequence information given in Fig 3.1. For the PCR oligos, the non-annealing, engineered regions are shown in bold. For the mutagenic oligos, the mutagenic bases are shown in bold and sequences complimentary to vector sequence are in italic.

2.2.10 DNA sequencing

Sequencing was performed on an ABI 377 automated sequencer after carrying out the reactions using the Taq FS Dye-terminator kit (PE Applied Biosystems), following the manufacturers' instructions precisely. For all sequencing reactions carried out in this project a Hybaid 'Touchdown' PCR thermal cycler was programmed with 25 of the following cycles: 96°C for 30 sec; 50°C for 15 sec; 60°C for 4 min.

2.3 Protein manipulation protocols

2.3.1 Immunoprecipitation

Immunoprecipitation was carried out using a modified version of that described by Sambrook *et al.*, 1989. For each incubation, 0.1 ml of extract (section 2.5.10) was used. An appropriate amount of antibody in 0.4 ml of ice-cold NET-gel buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.1% Nonidet P-40; 1 mM EDTA, pH 8; 0.25% gelatin; 0.02% NaN_3) was added to each extract, mixed and incubated on a rotating wheel at 4°C for 1 hr. For each extract, 2.5 mg of Protein A-Sepharose was weighed out and incubated in 1 ml of dH_2O for 10 min on a rotating wheel at 4°C. The swollen Sepharose beads were collected at 14,000 rpm for 30 sec at 4°C, resuspended in another 1 ml of dH_2O and centrifuged again. Finally, the beads were resuspended in 1 ml of NET-gel buffer and collected at 14,000 rpm for 30 sec at 4°C. 10 µl of NET-gel buffer was added to the swollen Sepharose beads to create a 50/50 Protein A-Sepharose/buffer mix and 20 µl of this was added to each extract. The mixture was incubated on a rotating wheel at 4°C for 1 hr. The Sepharose beads were then centrifuged at 14,000 rpm for 30 sec at 4°C, resuspended in 1 ml of NET-gel buffer and incubated on a rotating wheel for 20 min at 4°C. The Sepharose beads were again collected by centrifugation at 14,000 rpm for 30 sec at 4°C, resuspended

in 1 ml of NET-gel buffer and incubated on a rotating wheel for 20 min at 4°C. After another centrifugation at 14,000 rpm for 30 sec at 4°C, the Sepharose beads were resuspended in 1 ml 10 mM Tris-HCl, pH 7.5; 0.1% NP-40 and incubated on a rotating wheel for a further 20 min at 4°C. The washed Sepharose beads were collected at 14,000 rpm for 30 sec at 4°C and the final wash completely removed. Finally, the Sepharose pellet was resuspended in 20 µl in 1x SDS sample buffer (50 mM Tris-HCl, pH 6.8; 100 mM DTT; 2% (w/v) SDS; 0.1% (w/v) bromophenol blue; 10% glycerol) before loading on an SDS-polyacrylamide gel for electrophoresis.

2.3.2 SDS-polyacrylamide gel electrophoresis of proteins

The electrophoresis of proteins followed the method of Laemmli, 1970, with minor modifications. SDS-polyacrylamide gels were poured using the BioRad Mini-PROTEAN II system, following the manufacturers' instructions precisely. The 12.5% resolving gel was poured between the glass plates of the gel apparatus and overlaid with deionised water, made up as shown below, in the order shown:

Component	*ml*
Distilled water	1.57
1.5 M Tris-HCl, pH8.8	1.25
10% (w/v) SDS	0.05
30% acrylamide mix (Severn Biotech)	2.1
10% (w/v) ammonium persulphate	0.025
TEMED	0.005

After the resolving gel had polymerised, the overlay was removed and the stacking gel was poured over. Before the stacking gel polymerised, the comb was inserted to

allow the wells to form. The 4% stacking gel was prepared by assembling the following in the order shown:

Component	*ml*
Distilled water	1.525
0.5 M Tris-HCl, pH6.8	0.625
10% (w/v) SDS	0.025
30% acrylamide mix (Severn Biotech)	0.325
10% (w/v) ammonium persulphate	0.025
TEMED	0.005

The samples were denatured by heating at 100°C for 5 min in 1x SDS sample buffer (50 mM Tris-HCl, pH 6.8; 100 mM DTT; 2% (w/v) SDS; 0.1% (w/v) bromophenol blue; 10% glycerol). Once the stacking gel had polymerised, the comb was carefully removed and the wells flushed first with deionised water and then with running buffer (25 mM Tris; 250 mM glycine, pH 8.3; 0.1% (w/v) SDS) to remove unpolymerised acrylamide. After heat denaturation, the samples were loaded and voltage applied to the gel at 8 V/cm until the dye front had moved into the stacking gel and then 15 V/cm until the dye front reached the bottom of the resolving gel.

2.3.3 Staining SDS-polyacrylamide gels with Coomassie Brilliant Blue

SDS-polyacrylamide gels were immersed in Coomassie dye (0.005% (w/v) Coomassie Brilliant Blue R250 in 45% (v/v) methanol; 10% (v/v) glacial acetic acid) for 4 hours at room temperature. The gels were then destained by immersing in 5% (v/v) methanol/7% (v/v) glacial acetic acid for 24 hr, changing the destaining solution every few hours.

2.3.4 Filter hybridisation of proteins

2.3.4a Western blotting

The transfer of proteins from a gel to a membrane was achieved by electrophoretic elution (Towbin *et al.*, 1979). Semi-dry transfer was used for all blots in this study, using a BioRad Trans-Blot SD semi-dry blotter, following the manufacturers' instructions precisely. The gels were sandwiched between absorbent paper soaked in transfer buffer (48 mM Tris-HCl; 39 mM glycine; 20% (v/v) methanol; 0.0375% (w/v) SDS, pH 9.2) before being placed between electrodes, in contact with the PVDF membrane (BioRad). A current of 2.5 mA/sq. cm of gel was applied for 40 min, after which the blots were removed and stained to analyse transfer.

2.3.4b Staining of membranes for total protein

After electrophoretic transfer, PVDF membranes were stained in an aqueous solution of Ponceau S to reveal the transferred protein and to monitor blotting efficiency and completeness. A concentrated stock of Ponceau S was prepared (2% (w/v) Ponceau S in 30% (w/v) trichloroacetic acid) which was diluted ten times in deionised water before immersion of the blots. The blots were allowed to stain for 5 min before being washed gently in deionised water to remove unbound dye.

2.3.4c Blocking, washing and antibody incubations

The blots were blocked by immersing in 1x PBS (50 mM sodium phosphate, pH 7.4; 150 mM NaCl) containing 0.25% Tween 20 and 5% (w/v) non-fat milk ("Marvel" powder) overnight with gentle overnight. After blocking, the blocking solution was

removed and the first antibody added, diluted to an appropriate concentration in 1x PBS containing 0.05% Tween 20 and 1% Marvel. Incubation with the first antibody was for at least 1 hr at room temperature with gentle agitation. After this incubation, the first antibody was removed and the blots washed four times in large volumes of 1x PBS containing 0.5% Tween 20 for 5 min each wash. After washing, the second antibody was added; either horseradish peroxidase conjugated donkey anti-rabbit IgG, or donkey anti-mouse IgG (both from Diagnostics Scotland), diluted 1:1000 in the same buffer as for the first antibody. Incubations with the second antibodies were for 1 hr at room temperature, followed by extensive washing as before.

2.3.4d Detection of immune complexes on membranes

The horseradish peroxidase conjugated second antibodies were detected using an enhanced chemiluminescent reagent (ECL™, Amersham), following the manufacturers' instructions precisely. Hyperfilm™ECL™ chemiluminescent film (Amersham) was used to reveal the bands and the films were developed in a Konica SRX-101A automatic developer.

2.4 Manipulation and handling of *Escherichia coli*

2.4.1 *Escherichia coli* strains used

DH10B: F⁻ *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), φ80d*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *end*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, λ⁻, *rps*L, *nup*G.

MV1190: Δ(*lac-pro*AB), *thi*, *sup*E, Δ(*srl-rec*A)306::Tn10(tet^r) [F':*tra*D36, *pro*AB, *lac* I^{q}ZΔM15].

CJ236: *dut*-1, *ung*-1, *thi*-1, *rel*A1; pCJ105 (Cm^r).

XL1-Blue: *recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac* [F' *proAB* $lacI^{q}ZΔM15$Tn10(Tet^{r})]^{c}

2.4.2 Media and growth conditions for *Escherichia coli*

2.4.2a Temperature for growth of *Escherichia coli*

Cells were grown at 37°C unless otherwise stated. Cell growth in liquid cultures was estimated by measurement of optical density at 600 nm on a Hitachi U-2000 spectrophotometer.

2.4.2b Media for growth of *Escherichia coli*

(i) L-Broth (LB)

E. coli strains were routinely grown in rich medium LB:

Bacto-tryptone (Difco)	10 g/l
NaCl	10 g/l
Yeast extract	5 g/l
Glucose	1 g/l

(ii) 2x YT

Bacto-peptone (Difco)	16 g/l
Yeast extract	10 g/l
NaCl	5 g/l

For solid medium, 15 g/l Bacto-agar (Difco) was added.

2.4.2c Antibiotics

(i) Ampicillin

The sodium salt was dissolved in distilled water at 30 mg/ml to make the stock solution. This was added to the media at a final concentration of between 25-100 μg/ml.

(ii) Chloramphenicol

Solid chloramphenicol was dissolved in absolute ethanol at 34 mg/ml to make the stock solution. This was added to the media at a final concentration of between 15-34 μg/ml.

(iii) Kanamycin

Kanamycin sulphate was dissolved in distilled water at 50 mg/ml to make the stock solution. This was added to the media at a final concentration of between 50-70 μg/ml.

All antibiotic stocks were stored in aliquots at -20°C and were added to autoclaved media cooled to ~50°C.

2.4.2d X-gal and IPTG for blue/white screening

(i) X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)

A stock solution of X-gal was made up by adding it to dimethylformamide at 50 mg/ml and stored at -20°C. It was added to autoclaved media, cooled to ~50°C, at a final concentration of 40 μg/ml.

(ii) IPTG (isopropyl-β-D-thiogalactopyranoside)

A stock solution of IPTG was made up by adding it to distilled water at a concentration of 0.1 M and stored at 4°C. It was added to autoclaved media, cooled to ~50°C, at a final concentration of 0.1 mM.

2.4.2e Storage of *Escherichia coli*

Bacteria were stored on LB agar plates for periods up to 4 weeks at 4°C. Long term storage was in LB medium containing 20% (v/v) glycerol frozen at -70°C. Frozen bacteria were revived from long term storage by removing a small stab from the frozen culture and streaking out onto LB agar containing appropriate antibiotics.

2.4.3 Transformation of *Escherichia coli*

Competent *Escherichia coli* cells were prepared by a rubidium chloride procedure modified from a protocol described previously Hanahan, 1983. A single colony from an LB plate was used to inoculate 2.5 ml of LB medium and left to grow overnight at 37°C with shaking. The entire overnight culture was then used to inoculate 250 ml of LB medium containing 20 mM $MgSO_4$ and left to grow at 37°C with shaking until the OD_{600} reached 0.4-0.6. The cells were harvested by centrifugation at 6,000 rpm for 5 min at 4°C, resuspended in 0.4 vol of ice-cold TFB1 (30 mM K-acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15% glycerol, pH 5.8) and incubated on ice for 5 min. The cells were again harvested by centrifugation at 6,000 rpm for 5 min and resuspended in TFB2 (10 mM PIPES; 75 mM $CaCl_2$; 10 mM RbCl; 15% glycerol; pH 6.5). The resuspended cells were incubated on ice for 15-60 min and then frozen at -70°C in 200 µl aliquots.

The DNA to be transformed was incubated on ice with 200 µl competent cells for 20 min. The mixture was then incubated at 42°C for 45 sec before being replaced on ice for 2 min. 450µl of LB was added, mixed gently, and the mixture incubated at 37°C with shaking for 1 hr. An appropriate amount of the transformation mixture was plated on selective medium and left to grow overnight at 37°C.

2.4.4 Isolation of plasmid DNA from *Escherichia coli*

Plasmid DNA was isolated from transformants by the Alkaline Lysis method Sambrook *et al.*, 1989. 1.5 ml of an overnight culture of the transformant was centrifuged at 14,000 rpm for 1 min, the supernatant discarded, and the cell pellet was resuspended in 100 µl of BDI buffer (10 mM Tris-HCl pH 7.0; 1 mM EDTA). 200 µl of BDII solution (200 mM NaOH; 1% SDS) was added, the solution was mixed well and incubated on ice for 5 min. 150 µl of BDIII (3 M KOAc) was added, mixed well and centrifuged for 5 min at 14,000 rpm. The supernatant was transferred to a fresh tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) added. The phases were mixed by vortexing and then centrifuged at 14,000 rpm for 2 min. The upper, aqueous layer was transferred to a fresh tube and 1 ml of absolute ethanol was added. After vortexing, the solution was incubated on ice for 10 min to precipitate the DNA. The precipitated DNA was recovered by centrifugation at 14,000 rpm for 10 min, drained, air-dried, and resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 7.0; 1 mM EDTA) containing 10 µg/ml RNase A.

The most commonly used procedure to analyse recombinants was to isolate the plasmid DNA from individual clones as described, and then identify the insert in the recombinant plasmid by restriction digestion and agarose gel electrophoresis.

2.4.5 Production of single-stranded DNA from *Escherichia coli*

The plasmids pTZ18 (Appendix 1F) contain the f1 origin of replication, so on infection with the helper phage M13K07, cells containing these plasmids will synthesise single-stranded DNA from this origin, which will then be released in the form of phage particles.

A colony of *E.coli* strain CJ236 containing pTZ18.*cdc37*$^{+}$ was used to inoculate 5 ml LBCUA (LB plus 100 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 0.25 μg/ml uridine) and grown overnight at 37°C. 0.5 ml of the overnight culture was used to inoculate 50 ml LBCU (no ampicillin) and was grown at 37°C until slightly turbid. M13 K07 helper phage was added to a final concentration of 1x 10^{8} pfu/ml and incubated at 37°C with vigorous aeration for 90 min. Kanamycin was then added to a final concentration of 70 μg/ml and culture was left overnight at 37°C with vigorous aeration. The cells were then harvested twice at 8,000 rpm for 10 min. 250 μg of RNase A was added to the resulting supernatant and incubated at room temperature for 30 min. 0.25 vol of 3.5 M ammonium acetate/20% PEG-6,000 was added, mixed well and incubated on ice for 30 min. The phagemids were then recovered by centrifugation at 12,000 rpm for 15 min. The supernatant was discarded, the pellet drained well and then resuspended in 200 μl of high salt buffer (300 mM NaCl; 100 mM Tris, pH 8.0; 1mM EDTA). The resuspended phagemids were chilled on ice for 30 min and then centrifuged at 14,000 rpm for 2 min to remove insolubles. The supernatant was extracted twice with neutralised phenol, once with phenol:chloroform:isoamyl alcohol (25:24:1), and three times with chloroform:isoamyl alcohol (24:1). 0.1 vol of 7.8 M ammonium acetate and 2.5 vol of absolute ethanol was added, mixed and incubated at -70°C for 1 hr. The single-stranded phagemid DNA was recovered by centrifugation at 12,000 rpm for 15 min, the pellet washed with 70% ethanol and resuspended in 20 μl of TE buffer (10 mM Tris-HCl, pH 7.0; 1 mM EDTA).

2.5 Manipulation and handling of *Schizosaccharomyces pombe*

2.5.1 *Schizosaccharomyces pombe* strains used

ED665 *ade6-M210 leu1-32 ura4-D18 h⁻*

ED668 *ade6-M216 leu1-32 ura4-D18 h⁺*

ED836 *cdc2-M26 leu1-32 ura4-D18 h⁺*

ED837 *cdc2-130 ura4-D18 h⁻*

ED838 *cdc2-18 leu1-32 ura4-D18 h⁺*

ED839 *cdc2-48 leu1-32 ura4-D18 h⁺*

ED840 *cdc2-17 leu1-32 ura4-D18 h⁺*

ED841 *cdc2-45 ura4-D18 h⁺*

ED881 *cdc2-33 leu1-32 h⁻*

ED908 *wee1-50 leu1-32 ura4-D18 h⁺*

ED918 *cdc13-117 leu1-32 ura4-294 h⁺*

ED1049 *leu1-32 h⁻*

ED1169 *cdc2-L7 ura4-D18 h⁻*

ED1340 *swo1-26 leu1-32 ura4-D18 h⁻*

ED1502 *cdc13-9 leu1-32 h⁻*

2.5.2 Media and growth conditions for *Schizosaccharomyces pombe*

2.5.2a Temperature for growth of *Schizosaccharomyces pombe*

Appropriate temperatures varying from 25°C to 37°C were used to grow the strains depending on their phenotype.

2.5.2b Media for growth of *Schizosaccharomyces pombe*

(i) Yeast Extract (YE)

S.pombe strains were routinely grown in YE medium supplemented with adenine and uracil:

Yeast extract (Difco)	5 g/l
Glucose	30 g/l
Adenine	75 mg/l
Uracil	75 mg/l

(ii) Edinburgh Minimal medium (EMM)

S.pombe strains were selectively grown in EMM medium, a modification of EMM2 (Mitchison, 1970, Nurse, 1975):

Glucose	20 g/l
KH phthalate	3 g/l
Na_2HPO_4	1.8 g/l
NH_4Cl	5 g/l
$NaSO_4$	100 mg/l
$CaCl_2$	15 mg/l
$MgCl_2$	1 g/l
Vitamins	1 ml
Minerals	100 µl

Vitamins: 5 g inositol; 5 g nicotinic acid; 0.5 g calcium pantathenate; 5 g biotin; all dissolved in 500 ml distilled water.

Minerals: 1 g H_3BO_3; 1.04 g $MnSO_4.4\ H_2O$; 800 mg $ZnSO_4.7\ H_2O$; 400 mg $FeCl_3.6\ H_2O$; 288 mg H_2MoO_4; 80 mg $CuSO_4.5\ H_2O$; 2 g citric acid; 20 mg KI; all dissolved in 200 ml distilled water.

Amino acids were added to the EMM medium as required for each strain or omitted for selection. Leucine, adenine, uracil or histidine were added when appropriate at 75 mg/ml.

(iii) Malt Extract (ME)

A nitrogen limiting medium ME consisting of 30 g/l malt extract was used to induce conjugation and sporulation.

For solid medium, 20 g/l Bacto-agar (Difco) was added to either YE, EMM or ME media.

2.5.2c Phloxin B

To check ploidy of cells, phloxin B was added to solid media to a final concentration of 20 μg/ml. Diploid cells die faster than haploid cells and accumulate the dye more quickly, and therefore can be differentiated by relative colour staining.

2.5.2d Storage of *Schizosaccharomyces pombe*

Strains were stored on solid media for periods up to 4 weeks at 4°C. Long term storage was in medium containing 30% (v/v) glycerol, frozen at -70°C. Frozen strains were revived from long term storage by removing a small stab from the frozen culture and streaking out onto appropriate media.

2.5.3 Genetical analysis of *Schizosaccharomyces pombe*

2.5.3a Crossing strains

For crossing strains, standard genetical procedures were followed (Gutz *et al.*, 1974; Kohli *et al.*, 1977). Strains were crossed by mixing together fresh isolates of two cell types (mating types h^+ and h^-) on the surface of an ME plate with a loopful of sterile water. The mating mix was incubated at 25°C for 2-3 days to allow zygotes to form and sporulation to proceed. The progeny of the crosses were examined either by random spore analysis or by tetrad analysis.

2.5.3b Random spore analysis

A loopful of mating mix was suspended in 1 ml of 0.2% helicase (Suc d'Helix pomatia, Industrie Biologique, France) and incubated overnight at 35°C. The spore concentration was estimated using a haemocytometer and an appropriate dilution, to give about 200 viable spores per plate, was spread onto appropriate solid media.

2.5.3c Tetrad analysis

A loopful of mating mix was streaked onto a thin YE plate. Single asci were isolated using a fine glass needle attached to a Leitz micromanipulator and the plate was incubated at 20°C overnight to allow the asci walls to break down. The spores were then separated on the surface of the plate with the micromanipulator and incubated at an appropriate temperature until visible colonies were formed.

2.5.3d Analysis of growth phenotypes

Growth phenotypes of cells were tested by replica plating or by streaking from a master plate onto EMM plus or minus growth supplements for auxotrophs, and onto YE at the restrictive and permissive temperatures for temperature-sensitive strains.

2.5.3e Diploid construction

For this project, sporulation competent diploids were always constructed using h^+/h^- strains with complementing alleles of *ade6*. The *ade6-M210* and *ade6-M216* both confer adenine requirement for growth, but heterozygous diploids *ade6-M210 ade6-M216* are prototrophic. Strains carrying the *ade6* mutant alleles were crossed as previously described, left to conjugate overnight and then streaked onto selective media for adenine prototrophs. Diploid colonies were organised by their colour on plates containing phloxin, and their ability to sporulate checked microscopically.

2.5.4 Cell physiology of *Schizosaccharomyces pombe*

2.5.4a Growth of liquid cultures

Exponentially growing cells were obtained by firstly inoculating a single colony into a 10 ml pre-culture of YE or selective media. The pre-culture was incubated for 1-2 days at the appropriate temperature until the cells had entered the stationary phase growth. An aliquot of the pre-culture was inoculated into an appropriate volume of YE or selective media in a conical flask, and incubated with shaking at an appropriate

temperature until the correct density of cells was reached. The volume of pre-culture used to inoculate the culture was calculated as follows:

Volume to add = required density/density of preculture x volume of culture/2^n

where n is the number of doublings

The cell density of liquid cultures was estimated by measuring the optical density at 595 nm on a Hitachi U-2000 spectrophotometer.

2.5.4b Cell number count

A 100 µl sample of cell culture was mixed in 10 ml of ISOTON II solution and sonicated for 5 sec at setting 6 on a Lucas Soniprobe Sonicator. Two counts of 0.5 ml volume were taken and combined to give a cell count per ml. Cell numbers were counted using a Coulter counter (Coulter Electronics).

2.5.4c Cell length measurement

The cell length of samples was measured using the *measure length* tool in the IPLab scientific imaging software following the manufacturers' instructions precisely. The cells were stained with Calcofluor (section 2.5.4e) to aid visualisation of the outline of the cells. Approximately 200 cells were measured for each sample.

2.5.4d DAPI staining

DAPI staining was carried out using a variation of the procedure previously described (Toda *et al.*, 1981). 1 ml of culture was added to 100 µl of ice-cold 25%

gluteraldehyde and incubated on ice for 15 min. The cells were then washed three times in 250 µl of ice-cold distilled water. After each wash the cells were harvested by centrifugation at 14,000 rpm for 1 min. After the final wash, the cells were resuspended in the residual water and 1 µl was mixed with 1 µl of DAPI (20 µg/ml) on the microscope slide. A coverslip was used and the cells examined immediately under oil using an Axioskop 2 fluorescent microscope (Zeiss) with the 63x objective lens. Photos were taken on a digital camera (Princeton Instruments) and IPLab scientific imaging software (Scanalytics) was used to analyse the data.

2.5.4e Calcofluor staining

Calcofluor staining was carried according to a method previously described (Mitchison and Nurse, 1985). 1 ml of culture was added to 100 µl of ice-cold 25% gluteraldehyde and incubated on ice for 15 min. The cells were then washed three times in 250 µl of ice-cold distilled water. After each wash the cells were harvested by centrifugation at 14,000 rpm for 1 min. After the final wash, the cells were resuspended in the residual water and 1 µl was mixed with 1 µl of Calcofluor (1 mg/ml) on a microscope slide. A coverslip was used and the cells examined immediately as previously described.

2.5.4f Cell DNA content measurement (FACS analysis)

The DNA content of cell samples was determined by flow cytometry after DNA was stained with propidium iodide (Alfa *et al.*, 1993). Approximately 10^7 cells for each sample were harvested by centrifugation at 2,000 rpm for 5 min. The cell were resuspended in 1 ml distilled water, centrifuged for 15 sec in a microfuge and resuspended in 1 ml cold 70% ethanol. The cells were stored at this stage at 4°C. To

process the cells, 300 µl (approx. $2x10^6$ cells) of the preparation was added to 3 ml 50 mM Na citrate, mixed and centrifuged at 2,000 rpm for 5 min. The cell pellet was resuspended in 500 µl 50 mM Na citrate containing 0.1 mg/ml RNase A and incubated at 37°C for 4 hr. 500 µl 50 mM Na citrate containing 4 µg/ml propidium iodide was added and the cells processed immediately. Just before processing, the cells were sonicated for 45 sec at setting 6 on a Lucas Soniprobe Sonicator. The DNA content of the cell samples were measured using a Becton Dickinson FACScan.

2.5.5 Transformation of *Schizosaccharomyces pombe*

S.pombe strains were transformed by electroporation as described previously (Prentice, 1991), which achieves a high transformation efficiency. Cells to be transformed were grown in 200 ml of YE or selective media to a density of approximately 0.5-$1x10^7$ cells/ml. They were then harvested by centrifugation at 5,000 rpm for 10 min. The cells were washed three times in 10 ml of ice-cold 1.2 M sorbitol and then resuspended in 1.2 M sorbitol to a concentration of $1x10^9$ cells/ml. The DNA to be transformed was mixed with 200 µl of the cell suspension and immediately transferred to an ice-cold 0.2 cm cuvette. This suspension was pulsed at 2.25 kV (11.25 kV/cm), 200 Ω and 25 µF. Immediately after the pulse, 500 µl of 1.2 M sorbitol was added to the cuvette. Electroporated cells were spread onto selective media and incubated at the appropriate temperature until visible colonies were formed (4-6 days).

2.5.6 Stability test of transformants

For various reasons, the transformants had to be tested for stability of a selectable marker, therefore distinguishing between an autonomously replicating plasmid and an

integration event. If a plasmid carrying a selectable marker replicates autonomously it will be lost in the absence of selection, however, if the plasmid has integrated, or there has been a reversion or gene conversion event, the selectable phenotype will be maintained even after relaxing the selection. To carry out a stability test, a transformant colony was streaked out to single colonies on YE and incubated at an appropriate temperature with no selection until visible colonies were formed. 20-50 colonies from this plate were then patched out on to a second YE plate and allowed to grow overnight. This second plate was then replica plated onto selective media (YE plates at restrictive temperature or EMM plates minus the required supplements) and incubated until growth or no growth of the patches could be scored.

2.5.7 Preparation of chromosomal DNA from *Schizosaccharomyces pombe*

S. pombe chromosomal DNA was prepared by a large-scale version of a method described previously (Durkacz *et al.*, 1985). This method yields DNA of sufficient quantity and quality for a variety of purposes such as Southern blotting and PCR amplification. 100 ml cultures of YE or selective media were inoculated and incubated until the cells reached stationary phase (OD_{595} of 2-3). The cells were harvested by centrifugation at 3,000 rpm for 10 min and the cells resuspended in 1.5 ml of CPS buffer (50 mM citrate-phosphate buffer, pH 5.6; 40 mM EDTA, pH 8.0; 1.2 M sorbitol). 15 mg of zymolyase-20T (ICN) was added and incubated at 37°C for 5-30 min until the cell walls had been digested. The cells were then centrifuged at 3,000 rpm for 5 min and resuspended in 15 ml of 5x TE buffer (50 mM Tris-HCl, pH 7.0; 5 mM EDTA). 1.5 ml of 10% SDS was added, mixed well and incubated at 65°C for 5 min. 5 ml of 5 M KOAc, pH 5.6 was added and incubated on ice for 30 min. The mixture was then centrifuged at 5,000 rpm for 15 min, the supernatant passed through a gauze and then 20 ml of ice-cold isopropanol was added and left for

5 min at -70°C. The precipitate was recovered by centrifugation at 10,000 rpm for 10 min, drained well and air-dried. The pellet was then resuspended in 3 ml of 5x TE buffer (50 mM Tris-HCl, pH 7.0; 5 mM EDTA), RNase A added to a final concentration of 20 mg/ml, and incubated at 37°C for 2 hr. The preparation was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), mixed well, and centrifuged at 10,000 rpm for 10 min. The upper, aqueous phase was transferred and 0.1 vol of 3 M NaOAc and 2.5 vol of absolute ethanol were added, mixed and incubated on dry ice for 1 hr. The DNA was recovered by centrifugation at 10,000 rpm for 10 min, washed with 70% ethanol and air-dried. Finally, the DNA was resuspended in 200 µl of TE buffer (10 mM Tris-HCl, pH 7.0; 1 mM EDTA).

2.5.8 Preparation of total RNA from *Schizosaccharomyces pombe*

S. pombe total RNA was prepared as described previously (Kaufer *et al.*, 1985). This method yields RNA of good quantity and quality, suitable for Northern blotting. 100 ml cultures of YE or selective media were inoculated and incubated until the cells reached mid log phase (OD_{595} of ~0.5). The cells were harvested by centrifugation at 3,000 rpm for 10 min and the cells resuspended in 2 ml of 0.9% NaCl. The cells were divided into two screw-cap tubes, harvested again at 14,000 rpm for 1 min, and each pellet resuspended in 75 µl of STE buffer (0.32 M Sucrose; 20 mM Tris, pH 7.5; 10 mM EDTA, pH 8). Acid-washed beads (Sigma) were added to just below the meniscus and vortexed for 30 sec. 600 µl of NTES buffer (100 mM NaCl; 50 mM Tris, pH 7.5; 5 mM EDTA, pH 8; 1% SDS) was added and again vortexed for 30 sec. The preparation was then extracted with 500 µl of hot phenol three times, each time incubating at 65°C for 2 min with frequent vortexing. After each extraction, the phases were separated by centrifugation at 14,000 rpm and the upper, aqueous layer was transferred to a fresh tube. The preparation was then

extracted with phenol:chloroform:isoamyl alcohol (25:24:1) at room temperature by vortexing and then recovering the nucleic acids by centrifugation at 14,000 rpm for 1 min. 0.1 vol of 3 M NaOAc and 2.5 vol of absolute ethanol was then added to the final aqueous phase, vortexed and then precipitated at -70°C for about 30 min. The precipitated RNA was recovered by centrifugation at 14,000 rpm for 10 min, washed with 70% ethanol, air-dried, and each pellet resuspended in 22 µl of distilled water (treated with DEPC) before combining the two.

2.5.9 Preparation of denatured protein extract from *Schizosaccharomyces pombe*

S. pombe total protein extract was prepared using a modified (Shiozaki and Russell, 1996) version of that described previously (Moreno *et al.*, 1991). This method yields protein of good quantity and quality, suitable for Western blotting. 50 ml cultures of YE or selective media were inoculated and incubated until the cells reached mid log phase (OD_{595} of ~0.25). The cells were harvested by centrifugation at 3,000 rpm for 10 min and resuspended in 1.5 ml of STOP buffer (150 mM NaCl; 50 mM NaF; 10 mM EDTA; 1 mM NaN_3, pH 8.0). The cells were harvested again at 3,000 rpm for 10 min and resuspended in 2 vol extraction buffer (50 mM Tris-HCl, pH 7.5; 1mM EDTA; 10% glycerol; 50 mM NaF; 1 mM Na vanadate; 1 mM PMSF; 1 mM DTT; 1x protease inhibitors (*complete*™-Boehringer Mannheim)) on ice. The preparation was then transferred to a 1.5 ml tube and acid washed beads (Sigma) added to just below the meniscus. Each preparation was vortexed for five 20 sec intervals, chilling on ice for 1 min in between. Cell breakage was monitored microscopically. Each tube was punctured at the bottom with a red hot needle and placed inside another 1.5 ml tube inside a 15 ml tube. The tubes were centrifuged for 2 min at 2,500 rpm and the flow-through collected. The extracts were then centrifuged at 14,000 rpm for 15 min. The supernatants were transferred to fresh tubes and stored at -20°C.

2.5.10 Preparation of protein extract from *Schizosaccharomyces pombe* for immunoprecipitation

S. pombe total protein extract for immunoprecipitation was prepared using a modified version of an enzymatic lysis protocol described by Sambrook *et al.*, 1989. 50 ml cultures of YE or selective media were inoculated and incubated until the cells reached mid log phase (OD_{595} of ~0.25). The cells in 10 ml of the culture were harvested by centrifugation at 5,000 rpm for 10 min at 0°C and resuspended in 10 ml of ice-cold PBS (50 mM sodium phosphate, pH 7.4; 150 mM NaCl). The cells were harvested again at 5,000 rpm for 10 min at 0°C, resuspended in 10 ml stabilising buffer A (1 M sorbitol; 10 mM $MgCl_2$; 2 mM DTT; 50 mM K_2HPO_4, pH 7.8; 100 µg/ml PMSF) and incubated at 30°C for 10 min. The cells were collected again by centrifugation at 5,000 rpm for 10 min at 0°C before being resuspended in 10 ml stabilising buffer B (1 M sorbitol; 10 mM $MgCl_2$; 2 mM DTT; 25 mM K_2HPO_4, pH 7.8; 25 mM sodium succinate; 100 µg/ml PMSF) and incubated at 30°C for 2 min. 2.5 ml of a 50 mg/ml solution of Zymolyase 20T was added and the preparation incubated at 30°C for 20-30 min until the cell walls were digested. The protoplasts were then collected at 2,500 rpm for 15 min at 0°C. The pellet was resuspended in 0.1 ml lysis buffer (50 mM HEPES, pH 7; 1% Nonidet P-40; 100 µg/ml PMSF; 1x protease inhibitors) and incubated on ice for 30 min. Finally, the cell debris was collected by centrifugation at 14,000 rpm for 10 min at 4°C and the soluble extract transferred to a fresh tube and stored at -20°C.

2.6 Manipulation and handling of *Saccharomyces cerevisiae*

2.6.1 *Saccharomyces cerevisiae* strains used

ED1373 *cdc37-1 leu2-3,112 his3Δ ura3-52 lys- a*

ED1375 *cdc37-2 aro1 ura3-52 trp1 a*

2.6.2 Media and growth conditions for *Saccharomyces cerevisiae*

(i) YPD

S.cerevisiae strains were routinely grown in YPD medium:

Yeast extract	10 g/l
Glucose	20 g/l
Peptone	20 g/l

(ii) Synthetic Dropout medium (SD)

S.cerevisiae strains were selectively grown in SD medium:

Yeast nitrogen source (w/o amino acids)	6.7 g/l
Glucose	3 g/l

Amino acids were added to the SD medium or omitted for selection using 0.1 vol of a 10x Dropout solution:

L-Isoleucine	300 mg/ml
L-Valine	1500 mg/ml
L-Adenine hemisulphate salt	200 mg/ml
L-Arginine HCl	200 mg/ml
L-Histidine HCl monohydrate	200 mg/ml
L-Leucine	1000 mg/ml

L-Lysine HCl	300 mg/ml
L-Methionine	200 mg/ml
L-Phenylalanine	500 mg/ml
L-Threonine	2000 mg/ml
L-Tryptophan	200 mg/ml
L-Tyrosine	300 mg/ml
L-Uracil	200 mg/ml

For solid medium, 20 g/l Bacto-agar (Difco) was added to either YPD or SD media.

2.6.3 Analysis of growth phenotypes

Growth phenotypes of cells were tested by replica plating or by streaking from a master plate onto EMM plus or minus growth supplements for auxotrophs, and onto YPD at the restrictive and permissive temperatures for temperature-sensitive strains.

2.6.4 Transformation of *Saccharomyces cerevisiae*

S.cerevisiae strains were transformed by the lithium acetate procedure as developed by Ito *et al.*, 1983 and modified by Schiestl and Gietz, 1989, Hill *et al.*, 1991 and Gietz *et al.*, 1992. Cells to be transformed were grown in 300 ml of YPD or selective media until the OD_{600} reached 0.4-0.6. They were harvested by centrifugation at 2,500 rpm for 5 min and resuspended in 30 ml of TE buffer (10 mM Tris-HCl, pH 7.0; 1 mM EDTA). The cells were harvested again at 2,500 rpm for 5 min and resuspended in 1.5 ml of 100mM LiOAc in TE buffer. The DNA to be transformed was added to 100 µl of the cell suspension along with 0.1 mg of herring testes carrier

DNA (Clontech) and mixed well. 600 µl of 100 mM LiOAc, 40% PEG-4000 in TE buffer was added and vortexed at high speed for 10 sec to mix. The mixture were then incubated at 29°C for 1 hr with constant agitation. 70 µl of DMSO was added and mixed well by gentle inversion and then the mixture was heat shocked for 15 min at 42°C. The cells were chilled on ice for 2 min, centrifuged at 14,000 rpm for 5 sec and then resuspended in 500 µl of sterile TE buffer. Transformed cells were spread onto selective media and incubated at the appropriate temperature until visible colonies were formed.

Chapter 3

Cloning and sequence analysis of *cdc37*$^{+}$

3.1 Introduction

The initial aim of this project was to identify homologues of the *S.cerevisiae* gene *CDC37* in *S.pombe*. At the beginning of this project, the *S.pombe* genome sequencing project was underway, co-ordinated by the Sanger Centre in Cambridge. Most of the genomic DNA had been cloned into cosmids, and about 50% of these cosmids had been sequenced and the results entered into the database. Several strategies were employed to attempt to isolate putative *CDC37* homologues before one, named *cdc37*$^{+}$, was identified by use of the database at the Sanger Centre.

Fig 3.1 contains the nucleotide sequence of the region of *S.pombe* genomic DNA containing the putative *cdc37*$^{+}$ gene. Important regions of the sequence are annotated in this figure and are discussed throughout this chapter. This figure can be used as a map for the sequence analysis and cloning described in this chapter. The sequence used to design oligonucleotides can be also identified by using this figure together with the information contained in Table 2.1.

This chapter describes the identification of this gene, the analysis of the genomic sequence and the construction of a clone containing the entire ORF. It also investigates how the predicted *S.pombe* Cdc37 sequence compares to that of *S.cerevisiae* Cdc37p and other Cdc37 proteins identified in various organisms.

3.2 Approaches to identifying *CDC37* homologues in *S.pombe*

3.2.1 Degenerate PCR

The initial strategy that was used to identify potential *CDC37* homologues in *S.pombe* was to design degenerate primers using the *S.cerevisiae* Cdc37p sequence

*Pst*I
ctgcagcaaccattcttccgcctaacgc -1560

aagaatagcgattacagttgcgttcccaaggaaccctgttgacccgaagaaaataccgct -1500

agcaaaagcctctcgctttgccaggacaaagagatttcgaatgtaatcgttgtaacgatt -1440

tacttcttgtctttctcctagaaaagcctgtgtagtccgaacatttgcgagtttctcttc -1380

tgaaaccttgttaaatcaccgagagcatcttgtgtagtcctggaaagtttccgaacata -1320

ctctccataaaagaaagctcccaatgcaattgggggtacaattagggacatatatccagt -1260

gagtctcatggatacgtagagcatcatccctattccagcaattgcactaactgagctacg -1200

aagaccatcacttaagtacattgataaagacttaccgacaatactcgaatccgtcgtaag -1140

acgggatatgagatccccatgtttatgaaagtcaaaaaatgctccatctaatgacatgca -1080

tttagcaaacaagcgtgctcgaagacgactgacaattctttcactcagtagacgcaaggt -1020

gatgattctaccaaagttacaagctgaaccaaggaaaaataaacccaacaaaccaatata -960

*Eco*RI
aaacgtaccagaaggaattccatgatatgcgtgacagaagaatcgcctgacgagccagc -900

gtctaagattttgccaacaatgtagggaatggacattgtaacgccggaggaaactaagag -840

aagagatccagcaataaaaaaattccatccctgacccttgctagtgtgaagagacgaaa -780

aacattgactttcttgtttggtgtttgtaatgtaccatttgtatttatttttgagatatc -720

ctgtgcttgctcatcttttgatggaactgtagaattatgtcgcacacacaactttaaaaa -660

agcaagtgatcgtataggaagaatagatctagaaaaaggtaacggaaaactatttttaaa -600

aggatatatcttagaaccacttgaggccttttgagataacgagtttaggctcacattaac -540

ttcaatagaagtgtcactggtactataatattctgcatttggcgtgggaagtaatagagt -480

attcgtacttttgtaagccacgttgtttttcaaccaagtcaacggtactaaatagtttgc -420

tcgaaatattacgcgtttattgtagcagtgagcgaatggtactctcgaaagtccaaaacg -360

tattggatccatccatgaatgtgcctttcaaaggaaatgaacggaatcttttcgaaacgg -300

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ttcgatagaatttccacgaagtactctacattcgcataaggtaggtgctttggtacatac -120

taagatttcatttaccttaacacaaccacttctctacaacaagtcaaatcgacgagaata -60

gcaaagacatttgtgtcttttacttttttaaatttcttgaaattataatttgcttcgag 0

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tatgaagttaacagaattgacagGGATAAGCTAGAACTTAGTGATGACAGgtatgaaata 120

tacggtttggagaggaaatcggctctgtaaggacaatggttaaatactctacatgatgcc 180

tttgattggagtacaataaaagaagtcgaaatgaaatttcattagtaggatggaataaat 240

tcaaaaataaaattcagactcatctttttatcattttgaaggacatgttgggttcaaaaa 300

ggcaatcattaatttgcagtttgtatatttgatgccaattgggttcttttagctaataaa 360

caaccattgtcaaaacattcctttctttttccttcttcatttatttttcgttttgataaat 420

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aaccacatgcagTTCATCCAAATGTCGATAAAAAGTCTTTTATTAGATGGCGCCAACGTG 540

ATATCCACGAAAAACGGGCTGTTAGAAAGCAAAAAATGGAAGATATCAAAGGTGCAATGG 600

CGATGAATCGTAGACTCTTGTCTCGCATCTCCGAAATGGAGACTGTTCTTGAGAAGGAAT 660

*Bam*HI

CTCCTTCGGATCCTTATGTTTTATTGGGTTCATTTTTAGAGGCAAAAAAGTCTGAAGATA 720

*Nde*Ia

TGGATAGTGCCATTCCTGGAGGCATGTCGTATCATCATATGCTTATGTCTTTATTAAAAG 780

TAATTAAAGATGCCGAAGACACTACCGAAGAAAAATCTATGGATGATTCGGATAAATGCT 840

TGAGACGATTAAAGTCACACAAAGAGCGTCTTCTGAAGTTGCTAGAAGATGCACAAAAGG 900

AGTATGATACTTTAGAGGCTGAGAGTAAAAATTACATCACTTCTGAAGACTTGCATTTGG 960

GCTTTGATTCCACCTATGTTCAGAAAAAGGAGCCTGAAAAACCTAAAAAGACTAAAACCA 1020

AAAAGGAGACCATCCAGGTCATTGAATCTCTTAATAACCCTACACCTCCTACTGATTTTC 1080

CCGGCGCTAAAGAGCAAGCTTCTACTGGTAATGCCCCTAAAAATCCTGTCAATGAGAATG 1140

AGTCGGAAGATGAAGAAGGGTTGTCTCTTTCTGAAGATGGTAAGAAGTTTGCCAATATTG 1200

ATTTTGGTGATTATTCTTCATCGGAAGAGTTTTTGAAAGAACATTTAAATATCTTGGCAG 1260

ATGAGGAAGAATCTGATGCTATTCTTTTGGAAGCCTTTAATGCTGAACTAGAAGGGAAGC 1320

CGTCCTTAGCAAAACAATATGTTCATCAAGCCCTTTTGATCAGCTATTGCAGACAGCTTG 1380

GACCAAATGGTTTATCAATATTTTTTCAAAAAATTAAAGACCCAAATCACCAATCTCAGC 1440

*Nde*Ib

GGCTATTTTTAGAGGATGTTCATAATACATATGTCCGAATCCATGAACGTTCTGCTGCCA 1500

TCAGCAAAGAACAAGCTGAAAGCGGTGAAGGAGTAGAACAAATCCAGCTCTGTGCAGTTG 1560

ATCCTAATACGAAACTTTCCATCACCATCCCGGAGGCAGGTTCTACTGATCCAGAGACAC 1620

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AAAAAGCTCGTGCAGCTTTTGAGTCTTTTCCTCCTAACCTCCAAAAAGCTCTTATGACCA   1680

ATGATTTAGACAAGATTAATGTAGTTCTTGGAAAAATGGCAGTTGAAAATGCAGAAGAAG   1740

TTGTGGAAAAGCTCAGCAGTACTGGTATGTTGAGTATCGAAGAAGGAATCATCGACACAA   1800

CGAAAGGTGAGACTATTCCTCAATTGTCTTAAatttttctgcttacgggtgtgcttttag   1860

taagcgaaaacaccaaagttacatgtttgccgttggcatttttaaatggataaacaacaa   1920

tgaaattttatttattgatttaccatacctttgtgtgtaccgtataataaaataagtattt   1980

aaacaatttgtacatttgccatcaaagcaacgtggtcgctgggatatcttccttctaaag   2040

gctccgcttccttcatctcactgggtaaaggcacacgtaacagtgcctttaattttatac   2100

cttcaacgacttgattttcgggagtttgaagtgaggtatcacgattcataacaaatatgt   2160

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tcttgtagaaattaggaacaaattcagcatcaacttcttgcatacatcccaaagttggtg   3000

aataataggttaattcattagcgagcattcgtgagcgatttttccattttaatgcctcac   3060

cactgtgtggaaacattgaacgacgaatatttgtctgagcaagtacgttgtaagtcataa   3120

tagtaatatccaatgcagacttttcagtttcaaacggtgcataattagggatggagagca   3180

tttcacgctttataaagtccgtattgaattcgggtggagcattgcttggtggagcaatag   3240
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*XhoI**

EcoRI

gttttcgagcagccttcttagctaataatcagttaataaaattgagcaacaaaaaattaa 3300

acaaaggagtagagattcggtcttaaaaaaagtttcacaacaaccttcttattgttgctc 3360

gactagatgaacgaaaatttttttcatacccattttctcaagttttttttggcgttgtt 3420

tctctatgtattcaggcgttacgaacgaaggagggcctttagctgagccttttttcttaa 3480

cgggtatcgaatctgccatcttgttcttttttctgatcaaagaatgatatagcaagtttgg 3540

ataatctataatcagtgtggaatcgatcgataaaaaacttcaaagtcaatgtacacgttt 3600

taggacaagtgtacatacaatgacttttgagtagactttatttaagttttcacagccgaa 3660

atggattaacgactgttggttcgtacaacctcaggtatatcatctaaaataccgcatact 3720

agcgtgctctccttgatgccttctgttcgcttcaagaaaaggagccattggagcttcttt 3780

gactgatttgaaaggatctgtttcaaaattatcttcaattgtatttttaccctcggcatt 3840

tgaagagccttgaatgaatgcaaaggcattaactaaatcttcgagttccaaatctttcca 3900

agccggttcatgcctaagctgtttgtttttcattaagattagatttgcactagaatgttg 3960

aatagtcatctcatcttctgtaacaggtggtaagtgttctactcgcacttcgacatcgtc 4020

aactggaagtctatcttttagccaatccgaggcaaacaaaactactcccggtttattcat 4080

atgaacataattgtaattcgaatccattaattgttttactgattccaagtaggagactta 4140

tggtgcttttatacggttggtgttattggagaagtcggtggtaagtgtgtaaacaaagca 4200

tttagcagcaaagtctactagttgggggggagggagatctttccatgaaaatcaattataa 4260

gaattctagattaatttcttattcacataattctatgaacttttaagtagaaataaaaat 4320

cagttaggtggtttgctactattcttctcaaaaccttcgtctcttaatctatttattcct 4380

attggcatgttatttttcttgataaataaggtaaatatatgtgcttgcttagcttacaaa 4440

tttcaaagaatgacactaatagtgcaatgttttacttaagctatgattgtgtccaaaacc 4500

ctagcaatactgacagagttataatgaaaaattaagcgtatatttcacaatcatataatc 4560

atattccccctttctgaaaaaatgtcaatggcaaatcatatactgttttgagttaaaga 4620

ggctttggcagatcataaatcagccatcctaattagtaaaaagaatacaaaattgtgatg 4680

gttatgttaaaagtattcctagtcattagtgcatcaccttagagtcatgtgctgtatata 4740

tatatgtgtctacctttgcataaacaaacttgtattccacagtcaaactagtacaataca 4800

catcgttctgcgaaaacgttctgtttatttttcagcaaaaaattaaagtttaagctatca 4860

```
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taaacaataactttcacggaagcaacaacacaaccaacaaccaacccttcagaagtaact   4980
ttcggaataattttgtattcaaacctatatcgtttatcattcctttggatttcttttaat   5040
ctttctttcgaacacgaaaaacttactcttcactaagcttcgaaatttccaatcaaacga   5100
tttaacaataagaaaattttgtgcttaaaatcaccaaaatggattctcatgttttagcat   5160
ttccaattcctgatccatctacacctcctcccgaagaggcatccctttatgctagtaaat   5220
gtgactattatgaaagcgatatttactctcaaattaggagattaaccgaagaaaagtgca   5280
ttgagacagaaaaagagttgccagttaatataaaactacatgtcaaacataaaggaatga   5340
gcatatcatcttcccgcaatgaactagtggttactgtttcaggaaattataaaaatgtgt   5400
acactgcaaaaataaagattttgcaagccattcccaaaattctaatcagcaaacttgtgc   5460
tcgaagaacccctcgaaaacctccttttttgatgaagatgggtacgtttatgaagacacta   5520
tgaaacatttggacaaaataacggaaataacgggagcaaaaatttatatcatgcatcgtt   5580
ccatgtgtcatgatatcgat                                           5640
```

ClaI

Fig 3.1 The genomic nucleotide sequence of the putative *cdc37*$^+$ gene in *S.pombe*

Shown here is the sequence of the 7188 bp *Pst*I-*Cla*I region of genomic sequence believed to contain the *S.pombe cdc37*$^+$ sequence. The predicted ORF is shown in black uppertcase, the predicted UTR in black lowercase and unrelated sequence in grey lowercase. Also included are the predicted translational 'start' and 'stop' codons in red, putative splice consensus sequences in blue (Table 3.1) and the relevant restriction sites in the sequence are underlined. The position at which the poly-A Tail begins in the mRNA is shown in bold with an asterix. Table 2.1 contains a list of all the oligonucleotides designed using the above sequence and the locations are referenced with respect to this figure. Numbering of the sequence is with respect to the first base of the ORF ie. the adenine residue within the predicted translational 'start' codon (A=1, T=2, G=3...etc). * *XhoI* site engineered by a single base pair change to create site for cloning the 3' flanking region in the deletion construct.

and to amplify from an *S.pombe* cDNA library (T. Gaskell, pers. comm.). Using the *S.cerevisiae* Cdc37p sequence and sequences of homologues from other species, the degenerate primers were designed complimentary to regions where the sequence is conserved among species. Primers were also used designed complimentary to the *S.cerevisiae ADH1* promoter and terminator sequences in the library vector pDB20 (Fikes *et al.*, 1990).

Various reaction conditions and primer combinations were used to attempt amplification from the cDNA library by PCR using *Taq* polymerase (section 2.2.8). However, this approach was unsuccessful in identifying potential *CDC37* homologues in *S.pombe*.

3.2.2 Complementation of the *S.cerevisiae ts* mutant *cdc37-2*

A second approach involved attempting to rescue the defect of a *S.cerevisiae cdc37 ts* mutant using two *S.pombe* cDNA libraries. There were two *cdc37 ts* mutants available, kindly donated by M. Winey; *cdc37-1* (Reed, 1980b) and *cdc37-2* (Reed, 1980a). In both of these mutants at 25°C the cells behave as wild-type *S.cerevisiae*, but at 36°C the cells cease to divide and become arrested at 'start'. For this study, the *cdc37-2* mutant was used, as the arrest phenotype was much tighter than that of the *cdc37-1* mutant. To transform these *ts* mutants, two different *S.pombe* cDNA libraries were used, both cloned into *S.cerevisiae* expression vectors. One library, expressed by the strong, constitutive *ADH1* promoter in pDB20 (Fikes *et al.*, 1990), was a gift from M. Yanagida, whilst the other, expressed by the PGK promoter in pFL61 (Lollier *et al.*, 1995), was donated by M. Minet.

The *cdc37-2* strain was transformed with the *S.pombe* cDNA libraries and a cDNA containing the *CDC37* ORF as a positive control (section 2.6.4), allowing colonies to

form at 25°C. The colonies were then tested for growth at the permissive and restrictive temperatures; 25°C and 36°C, respectively. Preliminary studies using this approach, where ~6.4x 10^7 transformants were screened, were also unsuccessful in identifying potential *CDC37* homologues in *S.pombe*.

3.2.3 Searching the *S.pombe* sequence database with the *S.cerevisiae* Cdc37 sequence

The translated DNA database at the Sanger Centre was searched with the Cdc37 protein sequence from *S.cerevisiae* using BLAST and sequences showing similarity were analysed further for potential genes. Only one cosmid in the translated DNA database contained a region which showed significant similarity to the sequence of Cdc37p. Cosmid c28E12 containing DNA from chromosome 2 contained three regions with significant similarity to Cdc37p. The largest region of 442 amino acids had an identity of 31% with the *S.cerevisiae* Cdc37, whilst in close proximity upstream, two smaller regions of 8 and 40 amino acids had identities of 100% and 37%, respectively. The regions of similarity in the database sequence were not in the same reading frame, suggesting that the sequence contained introns, so the genomic sequence was analysed to determine its structure. It is worth noting at this point, that subsequent searches at the end of this project, with the *S.pombe* genome database project almost complete, still only result in this single region of genomic DNA with significant similarity, suggesting that only one homologue of *CDC37* exists in *S.pombe*.

3.3 Analysis of *S.pombe cdc37*$^+$ sequence

Upon arrival of the cosmid c28E12, a 7182 bp region containing the putative *cdc37*$^+$ gene was cloned into pBluescript SK- using *Cla*I and *Pst*I sites predicted from the genomic sequence (Fig 3.1). The region of the clone containing the putative *cdc37*$^+$ gene was subsequently sequenced and compared to the sequence published by the Sanger Centre. The sequences matched exactly except for one base pair. Since the discrepancy occurred at a region which was believed to be within the ORF, it was decided that sequencing a cDNA clone would reveal the correct sequence (section 3.4). Meanwhile, the genomic structure of the *cdc37*$^+$ gene was predicted using splicing consensus sequences from previously analysed *S.pombe* genes (Zhang and Marr, 1994; Chen and Zhang, 1998) and by comparison with *S.cerevisiae* Cdc37p.

3.3.1 Prediction of the transcriptional and translational initiation/termination sites

As the N-terminal end of the Cdc37 proteins seem to be highly conserved in all species where they have been identified to date, the predicted 'start' translation site (ATG) was identified along with the subsequent few amino acids which were identical to the *S.cerevisiae* Cdc37p sequence. The translational start consensus sequence outside of the ATG codon does not appear to be particularly well conserved in *S.pombe* (Zhang and Marr, 1994), therefore, a definitive prediction of the site is impossible by sequence analysis alone and needed to be confirmed by analysis of a cDNA sequence. However, no other potential start codons exist in any reading frame within 250 bp upstream of the predicted start site.

Due to the limited sequence similarity throughout most of the length of the *S.cerevisiae* and *S.pombe* Cdc37 proteins and the possibility of further introns, it

was difficult to predict the translational stop codon. As there appeared to be no consensus splice sites within the 442 amino acid largest region of similarity, which is furthest downstream, it was assumed that this was the final exon and that there were no more introns present. This region contained no stop codon, therefore the next stop codon within the same reading frame was predicted to be the translational stop.

Promoters in *S.pombe* are not well understood and in particular, very little information exists on the range of sequences that can serve as functional TATA elements. However, TATA sequence motifs have been suggested for genes in *S.pombe* that are transcribed at moderate or high levels (Russell, 1989). The *cdc37*$^{+}$ gene has no obvious TATA motif upstream, suggesting that *cdc37*$^{+}$ may only be expressed at moderate to low levels. The transcriptional initiation and termination consensus sequences in *S.pombe* are also not highly conserved and no obvious putative sequences exist.

With the start and stop codons predicted, it was clear that there were introns present at the 5' end, therefore the sequence would need to be analysed further for the presence of splice donor, acceptor and branch sites.

3.3.2 Prediction of exon-intron structure

The presence of introns is relatively common in *S.pombe* genes and tend to be characteristically short (Zhang and Marr, 1994). *S.pombe* introns, as with *S.cerevisiae*, tend to be located towards the 5' end of the gene (Fink, 1987) and consensus sequences for branch and splice sites have been determined (Zhang and Marr, 1994, Chen and Zhang, 1998). Using these consensus sequences and by lining up the genomic sequence with the *S.cerevisiae* Cdc37p sequence, 2 introns were predicted to be present at the 5' end of the gene. From analysing the genomic

sequence alone, the predicted structure of the gene suggested that the ORF was 1404 bp long, consisting of 3 exons of 23 bp, 41 bp and 1340 bp and 2 introns of 60 bp and 368 bp in length (Fig. 3.2). After this extensive sequence analysis was carried out, the predictions had to be tested by sequence analysis of cDNAs containing the *cdc37*$^{+}$ ORF.

3.4 PCR amplification of cDNAs containing the *cdc37*$^{+}$ ORF

Since the accuracy of the sequence was obviously important in the following PCR amplification, *Taq* polymerase was considered not to yield high enough fidelity. However, high fidelity polymerases, such as *Pwo*, usually possess an integral 3'→ 5' proof-reading exonuclease activity which result in PCR products that are blunt-ended and thus difficult to clone. For this project a system was used, called Expand™ High Fidelity PCR system, which reaches a compromise between fidelity and ease of cloning. It is composed of a mix containing *Taq* and *Pwo* DNA polymerases, designed to give higher fidelity PCR products that resemble a mixture of 3' single A overhang products similar to those generated by *Taq* DNA polymerase alone and blunt-ended products. This meant that the higher-fidelity PCR products could still be sub-cloned easily into the pGEM-T vector.

The two cDNA libraries previously described (section 3.2.2) were used as templates for PCR amplification. However, amplification was only consistently achieved with one of them, in the library vector pDB20 containing the *ADH1* promoter for expression. This was an oligo d(T)-primed library which was non-directionally cloned into the vector. Oligos were designed that were complementary to a region of either the promoter (5' forward - TG1) or terminator (3' reverse – TG2) sequences of the library vector.

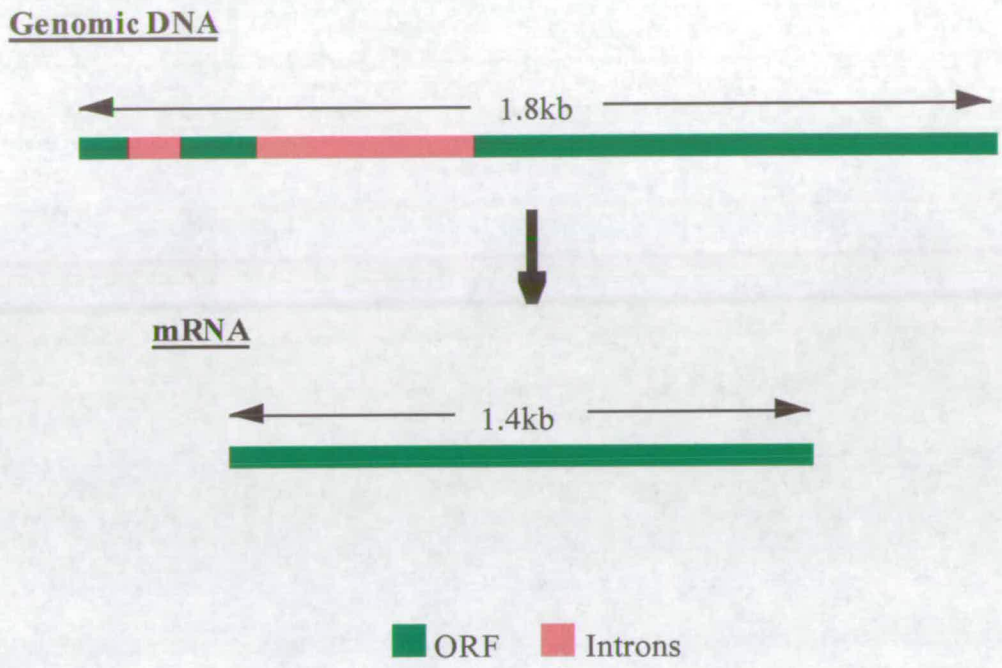

Fig 3.2 Original prediction of the genomic structure of the *cdc37*$^+$ gene in *S.pombe* by analysis of the genomic sequence

The genomic structure of the *cdc37*$^+$ gene was predicted by sequence analysis of a genomic clone and alignment with the *S.cerevisiae* Cdc37p sequence. Shown here is the predicted structure of the *cdc37*$^+$ gene (not to scale). The gene contains 2 introns of 60 bp and 368 bp in length. The 1404 bp ORF is made up of 3 exons of 23 bp, 41 bp and 1340 bp.

3.4.1 Analysis of the structure of the 5' end of the gene

Initially, in order to test the prediction of the structure of the 5'end of the *cdc37*$^+$ gene and to identify any 5' UTR, a reverse primer (PW3) was designed using the sequence at the 5' end of the final exon, together with both forward (TG1) and reverse (TG2) primers complimentary to the *ADH1* promoter vector sequence. The DNA was amplified from the library by PCR (section 2.2.8) and the reactions were run on a 1% agarose gel (section 2.2.3). The reactions with primer PW3 and primer TG1 resulted in a band of ~350 bp being amplified from the library. However, no amplification was seen with PW3 and the primer TG2. The band was excised from the gel and purified (section 2.2.4), before being cloned directly into pGEM-T. Once cloned into the vector the insert was sequenced using the T7 primer against the sequence of the vector adjacent to the polylinker site.

The sequencing results revealed that although our prediction was almost accurate, we had failed to notice another extremely small exon of only 11 bp (Fig 3.1). This changed the structure of the gene slightly, resulting in a sequence with stronger consensus sequences for splicing and which lined up more readily with the *S.cerevisiae* Cdc37p sequence. The confirmed ORF from the cDNA is 1401 bp, consisting of 4 exons of 23 bp, 27 bp, 11 bp and 1340 bp and 3 introns of 60 bp, 329 bp and 42 bp in length (Fig 3.3). As has been observed in the large majority of *S.pombe* genes examined, the longest region of the *cdc37*$^+$ ORF is present in the final exon (Chen and Zhang, 1998). The splice donor, acceptor and branch sequences for the introns are shown in Table 3.1.

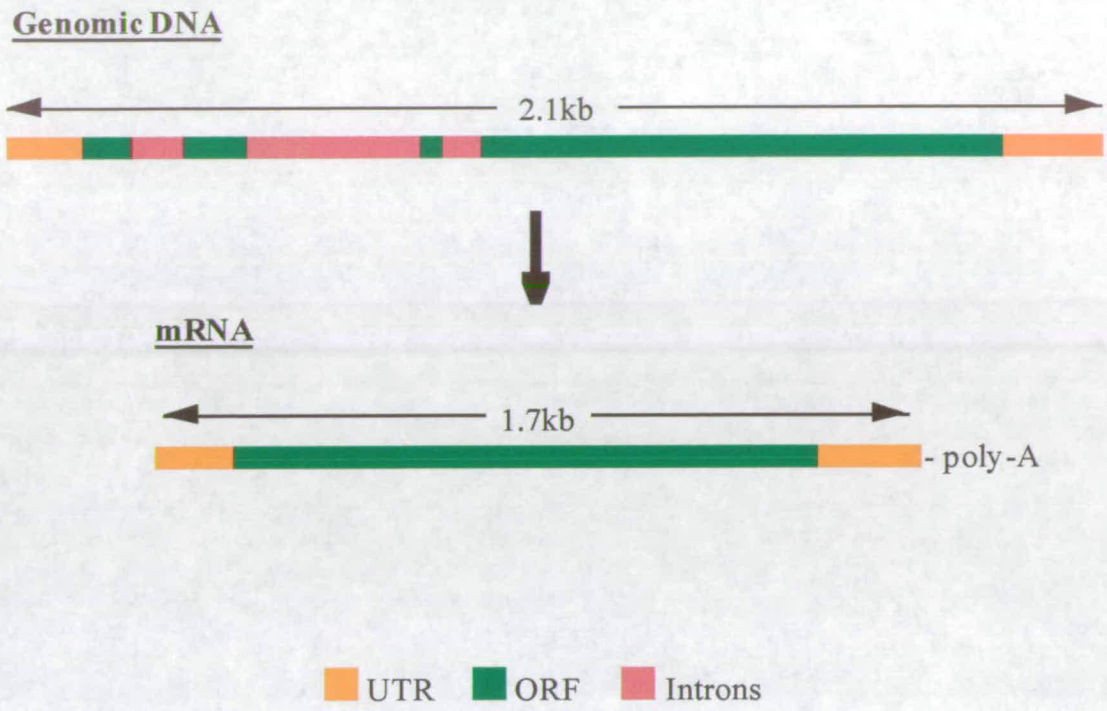

Fig 3.3 Genomic structure of the *cdc37*$^{+}$ gene in *S.pombe*

The genomic structure of the *cdc37*$^{+}$ gene was predicted by sequence analysis of a genomic clone and confirmed by sequence analysis of cDNAs amplified from a cDNA library. Shown here is the confirmed structure of the *cdc37*$^{+}$ gene (not to scale). The gene contains 3 introns of 60 bp, 329 bp and 42 bp in length. The 1401 bp ORF is made up of 4 exons of 23 bp, 27 bp, 11 bp and 1340 bp. Also shown in the diagram is the 78 bp of 5' UTR and all of the 114 bp of 3' UTR established by sequence analysis of clones amplified from a cDNA library.

Table 3.1 Sequence of the predicted splice donor, branch and acceptor sites within the putative *S.pombe cdc37*$^{+}$ sequence

	Donor site	Branch/acceptor site
Intron 1	**GT**AAGT	TTA**A**C..(8 bp)..C**AG**
Intron 2	**GT**ATGA	CTA**A**C..(6 bp)..T**AG**
Intron 3	**GT**TTGT	TTA**A**C..(6 bp)..C**AG**

Bases shown in bold and underlined are those that are present in all *S.pombe* genes to date. Those shown underlined are those which are present in over 70% of genes to date (Zhang and Marr, 1994).

3.4.2 Elucidating the 5' and 3' UTR sequences

As well as confirming the intron-exon structure of the 5' end of the gene, the ~340 bp PCR product generated in section 3.4.1 also contained a region of 5' UTR upstream of the predicted translation start codon. Sequencing showed that this clone appeared to contain 78 bp of 5' UTR (Fig. 3.4). Without being able to confirm that this is completely all of the 5' UTR, this is probably the majority of it, supported by the results of Northern analysis (section 4.3.3).

To discover if any 3' UTR was present and to confirm the structure of the remainder of the gene, a forward internal primer (PW4) was designed complimentary to the 5' end and PCR carried out (section 2.2.8) with the 3' reverse vector primer (TG2). This resulted in amplification of a ~1400 bp band which was subsequently cloned into pGEM-T and sequenced. The sequencing confirmed that no more introns were present and identified the predicted stop codon. It also shows that a cDNA containing all 114 bp of 3' UTR is present in the library, as the sequence extends to the poly(A)-tail (Fig 3.4).

3.4.3 Constructing a clone containing the entire ORF

In order to compare the cDNA sequence with the genomic sequence and to create a reagent for further investigations, clones containing the whole ORF were amplified by PCR from an *S.pombe* cDNA library constructed in the *S.cerevisiae* multicopy vector pDB20 (Fikes *et al.*, 1990). To carry this out, two further primers were designed complimentary to the *cdc37*$^{+}$ sequence; one at each terminus of the ORF, containing engineered recognition sites for the restriction enzymes *Nde*I (5' end - PW5) and *Sma*I (3' end - PW6) for cloning the ORF into the pREP series of *S.pombe* expression vectors (section 4.3.1). Six independent PCR products were cloned into

```
gtcaaatcgacgagaatagcaaagacatttgtgtcttttacttttttaaatttcttgaa   -18

attataatttgcttcgagATGGCAATTGATTACAGCAAGTGGGATAAGCTAGAACTTAGT    42
                    M  A  I  D  Y  S  K  W  D  K  L  E  L  S     14

GATGACAGTGACATTGAAGTTCATCCAAATGTCGATAAAAAGTCTTTTATTAGATGGCGC   102
 D  D  S  D  I  E  V  H  P  N  V  D  K  K  S  F  I  R  W  R     34

CAACGTGATATCCACGAAAAACGGGCTGTTAGAAAGCAAAAAATGGAAGATATCAAAGGT   162
 Q  R  D  I  H  E  K  R  A  V  R  K  Q  K  M  E  D  I  K  G     54

GCAATGGCGATGAATCGTAGACTCTTGTCTCGCATCTCCGAAATGGAGACTGTTCTTGAG   222
 A  M  A  M  N  R  R  L  L  S  R  I  S  E  M  E  T  V  L  E     74
          BamHI
AAGGAATCTCCTTCGGATCCTTATGTTTTATTGGGTTCATTTTTAGAGGCAAAAAAGTCT   282
 K  E  S  P  S  D  P  Y  V  L  L  G  S  F  L  E  A  K  K  S     94
                                        NdeIa
GAAGATATGGATAGTGCCATTCCTGGAGGCATGTCGTATCATCATATGCTTATGTCTTTA   342
 E  D  M  D  S  A  I  P  G  G  M  S  Y  H  H  M  L  M  S  L    114

TTAAAAGTAATTAAAGATGCCGAAGACACTACCGAAGAAAAATCTATGGATGATTCGGAT   402
 L  K  V  I  K  D  A  E  D  T  T  E  E  K  S  M  D  D  S  D    134

AAATGCTTGAGACGATTAAAGTCACACAAAGAGCGTCTTCTGAAGTTGCTAGAAGATGCA   462
 K  C  L  R  R  L  K  S  H  K  E  R  L  L  K  L  L  E  D  A    154

CAAAAGGAGTATGATACTTTAGAGGCTGAGAGTAAAAATTACATCACTTCTGAAGACTTG   522
 Q  K  E  Y  D  T  L  E  A  E  S  K  N  Y  I  T  S  E  D  L    174

CATTTGGGCTTTGATTCCACCTATGTTCAGAAAAAGGAGCCTGAAAAACCTAAAAAGACT   582
 H  L  G  F  D  S  T  Y  V  Q  K  K  E  P  E  K  P  K  K  T    194

AAAACCAAAAAGGAGACCATCCAGGTCATTGAATCTCTTAATAACCCTACACCTCCTACT   642
 K  T  K  K  E  T  I  Q  V  I  E  S  L  N  N  P  T  P  P  T    214

GATTTTCCCGGCGCTAAAGAGCAAGCTTCTACTGGTAATGCCCCTAAAAATCCTGTCAAT   702
 D  F  P  G  A  K  E  Q  A  S  T  G  N  A  P  K  N  P  V  N    234

GAGAATGAGTCGGAAGATGAAGAAGGGTTGTCTCTTTCTGAAGATGGTAAGAAGTTTGCC   762
 E  N  E  S  E  D  E  E  G  L  S  L  S  E  D  G  K  K  F  A    254

AATATTGATTTTGGTGATTATTCTTCATCGGAAGAGTTTTTGAAAGAACATTTAAATATC   822
 N  I  D  F  G  D  Y  S  S  S  E  E  F  L  K  E  H  L  N  I    274

TTGGCAGATGAGGAAGAATCTGATGCTATTCTTTTGGAAGCCTTTAATGCTGAACTAGAA   882
 L  A  D  E  E  E  S  D  A  I  L  L  E  A  F  N  A  E  L  E    294

GGGAAGCCGTCCTTAGCAAAACAATATGTTCATCAAGCCCTTTTGATCAGCTATTGCAGA   942
 G  K  P  S  L  A  K  Q  Y  V  H  Q  A  L  L  I  S  Y  C  R    314

CAGCTTGGACCAAATGGTTTATCAATATTTTTTCAAAAAATTAAAGACCCAAATCACCAA  1002
 Q  L  G  P  N  G  L  S  I  F  F  Q  K  I  K  D  P  N  H  Q    334
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                                    NdeIb
TCTCAGCGGCTATTTTTAGAGGATGTTCATAATACATATGTCCGAATCCATGAACGTTCT  1062
 S  Q  R  L  F  L  E  D  V  H  N  T  Y  V  R  I  H  E  R  S    354

GCTGCCATCAGCAAAGAACAAGCTGAAAGCGGTGAAGGAGTAGAACAAATCCAGCTCTGT  1122
 A  A  I  S  K  E  Q  A  E  S  G  E  G  V  E  Q  I  Q  L  C    374

GCAGTTGATCCTAATACGAAACTTTCCATCACCATCCCGGAGGCAGGTTCTACTGATCCA  1182
 A  V  D  P  N  T  K  L  S  I  T  I  P  E  A  G  S  T  D  P    394

GAGACACAAAAAGCTCGTGCAGCTTTTGAGTCTTTTCCTCCTAACCTCCAAAAAGCTCTT  1242
 E  T  Q  K  A  R  A  A  F  E  S  F  P  P  N  L  Q  K  A  L    414

ATGACCAATGATTTAGACAAGATTAATGTAGTTCTTGGAAAAATGGCAGTTGAAAATGCA  1302
 M  T  N  D  L  D  K  I  N  V  V  L  G  K  M  A  V  E  N  A    434

GAAGAAGTTGTGGAAAAGCTCAGCAGTACTGGTATGTTGAGTATCGAAGAAGGAATCATC  1362
 E  E  V  V  E  K  L  S  S  T  G  M  L  S  I  E  E  G  I  I    454

GACACAACGAAAGGTGAGACTATTCCTCAATTGTCTTAAattttctgcttacgggtgtg  1422
 D  T  T  K  G  E  T  I  P  Q  L  S  •                        466

Cttttagtaagcgaaaacaccaaagttacatgtttgccgttggcatttttaaatggataa  1482


acaacaatgaaattttatttattgatttaaaaaaaaaaaaaaaaa                 1527
```

Fig 3.4 The predicted nucleotide and deduced amino acid sequence of the *cdc37*$^+$ gene in *S.pombe*

Shown here is the predicted sequence of *cdc37*$^+$ mRNA sequenced by amplification from a cDNA library. All of the 3' UTR is included but the existence of additional 5' UTR within *cdc37*$^+$ mRNA cannot be ruled out. The DNA sequence is shown in grey, the predicted 'start' and 'stop' translation sites in red and the deduced amino acid sequence in black. The 1401 bp predicted ORF is shown in upper case and the UTR in lower case.

pGEM-T and sequenced. All 6 clones had PCR-induced mutations somewhere in their sequences but by comparison to each other, a consensus sequence was constructed which agreed exactly with my genomic sequence. The single base pair difference with the database sequence was communicated to the Sanger Centre and subsequently corrected.

The pREP vectors that were to be used for expression in *S.pombe* all required a 5' terminal *Nde*I site, which was introduced into the ORF by PCR. However, the *cdc37*$^+$ ORF contains two internal *Nde*I sites at positions 326 (labelled *a*) and 1038 (labelled *b*) of the ORF. This meant that a two-step cloning strategy was necessary to insert the full-length ORF into the expression vectors (Fig 3.5). The presence of an internal *Bam*HI site at position 237 of the ORF gave a convenient method for piecing together clones which between them contained an error-free full-length ORF.

One of the clones containing the full-length ORF generated by the PCR just described, contained correct sequence from the internal *Nde*I sites out to either termini, but contained a PCR-induced mutation in the middle region between the two *Nde*I sites. The *Bam*HI-*Sma*I region of this clone was inserted into pBluescript SK-. The middle *Nde*I fragment was then replaced by the corresponding fragment (which contains no introns) from the genomic cosmid, resulting in an error-free region of the 3' end of the *cdc37*$^+$ ORF from the *Bam*HI site to the engineered *Sma*I site at the extreme C-terminus. This construct was used for further cloning described in other sections (Appendix 1B). A second PCR product containing the 5' end of the ORF from the engineered *Nde*I site at the extreme N-terminus to the internal *Bam*HI site was also cloned into pGEM-T. This construct with the *Nde*I-*Bam*HI 5' end was also used for further cloning (Appendix 1C). Sequential cloning of these two fragments allowed the full-length *cdc37*$^+$ ORF to be cloned into the *Nde*I-*Sma*I sites of the expression vectors.

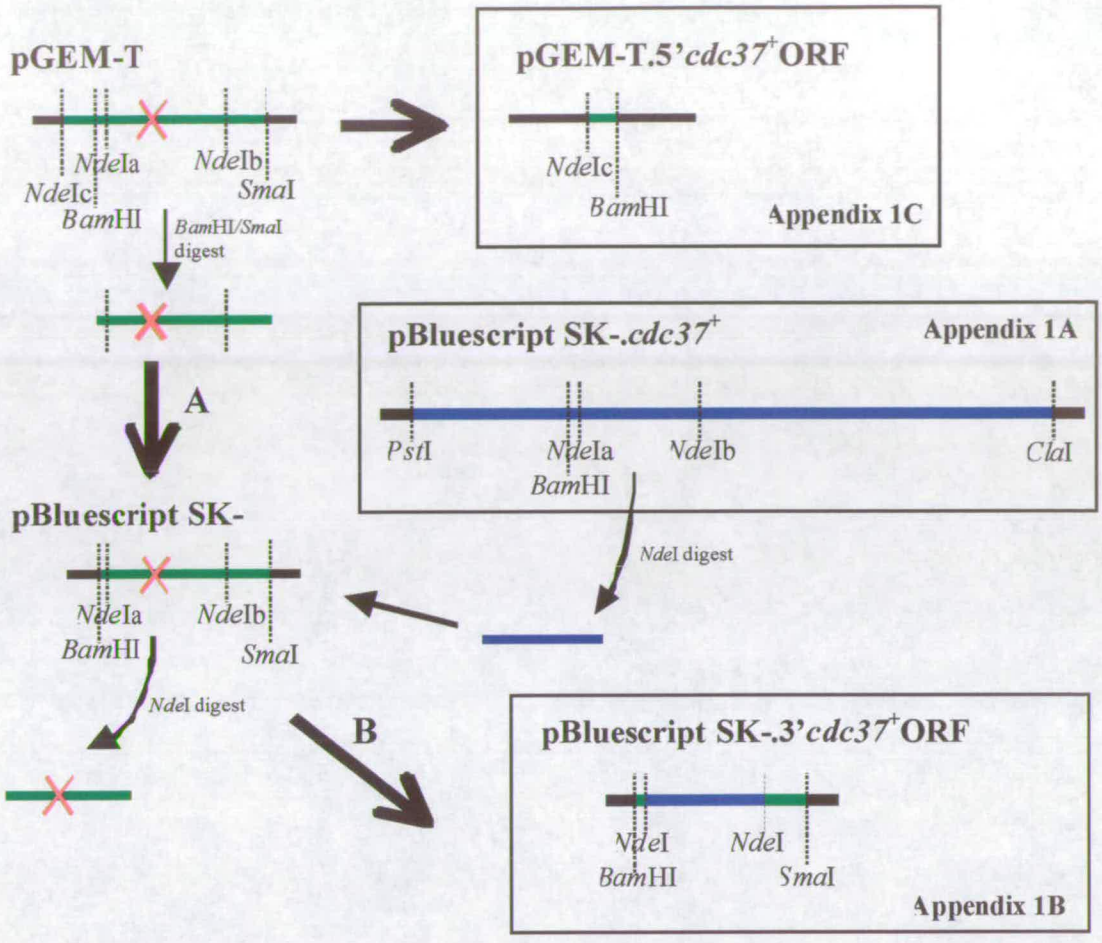


Fig. 3.5 Strategy for cloning the full-length *cdc37*$^{+}$ ORF in *S.pombe*

Two clones were assembled that between them contained the full-length ORF of *cdc37*$^{+}$. A 1417 bp PCR product (using primers PW5 and PW6) was cloned into pGEM-T containing the full-length ORF. This clone had no errors between the engineered *Nde*Ic site and the *Bam*HI site or between the *Nde*Ib site and the *Sma*I site, but a PCR-induced error between the *Nde*Ia and *Nde*Ib sites (represented by a red cross). **A.** The 1167 bp *Bam*HI-*Sma*I fragment was then cloned into pBluescript SK-. **B.** The fragment in pBluescript SK- was digested with *Nde*I, removing the 712 bp region which was replaced with the corresponding fragment from a genomic clone (Fig 3.1, Appendix 1A), thus removing the PCR-induced mutation. This resulted in a construct with the 1167 bp error-free *Bam*HI-*Sma*I 3' end ready for further cloning (Appendix 1B). Also, a 253 bp PCR product (using primers PW3 and PW5) containing the 5' end of the ORF from the engineered *Nde*Ic site to the internal *Bam*HI site was cloned into pGEM-T. This resulted in a construct with the 238 bp *Nde*I-*Bam*HI 5' end ready for further cloning (Appendix 1C). These two constructs (B and C) were used for sequentially cloning the ORF into various vectors described in later chapters. Amplified DNA is shown in green, genomic DNA is shown in blue and vector sequence in black.

3.5 Predicted amino acid sequence of *cdc37*$^+$

Translation of the ORF of *cdc37*$^+$ is predicted to encode a polypeptide of 52 kDa containing 466 amino acids (Fig. 3.4). Although the similarity between *S.pombe* Cdc37 and *S.cerevisiae* Cdc37p is only moderate overall (34% identical), the two proteins are ~85% identical over the first 40 amino acids and conserved motifs exist in several regions throughout the length of the proteins (Fig. 3.6). In fact, homologues previously identified from other species also have only moderate similarity with the Cdc37p sequence overall but high identity at the N-terminus (Fig. 3.7). However, despite this, the *Drosophila cdc37*$^+$ gene rescues the temperature-sensitive defect of the *S.cerevisiae* mutant allele *cdc37-1* (Cutforth and Rubin, 1994).

3.6 Summary

A homologue of the *S.cerevisiae* gene *CDC37* has been identified in *S.pombe*. The gene contains 3 introns, a predicted ORF of 1401bp and has been named *cdc37*$^+$. The *S.pombe* Cdc37 protein has a predicted molecular weight of ~52 kDa, similar to that of the Cdc37 proteins found in other species. All of the Cdc37 protein sequences identified from a variety of species, including the *S.pombe* protein identified here, show high conservation at the N-terminus but only moderate sequence similarity overall. The *S.pombe* Cdc37 sequence is most similar to the *S.cerevisiae* Cdc37p sequence. The sequences of the Cdc37 proteins identified so far contain no consensus motifs that give any clue as to their function.

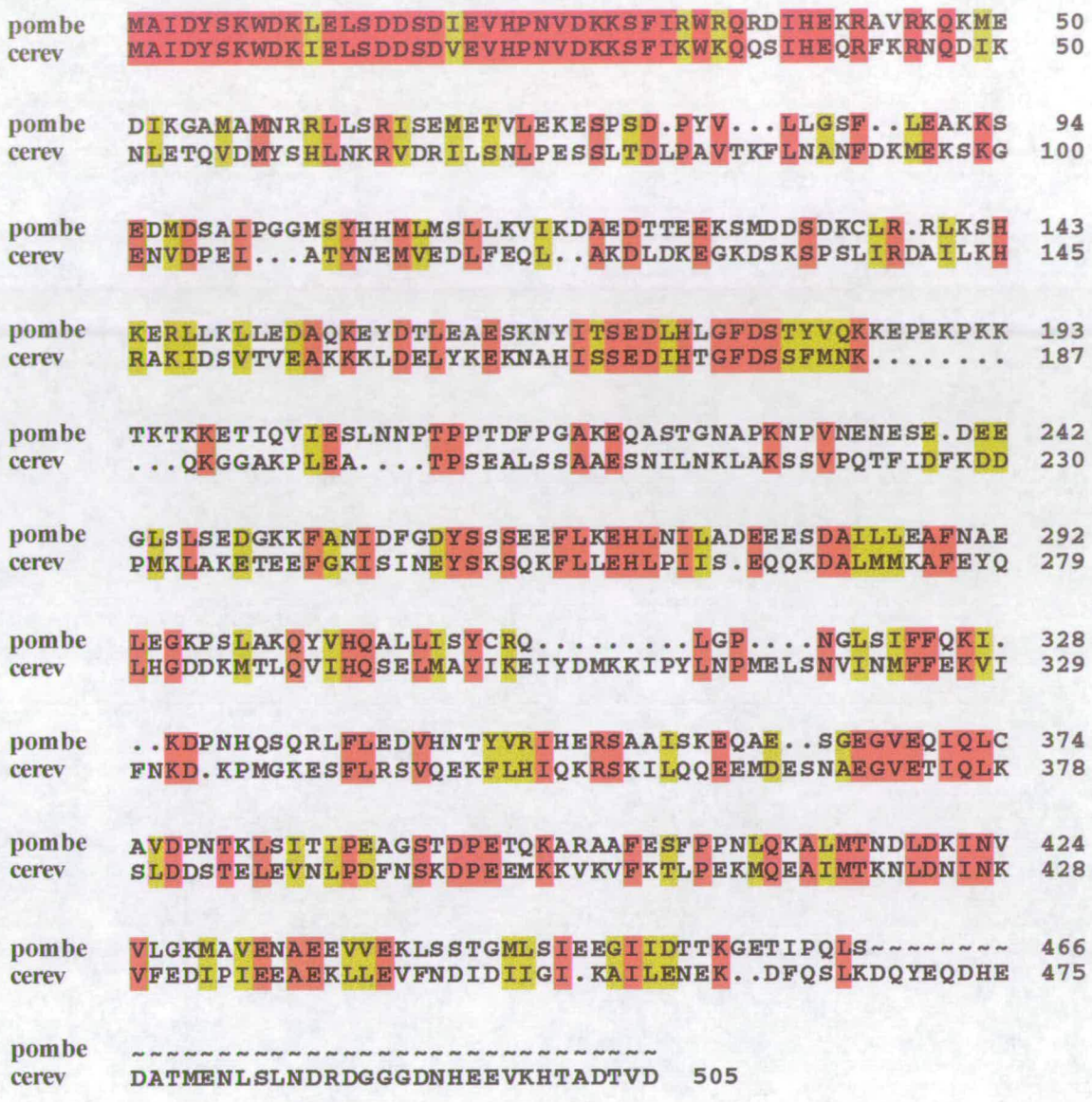
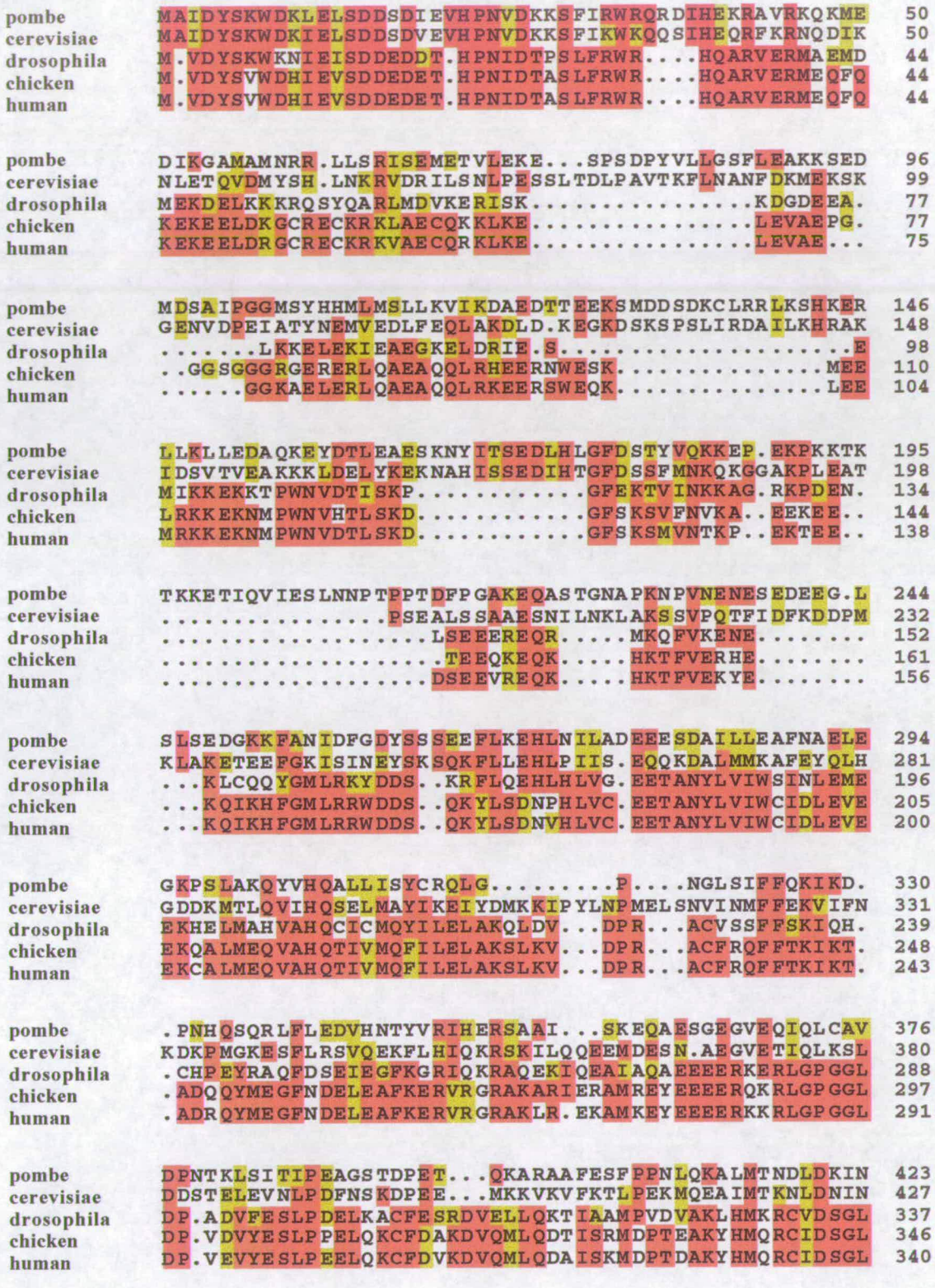


Fig. 3.6 Sequence comparison of *S.cerevisiae* Cdc37p and *S.pombe* homologue

Shown is an optimal alignment of *S. cerevisiae* Cdc37 (from Gerber *et al.*, 1995) with predicted amino acid sequence of *S. pombe* homologue. Residues identical are shaded in red and those similar are shaded in yellow. Alignment was performed using the default settings in the *gcg pileup* program.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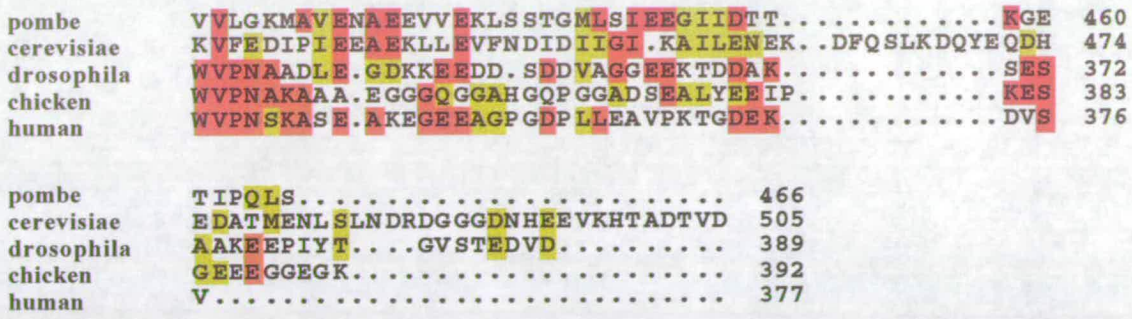
cerevisiae MAIDYSKWDKIELSDDSDVEVHPNVDKKSFIKWKQQSIHEQRFKRNQDIK 50
drosophila M.VDYSKWKNIEISDDEDDT.HPNIDTPSLFRWR....HQARVERMAEMD 44
chicken M.VDYSVWDHIEVSDDEDET.HPNIDTASLFRWR....HQARVERMEQFQ 44
human M.VDYSVWDHIEVSDDEDET.HPNIDTASLFRWR....HQARVERMEQFQ 44
pombe DIKGAMAMNRR.LLSRISEMETVLEKE...SPSDPYVLLGSFLEAKKSED 96
cerevisiae NLETQVDMYSH.LNKRVDRILSNLPESSLTDLPAVTKFLNANFDKMEKSK 99
drosophila MEKDELKKKRQSYQARLMDVKERISK.....KDGDEEA. 77
chicken KEKEELDKGCRECKRKLAECQKKLKE.....LEVAEPG. 77
human KEKEELDRGCRECKRKVAECQRKLKE.....LEVAE... 75
pombe MDSAIPGGMSYHHMLMSLLKVIKDAEDTTEEKSMDDSDKCLRRLKSHKER 146
cerevisiae GENVDPEIATYNEMVEDLFEQLAKDLD.KEGKDSKSPSLIRDAILKHRAK 148
drosophila .....LKKELEKIEAEGKELDRIE.S.....E 98
chicken ..GGSGGGRGERERLQAEAQQLRHEERNWESK.....MEE 110
human .....GGKAELERLQAEAQQLRKEERSWEQK.....LEE 104
pombe LLKLLEDAQKEYDTLEAESKNYITSEDLHLGFDSTYVQKKEP.EKPKKTK 195
cerevisiae IDSVTVEAKKKLDELYKEKNAHISSEDIHTGFDSSFMNKQKGGAKPLEAT 198
drosophila MIKKEKKTPWNVDTISKP.....GFEKTVINKKAG.RKPDEN. 134
chicken LRKKEKNMPWNVHTLSKD.....GFSKSVFNVKA..EEKEE.. 144
human MRKKEKNMPWNVDTLSKD.....GFSKSMVNTKP..EKTEE.. 138
pombe TKKETIQVIESLNNPTPPTDFPGAKEQASTGNAPKNPVNENESEDEEG.L 244
cerevisiae .....PSEALSSAAESNILNKLAKSSVPQTFIDFKDDPM 232
drosophila .....LSEEEREQR.....MKQFVKENE..... 152
chicken .....TEEQKEQK.....HKTFVERHE..... 161
human .....DSEEVREQK.....HKTFVEKYE..... 156
pombe SLSEDGKKFANIDFGDYSSSEEFLKEHLNILADEEESDAILLEAFNAELE 294
cerevisiae KLAKETEEFGKISINEYSKSQKFLLEHLPIIS.EQQKDALMMKAFEYQLH 281
drosophila ...KLCQQYGMLRKYDDS..KRFLQEHLHLVG.EETANYLVIWSINLEME 196
chicken ...KQIKHFGMLRRWDDS..QKYLSDNPHLVC.EETANYLVIWCIDLEVE 205
human ...KQIKHFGMLRRWDDS..QKYLSDNVHLVC.EETANYLVIWCIDLEVE 200
pombe GKPSLAKQYVHQALLISYCRQLG.....P....NGLSIFFQKIKD. 330
cerevisiae GDDKMTLQVIHQSELMAYIKEIYDMKKIPYLNPMELSNVINMFFEKVIFN 331
drosophila EKHELMAHVAHQCICMQYILELAKQLDV...DPR...ACVSSFFSKIQH. 239
chicken EKQALMEQVAHQTIVMQFILELAKSLKV...DPR...ACFRQFFTKIKT. 248
human EKCALMEQVAHQTIVMQFILELAKSLKV...DPR...ACFRQFFTKIKT. 243
pombe .PNHQSQRLFLEDVHNTYVRIHERSAAI...SKEQAESGEGVEQIQLCAV 376
cerevisiae KDKPMGKESFLRSVQEKFLHIQKRSKILQQEEMDESN.AEGVETIQLKSL 380
drosophila .CHPEYRAQFDSEIEGFKGRIQKRAQEKIQEAIAQAEEEERKERLGPGGL 288
chicken .ADQQYMEGFNDELEAFKERVRGRAKARIERAMREYEEEERQKRLGPGGL 297
human .ADRQYMEGFNDELEAFKERVRGRAKLR.EKAMKEYEEEERKKRLGPGGL 291
pombe DPNTKLSITIPEAGSTDPET...QKARAAFESFPPNLQKALMTNDLDKIN 423
cerevisiae DDSTELEVNLPDFNSKDPEE...MKKVKVFKTLPEKMQEAIMTKNLDNIN 427
drosophila DP.ADVFESLPDELKACFESRDVELLQKTIAAMPVDVAKLHMKRCVDSGL 337
chicken DP.VDVYESLPPELQKCFDAKDVQMLQDTISRMDPTEAKYHMQRCIDSGL 346
human DP.VEVYESLPEELQKCFDVKDVQMLQDAISKMDPTDAKYHMQRCIDSGL 340



% identity	pombe	cerevisiae	drosophila	chicken	human
pombe	-	34.7	23.9	25.3	24.6
cerevisiae	-	-	24.8	24.4	24.6
drosophila	-	-	-	51.9	50.8
chicken	-	-	-	-	84.1
human	-	-	-	-	-

Fig. 3.7 Sequence comparison of *S.cerevisiae* Cdc37p, a *S.pombe* homologue and homologues from other species

Shown is an optimal alignment of *S.cerevisiae* Cdc37p (Ferguson *et al.*, 1986; Gerber *et al.*, 1995) with predicted amino acid sequence of the *S.pombe* homologue identified in this project, together with homologues from *D.melanogaster* (Cutforth and Rubin, 1994), chicken (Huang *et al.*, 1998) and human (Stepanova *et al.*, 1996). Residues identical are shaded in red and those similar are shaded in yellow. Alignment was performed using the default settings in the *gcg pileup* program. Also included is a table showing the percentage amino acid identity between each homologue using the *Jotun Hein* default settings of the *MegAlign* program in *DNAStar*.

Chapter 4

Construction and analysis of a *cdc37* deletion mutant

4.1 Introduction

The classical route to investigating the function of a novel gene in yeast is to disrupt or delete the gene of interest at the chromosomal locus. These two techniques are very similar and their aim is to eliminate the function of the gene that is targeted. In a disruption experiment, a selectable genetic marker is inserted into the target gene using a convenient restriction site; in a deletion experiment, part or all of the target gene is replaced with the genetic marker. It is preferential to use a deletion strategy where possible, because removal of the gene assures that a null mutant is generated, whereas disruption does not always do so. Deletion or disruption strategies have been widely used to create null mutants of many yeast genes.

For this project, a *cdc37*$^{+}$ null mutant was created by deletion of the entire ORF using the one-step gene replacement method (Rothstein, 1983). The method relies upon a double recombination event taking place at the chromosomal locus of the gene of interest. The first step is to create a construct that contains a linear fragment containing the DNA normally flanking either side of the target gene and a selectable marker inserted in between. Next, the linear fragment is released from the plasmid vector, containing the selectable marker flanked by sufficient length of DNA sequence to allow homologous pairing with both sides of the chromosomal target sequence. Standard transformation techniques are then used to introduce the linear fragment into a suitable strain and transformants that show stable mitotic inheritance for the genetic marker are isolated and tested further for correct integration.

If the gene of interest is essential for cell growth or division, then loss of function will be lethal and no transformants will be isolated. To avoid this, the gene replacement is carried out in a diploid strain. The replacement results in a heterozygous diploid which may then be allowed to sporulate and the haploid progeny analysed. Where the gene of interest is essential for viability, the null mutant haploids created by

sporulation can be maintained by expression of the gene from an introduced plasmid and thus analysed further. For this study, we used a set of *S.pombe* expression vectors known as the pREP plasmids (Maundrell, 1990, Basi *et al.*, 1993), in which expression is regulatable by the addition (represses promoter) or omission (derepresses promoter) of thiamine from the medium.

4.2 Creation of a *cdc37* deletion mutant strain

4.2.1 Deletion of *cdc37*$^+$ at the chromosomal locus

Since the *cdc37*$^+$ ORF sequence had already been determined (Chapter 3), it was possible to construct a replacement experiment that would unambiguously create a null mutation of *cdc37*$^+$ by complete removal of the predicted ORF.

The deletion construct was created in the cloning vector pBluescript SK- (Fig 4.1). Eight hundred and twenty six bp of 5' and 804 bp of 3' flanking regions were amplified separately from the pBluescript.*cdc37*$^+$ plasmid by PCR and each was cloned into pGEM-T seperately. These regions were then cloned sequentially into pBluescript SK- using the appropriate restriction enzymes. Using a restriction site engineered by the PCR, the selectable marker, *ura4*$^+$, was inserted between these regions. The final construct contained a linearisable deletion fragment including two 827 bp regions of DNA normally either side of the *cdc37*$^+$ gene with the *ura4*$^+$ marker inserted between (Appendix 1D). The linear *cdc37*$^+$ deletion fragment could be released from the plasmid by digestion with the appropriate restriction enzymes.

Two haploid strains were used to construct a diploid of the genotype *ade6-M210/ade6-M216 ura4-D18/ura4-D18 leu1-32/leu1-32 h*$^+$*/h*$^-$ (section 2.5.3a,e). Approximately 2 μg of the gel-purified *cdc37*$^+$ deletion fragment was then used to

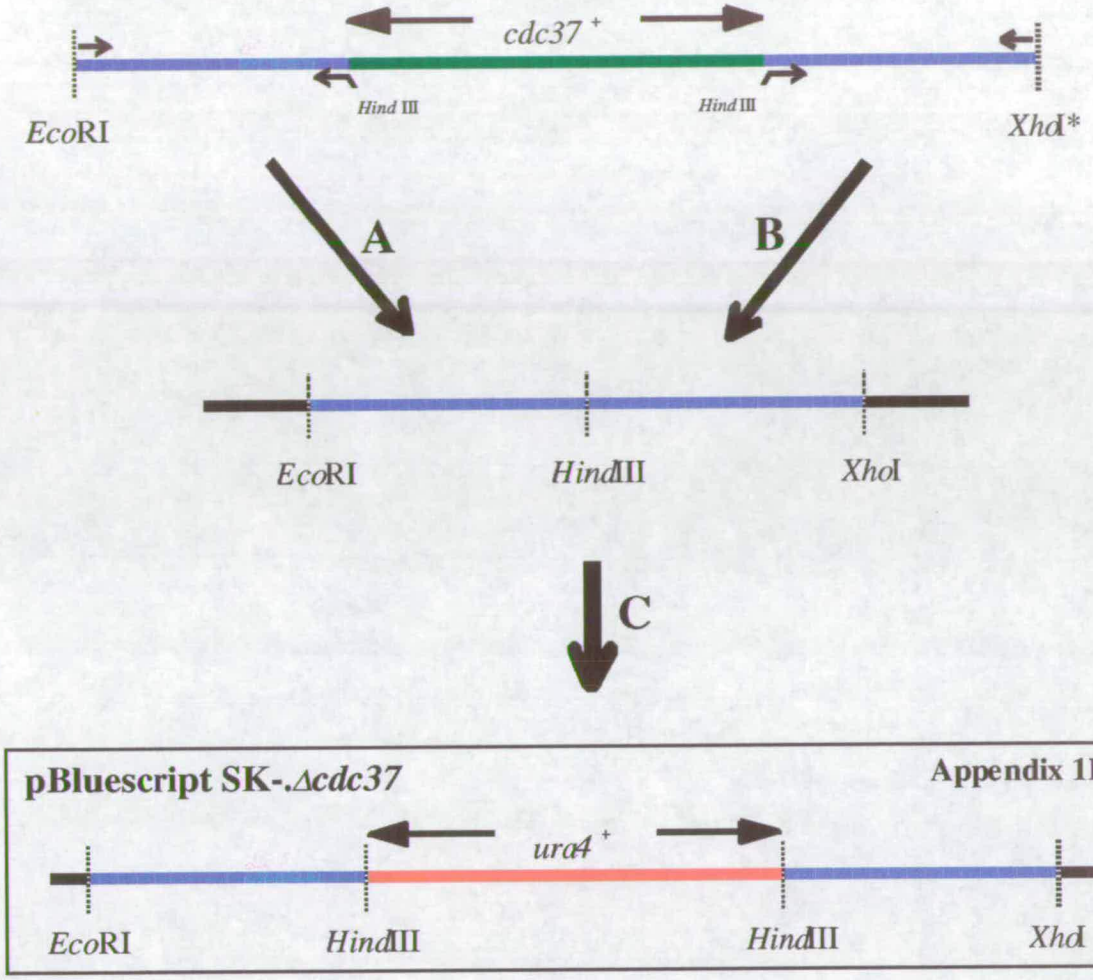


Fig 4.1 Constructing a fragment for *cdc37*$^{+}$ deletion in *S.pombe*

A 3388 bp *Eco*RI-*Xho*I linear *cdc37*$^{+}$ deletion fragment was constructed in pBluescript SK-. **A.** An 826 bp region of genomic DNA immediately upstream of the *cdc37*$^{+}$ gene was amplified by PCR using primers PW7 (containing an *EcoRI* site) and PW8 (containing an engineered *Hind*III site). The PCR product was cloned into pGEM-T, sequenced and then cloned into pBluescript SK- using *Eco*RI and *Hind*III. **B.** An 804 bp region of genomic DNA immediately downstream of the *cdc37*$^{+}$ gene was also amplified by PCR using primers PW9 (containing an an engineered *Hind*III site) and PW10 (containing a *Xho*I* site). This PCR product was also cloned separately into pGEM-T, sequenced and then cloned into pBluescript SK- (already containing 5' flanker) using *Hind*III and *Xho*I. **C.** Finally, a 1758 bp *Hind*III fragment, containing the *ura4*$^{+}$ gene, was digested from pBR322 and inserted into the engineered *Hind*III between the two flanking regions.

* 5 out of 6 bp of the *XhoI* recognition site were present in the genomic sequence of the 3' flanking region, therefore primer PW10 contained 1 engineered base to create a *Xho*I restriction site.

transform the diploid (section 2.5.5). Heterozygous diploid transformants were selected on minimal plates supplemented with leucine. These stable transformants needed to be tested further to ensure that homologous recombination had taken place as expected and indeed, a 2074 bp region of the *cdc37*$^+$ gene containing the entire ORF had been replaced by a 1758 bp fragment containing the selectable marker, *ura4*$^+$ (Fig 4.2).

4.2.2 Phenotype of *cdc37* deletion mutation

From the 32 diploid transformants tested, 9 showed stable mitotic inheritance of the *ura4*$^+$ marker (section 2.5.6) and were sporulation competent. Two of these transformants (1 and 25) were selected, allowed to sporulate and tetrad analysis carried out. Analysis of 20 tetrads from each transformant showed that only two viable spores were ever generated and in all cases the viable spores were unable to grow without uracil, suggesting that *cdc37*$^+$ is essential for viability in *S.pombe* and deletion of this gene results in a lethal phenotype. The inviable spores generated from meiosis in the diploids had varying phenotypes; mostly, the spores were able to divide 0-5 times (Fig 4.3). Some of these cells were elongated, indicating a cell cycle defect; some cells appeared as normal, whilst others were round and swollen, suggesting other biological roles for *cdc37*$^+$. However, a significant number of *Δcdc37* spores failed to germinate at all.

4.2.3 Confirmation of *ura4*$^+$ integration at the *cdc37*$^+$ chromosomal locus

Before further characterisation of the *cdc37*$^+$/*Δcdc37* strain took place, transformant 1 was tested by Southern analysis and PCR to show that the *ura4*$^+$ marker had integrated at the *cdc37*$^+$ locus as expected. Both the PCR and Southern blot analysis

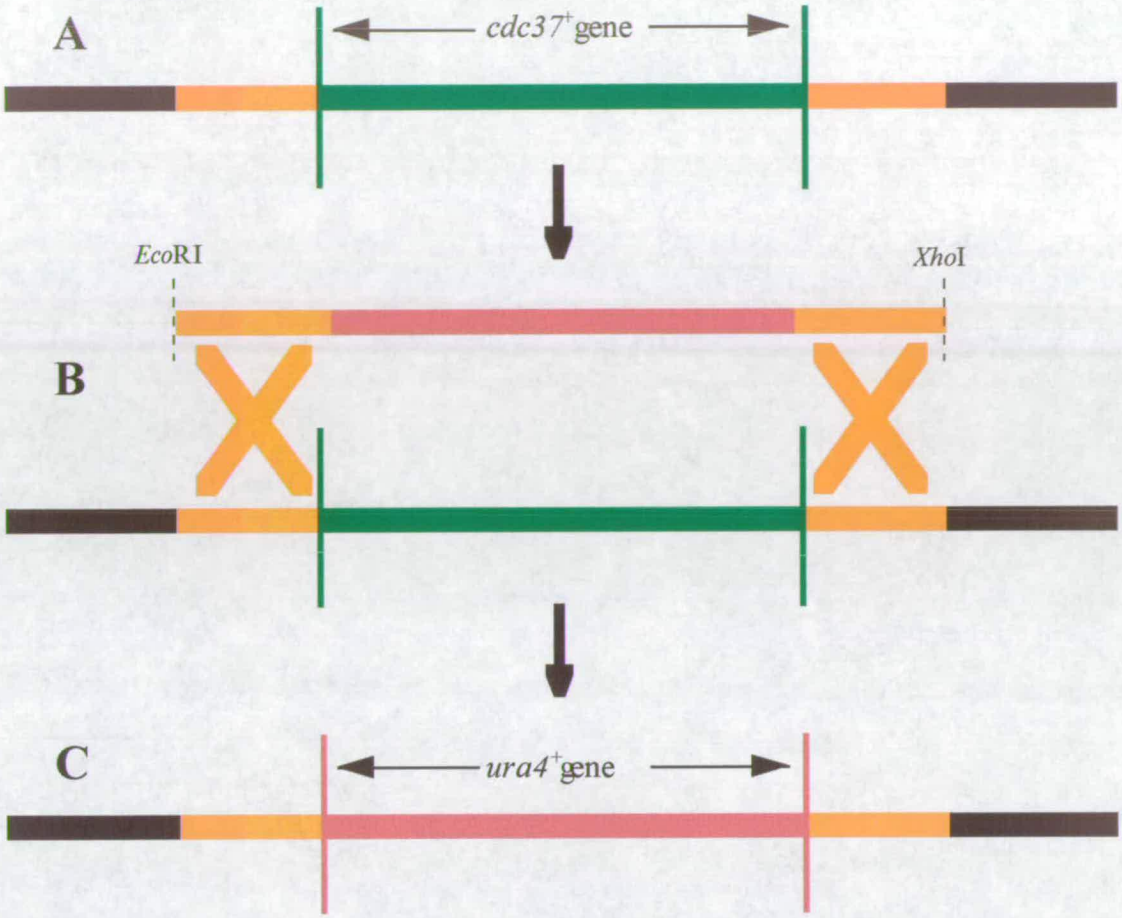
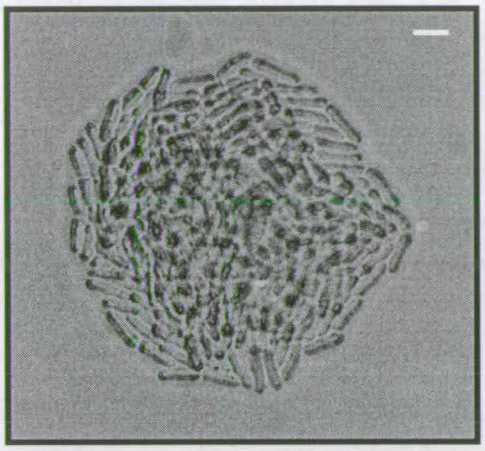


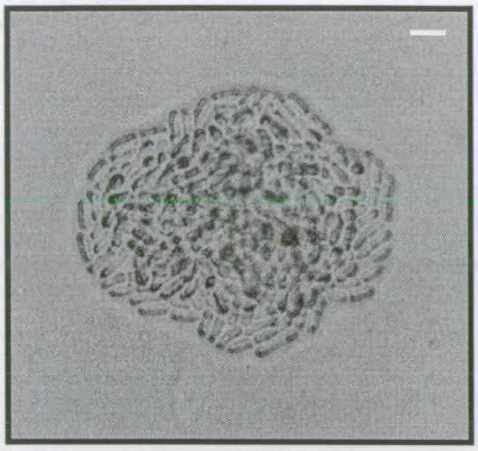
Fig 4.2 Strategy of one-step gene replacement for *cdc37*$^+$ deletion in *S.pombe*

The *cdc37*$^+$ gene was replaced in one chromosome in a *ura4-D18/ura4-D18* diploid by replacement with the *ura4*$^+$ gene using the one-step gene replacement method. **A.** Normal arrangement of *cdc37*$^+$ gene on chromosome 2 in *S.pombe*. **B.** The linear deletion fragment was released from plasmid pBluescript SK-.*cdc37*$^+$ (Appendix 1D) by digestion with *Eco*RI and *Xho*I. It was then transformed into a *ura-* diploid strain and integrated into one chromosome by homologous recombination. **C.** Final arrangement at *cdc37*$^+$ locus in one copy of chromosome 2 in transformed diploid strain. Transformants were selected for their ability to grow on minimal medium lacking uracil. Strains showing stable mitotic inheritance were analysed further by Southern blot.

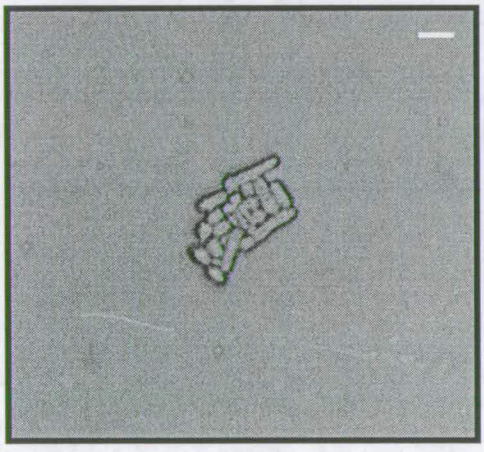
A. *cdc37*$^{+}$



B. *cdc37*$^{+}$



C. *Δcdc37*



D. *Δcdc37*

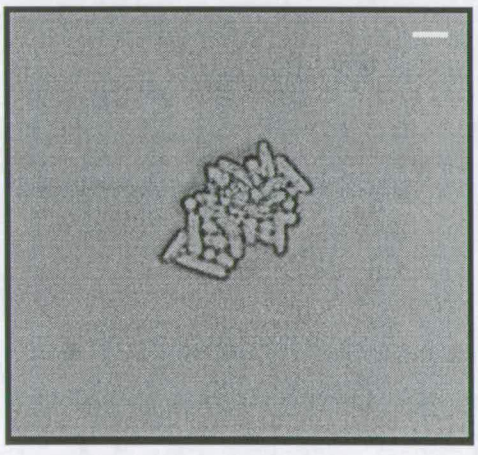


Fig 4.3 Phenotypes of progeny derived from *cdc37*$^{+}$/*Δcdc37* heterozygous diploid

A *cdc37*$^{+}$/*Δcdc37* heterozygous diploid was allowed to sporulate on ME medium at 20°C for 2 days. Tetrad analysis was then carried out and the progeny analysed. Shown is a typical set of cells grown at 30°C for 2 days from spores. Spores A and B are viable, containing the *cdc37*$^{+}$ gene and therefore do not carry the *ura4*$^{+}$ marker. Spores C and D are *Δcdc37* cells and are inviable, managing only between 0 and 5 divisions. The white bar in the top right hand corner represents 10 μm.

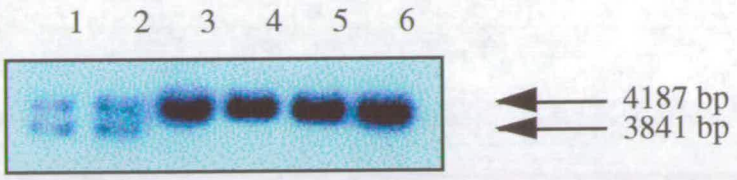
were carried out on the heterozygous diploid, the parent haploid strains and the viable progeny haploid cells. The chromosomal DNA was prepared from the strains after growing overnight in suitably supplemented minimal medium at 30°C (section 2.5.7). The Southern blot shows that bands corresponding to both the *cdc37*$^+$ locus and the *Δcdc37* locus are present in the *cdc37*$^+$/*Δcdc37* diploid, whilst a band corresponding only to the wild-type locus is visible in the parent haploids and viable progeny (Fig 4.4). The PCR confirms this observation; amplification is possible with both the *cdc37*$^+$-specific and *ura4*$^+$-specific primers in the *cdc37*$^+$/*Δcdc37* diploid, whilst only with the *cdc37*$^+$-specific primer in the parent haploids and viable progeny (Fig 4.5). These analyses confirmed that the *cdc37*$^+$ gene was deleted at one chromosomal locus in the diploid by a single integration event of the *ura4*$^+$ gene. Transformant 1 was used in all subsequent experiments.

4.3 Rescue of *cdc37* deletion lethality by regulatable expression of the *cdc37*$^+$ ORF

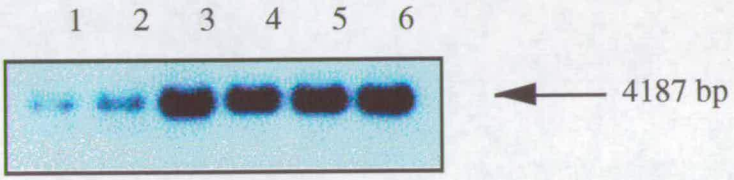
4.3.1 Cloning *cdc37*$^+$ into the pREP *S.pombe* expression vectors

Ectopic expression of *cdc37*$^+$ was carried out in the pREP1 vectors, which carry the *S.cerevisiae* marker *LEU2* and various strength versions of the thiamine-repressible *S.pombe nmt1* promoter. pREP1 carries the wild-type, full-strength *nmt1* promoter. By site-directed mutations in the TATA box, two lower strength versions of the *nmt1* promoter were generated. They are contained in pREP41 (mid-strength) and pREP81 (low-strength). For this project, these set of selectable plasmids allow regulatable expression of *cdc37*$^+$ at various levels. When thiamine is absent from the medium, the promoters are fully functional and *cdc37*$^+$ is expressed. When thiamine is added to the medium, expression of *cdc37*$^+$ is repressed.

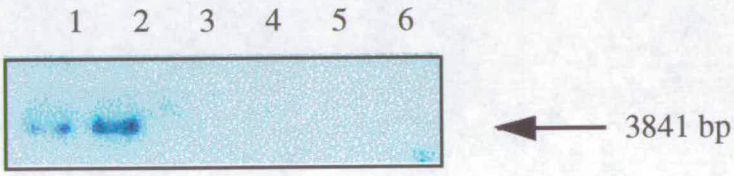
A. 5' flanking probe



B. *cdc37*$^{+}$ probe



C. *ura4*$^{+}$ probe



D. Probe location

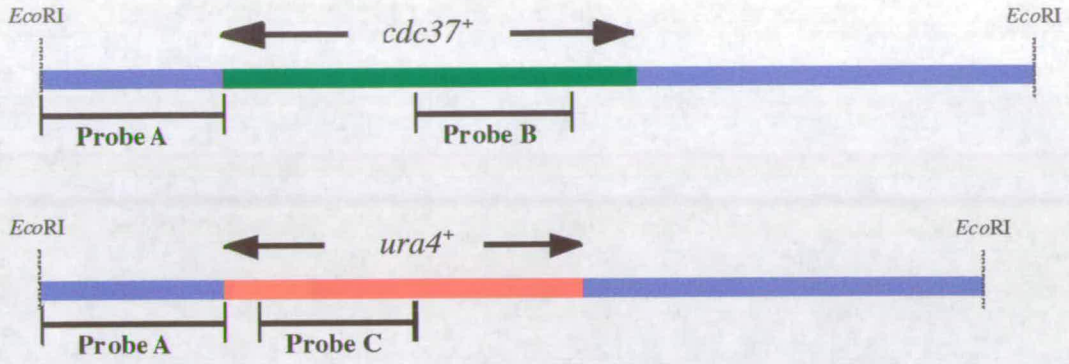
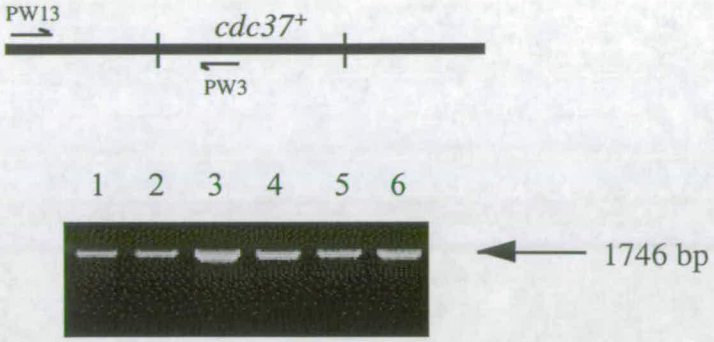


Fig 4.4 Southern analysis of *cdc37*$^{+}$/*Δcdc37* heterozygous diploid

Genomic DNA was extracted from a *cdc37*$^{+}$/*Δcdc37* heterozygous diploid, the parent haploids and the viable progeny derived from sporulation of the diploid. The genomic DNA was digested with *Eco*RI at 37°C for 8 hr before electrophoresis on a 1% agarose gel and Southern blotting on to a nylon filter. Panel A shows the filter hybridised with an 827 bp radiolabelled *Eco*RI-*Hind*III fragment of DNA normally flanking the 5' end of the *cdc37*$^{+}$ gene, panel B hybridised with a 712 bp radiolabelled *Nde*I-*Nde*I fragment of the *cdc37*$^{+}$ ORF and panel C hybridised with an 820 bp radiolabelled *Nsi*I fragment of the *ura4*$^{+}$ ORF. The 4187 bp band corresponds to the wild-type locus and the 3841 bp band to the deleted locus. Lanes 1 and 2 contain the digested DNA from two independent colonies of the diploid, lanes 3 and 4 the DNA of the two parent haploids and lanes 5 and 6 the DNA of the two viable spores derived from meiosis. Panel D shows the regions at which each of the three probes anneal.

A. *cdc37*$^+$ primer



B. *ura4*$^+$ primer

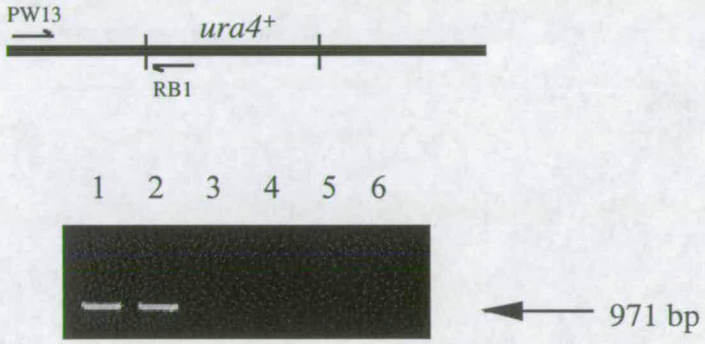


Fig 4.5 PCR analysis of *cdc37*$^+$/*Δcdc37* heterozygous diploid

Genomic DNA was extracted from *cdc37*$^+$/*Δcdc37* heterozygous diploid transformant 1, the parent haploids and the viable progeny derived from sporulation of the diploid. PCR was carried out using a forward primer against the sequence that flanks the *cdc37*$^+$ gene (primer PW13) and either a reverse primer against the *cdc37*$^+$ ORF (primer PW3), or a reverse primer against the *ura4*$^+$ ORF (primer RB1). The results were run on a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Gel A shows the results with the *cdc37*$^+$-specific primer and gel B shows the results with the *ura4*$^+$ primer. The 1746 bp bands are the amplification products from the wild-type locus and 971 bp bands from the deleted locus. Lanes 1 and 2 contain the PCR products from two independent colonies of the diploid, lanes 3 and 4 the products of the two parent haploids and lanes 5 and 6 the products of the two viable spores derived from meiosis of the diploid.

The gene was cloned in two stages into the pREP vectors because of the two internal *Nde*I sites in the *cdc37*$^+$ ORF as previously described (Fig 3.1). Firstly, a 238 bp fragment containing the translation initiation site was cloned into the corresponding restriction sites contained in the pREP vectors, pREP1, pREP41 and pREP81 (Fig 4.6). Subsequently, the remaining 1167 bp fragment of the ORF was then inserted into each vector. This resulted in the *cdc37*$^+$ gene cloned downstream of the *nmt1* promoter in the pREP vectors (Appendix 1E).

4.3.2 Phenotypes resulting from various expression levels of the *cdc37*$^+$ ORF in the *cdc37* deletion mutant

The pREP.*cdc37*$^+$ORF constructs were transformed into the *cdc37*$^+$/*Δcdc37* heterozygous diploid (section 2.5.5). Diploid transformants were selected on minimal plates with no supplements. The resultant diploids were then allowed to sporulate by incubating on ME plates at 25°C for 48 hr. Random spore analysis was then carried out (section 2.5.3b) and *Δcdc37* haploids (containing *ura4*$^+$) were selected for on minimal plates containing adenine and leucine. These haploids were then tested on minimal plates supplemented only with adenine for presence of the leucine marker carried on the plasmid. Every haploid isolated that carried the *ura4*$^+$ marker for *cdc37*$^+$ deletion, also contained the leucine marker from the pREP plasmid expressing *cdc37*$^+$. Thus, the *Δcdc37* haploids can only survive when maintained by the pREP1/41/81.*cdc37*$^+$ORF constructs; further evidence that the *cdc37*$^+$ gene is essential for viability in *S.pombe*.

The 3 new strains - *cdc37*$^+$::*ura4*$^+$[pREP1.*cdc37*$^+$ORF]; *cdc37*$^+$::*ura4*$^+$[pREP41.*cdc37*$^+$ORF]; *cdc37*$^+$::*ura4*$^+$[pREP81.*cdc37*$^+$ORF] - were maintained on minimal plates supplemented with adenine. In order to investigate the effects of repressing expression of *cdc37*$^+$ from the plasmids, the cells were incubated

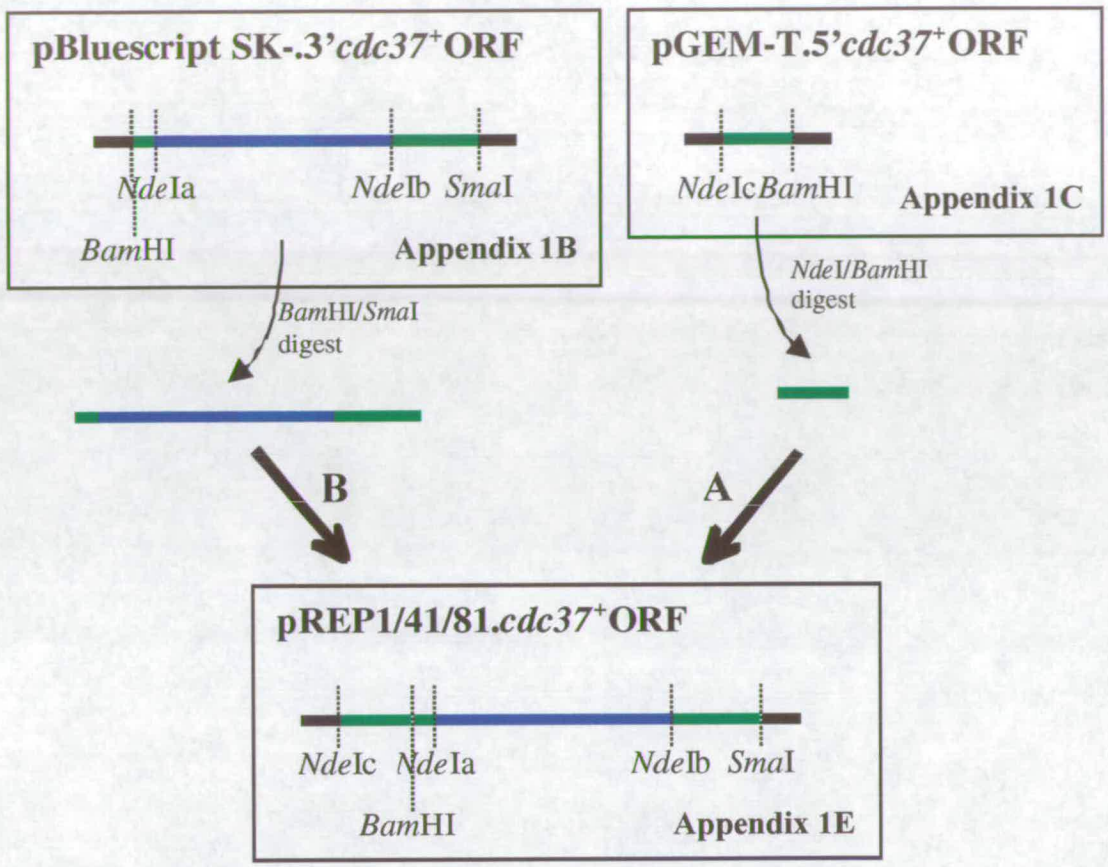


Fig 4.6 Cloning *cdc37*$^+$ into the pREP *S.pombe* expression vectors

The *cdc37*$^+$ ORF was cloned into the *S.pombe* expression vectors, pREP, in two stages. **A.** The 238 bp *Nde*I-*Bam*HI fragment (Fig 3.5) containing the 5' end of the *cdc37*$^+$ ORF was cloned into the corresponding sites in the pREP vectors. **B.** The 1167 bp *Bam*HI-*Sma*I fragment (Fig 3.5) containing the remaining 3' end of the *cdc37*$^+$ ORF was then cloned into the pREP vectors that already contained the *Nde*I-*Bam*HI fragment.

on minimal plates supplemented with adenine in the presence or absence of thiamine for 4 days at 30°C and scored for colony formation (Fig 4.7). All three *Δcdc37* strains formed normal-size colonies in the absence of thiamine after 4 days. The strain expressing *cdc37*$^+$ from pREP1 also formed normal-size colonies in the presence of thiamine. However, the strain expressing *cdc37*$^+$ from pREP41 only formed small colonies and the strain expressing from pREP81 barely formed colonies at all, with growth restricted to the formation of microcolonies. Both of these strains failed to form normal colony size even after extended incubation.

In order to gain some insight into what was causing this defect in colony formation in the strain expressing *cdc37*$^+$ from pREP81, the cells were examined more closely under the microscope after 24 and 48 hr at 30°C (Fig 4.8). After 24 hr, the cells grown in the presence of thiamine (promoter repressed) appeared normal and very similar to those incubated in the absence of thiamine (promoter derepressed). However, after 48 hr, whilst the cells incubated in the absence of thiamine remained as normal, many of those grown in the presence of thiamine became elongated, indicating that repressing expression of the *cdc37*$^+$ gene from pREP81 leads to a cell cycle defect. Phenotypes that were also visible included cells which were swollen and/or misshapen, suggesting biological roles for *cdc37*$^+$ other than its involvement in the cell cycle.

4.3.3 Relative RNA levels of *cdc37*$^+$ overexpression

To ensure that the *cdc37*$^+$ gene was being expressed from the various strength pREP vectors as expected and to compare the expression levels with that of a wild-type strain, the relative RNA levels were analysed by Northern blot (Fig 4.9). Each of the strains was grown in minimal medium supplemented with adenine (in the absence or

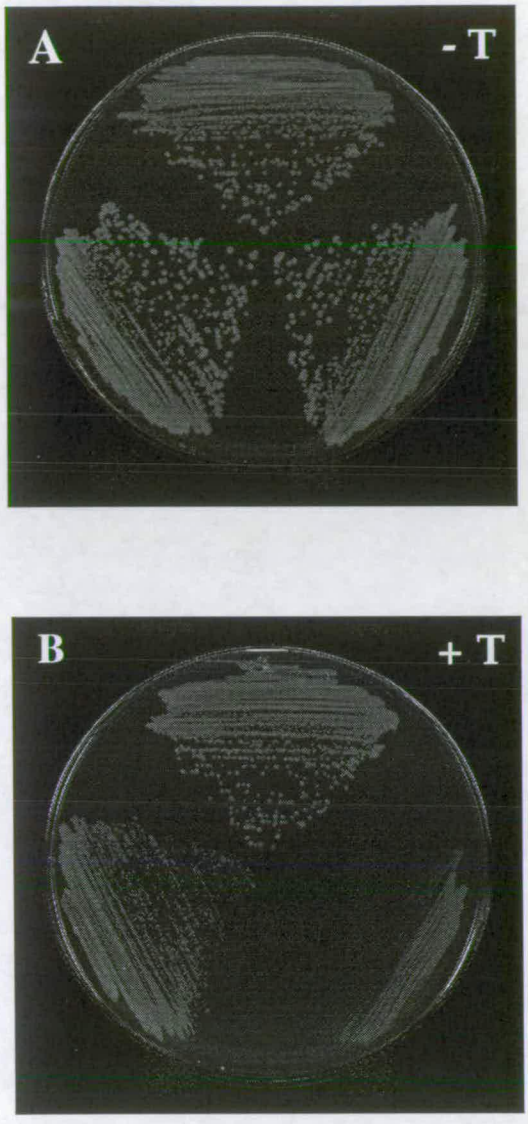


Fig 4.7 Effects of *cdc37*$^+$ overexpression in *Δcdc37* strain

Δcdc37 haploids surviving by expression of plasmid-borne *cdc37*$^+$ from various strength versions of the thiamine-repressible *nmt1*$^+$ promoter were allowed to grow in the presence (promoter repressed) or absence (promoter derepressed) of 4 μg/ml thiamine. The cells were streaked to single colonies on plates containing appropriately supplemented minimal media and incubated at 30°C for 4 days until visible single colonies were formed. Plate A shows the colony formation of the various strains in the absence of thiamine (-T) and plate B in the presence of thiamine (+T). *Δcdc37* strains are expressing *cdc37*$^+$ from, anti-clockwise from top, pREP1 (high expression), pREP41 (mid expression) and pREP81 (low expression).

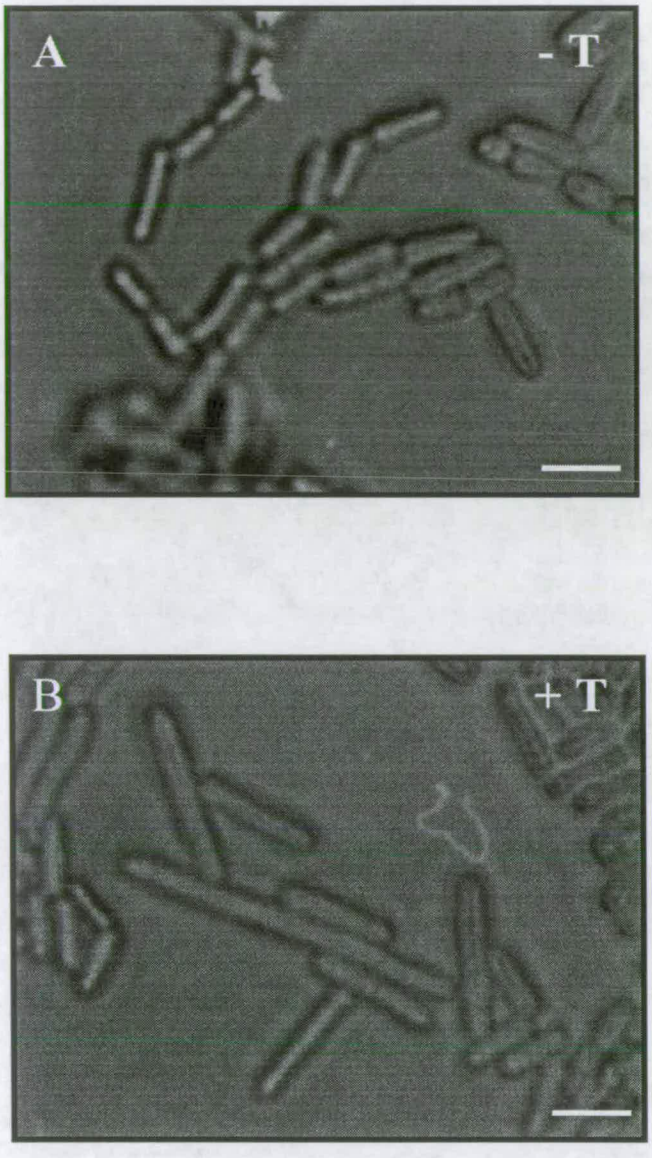


Fig 4.8 Effects of switching off *cdc37*$^+$ expression from pREP81 in *Δcdc37* strain

A *Δcdc37* haploid strain surviving by expression of plasmid-borne *cdc37*$^+$ from the low- strength version of the thiamine-repressible *nmt1*$^+$ promoter in pREP81 was allowed to grow in the presence (promoter repressed) or absence (promoter derepressed) of 4 μg/ml thiamine. The cells were streaked to single colonies on plates containing appropriately supplemented minimal media and incubated at 30°C for 2 days. Panel A shows the morphology of the cells in the absence of thiamine (-T) and panel B in the presence of thiamine (+T). The white bar in the bottom right hand corner represents 10 μm.

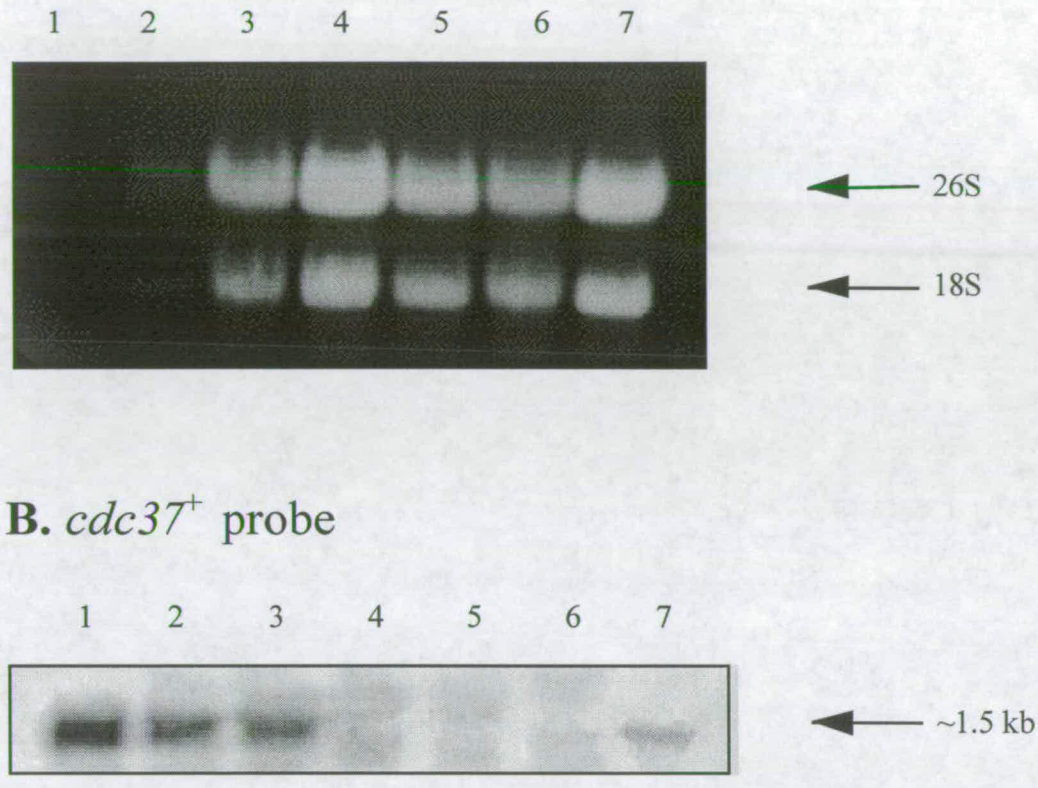


Fig 4.9 Northern analysis of *Δcdc37* haploids overexpressing plasmid-borne *cdc37*$^+$

Total RNA was extracted from *Δcdc37* haploids, expressing plasmid-borne *cdc37*$^+$ from various strength versions of the thiamine-repressible *nmt1*$^+$ promoter. The strains were allowed to grow in the presence (promoter repressed) or absence (promoter de-repressed) of 4 μg/ml thiamine for 24 hr at 30°C. The RNA was denatured, before electrophoresis on a 1% agarose gel and Northern blotting on to a nylon filter. Panel A shows the gel, stained with ethidium bromide to check loading, whilst panel B shows the filter hybridised with a 712 bp radiolabelled *Nde*I fragment of the *cdc37*$^+$ ORF. The ~1.5 kb bands correspond to the *cdc37* mRNA. Lanes 1 and 4 contain the RNA extracted from a *Δcdc37* strain expressing *cdc37*$^+$ from pREP1; lanes 2 and 5 contain the RNA from the *Δcdc37* expressing *cdc37*$^+$ from pREP41; lanes 3 and 6 contain the RNA from a *Δcdc37* strain expressing *cdc37*$^+$ from pREP81 and lane 7 contains RNA from a wild-type strain. Lanes 1-3 contain RNA extracted from cells grown in the absence of thiamine and lanes 4-6 contain RNA extracted from cells grown in the presence of thiamine. The RNA in lanes 1 and 2 were diluted 1:80 and 1:12 respectively, to reduce signal to levels comparable with that in the other lanes.

presence of thiamine) at 30°C with constant shaking for 24 hr. RNA was then extracted from each of the strains as previously described (section 2.5.8).

The derepressed level of *cdc37*$^+$ expressed from pREP81 is slightly higher than the level of expression in wild-type cells, whilst the derepressed expression from pREP41 and pREP1 is much higher. However, the repressed expression levels in all 3 strains are not high enough for the RNA to be visible on the blot, indicating that they are much lower than wild-type expression. Interestingly, the level of expression with the promoter repressed in the strain expressing *cdc37*$^+$ from pREP1 is much lower than that of wild-type, yet the cells continue to grow and appear as normal.

4.4 Summary

This chapter describes the investigation on the effects of the removal of *cdc37*$^+$ from the *S.pombe* genome. When a *cdc37*$^+$/*Δcdc37* diploid is allowed to sporulate only two of the four progeny are ever viable and they never carry the marker for *cdc37*$^+$ deletion. This shows that the *cdc37*$^+$ gene is essential for viability in *S.pombe*. A null mutant can be maintained by expression of *cdc37*$^+$ from the low-strength *nmt1* promoter in pREP81. With expression derepressed, the cells appear to grow as normal; when repressed, many of the cells become elongated. This elongation phenotype is indicative of a cell cycle role for *cdc37*$^+$ in *S.pombe*. This evidence agrees well with that from *S.cerevisiae* where the *CDC37* gene has also been shown to be essential and *ts* mutants predominantly display a cell cycle defect at the restrictive temperature. However, it appears that *cdc37*$^+$ also has other biological functions in *S.pombe*, as some of the cells appeared swollen and/or misshapen.

Since the *Δcdc37* strain expressing *cdc37*$^+$ from pREP81 had identified the cell cycle role of *cdc37*$^+$ in *S.pombe*, the next obvious step seemed to be to examine the

physiology of this strain more closely. Detailed experiments were planned to attempt to identify at which stage(s) of the cell cycle *cdc37*$^{+}$ is required. These experiments are described in Chapter 5.

Chapter 5

Effects of Cdc37 depletion

5.1 Introduction

The *cdc37::ura4*[pREP81.*cdc37*$^+$ORF] strain described in the previous chapter provides a convenient tool for analysing the physiological role of Cdc37. As described previously, this conditional mutant grows normally when the promoter is derepressed but when repressed (by the addition of thiamine to the growth medium), some of the cells become elongated, indicating a cell cycle defect.

S.pombe cells grow by tip elongation; they co-ordinate cell growth and cell division, therefore cell length is a good measure of the cell cycle stage of a cell. The detailed analyses described in this chapter, including cell length studies and FACS analysis, can provide useful information about the specific stage of a defect in the cell cycle.

In order to gain further understanding of the physiological role of Cdc37, a depletion experiment was carried out. The cells were allowed to grow in liquid culture overnight in the absence of thiamine, with the promoter derepressed, before they were moved into minimal medium containing thiamine, with the promoter repressed. Several physiological effects of Cdc37 depletion were then analysed at certain time-points throughout the experiment.

5.2 Effect of Cdc37 depletion on growth and division

In the expression experiments on plates described in the last chapter (section 4.3), little or no effect of repressing *cdc37*$^+$ expression in the *cdc37::ura4*[pREP81.*cdc37*$^+$ORF] strain was observed after 24 hr incubation. However, after 48 hr, the phenotypes were visible and severe. Because of this, both growth and cell number of a liquid culture were monitored between 24 and 48 hr after the addition of thiamine (Fig 5.1). Culture growth was estimated by measurement of

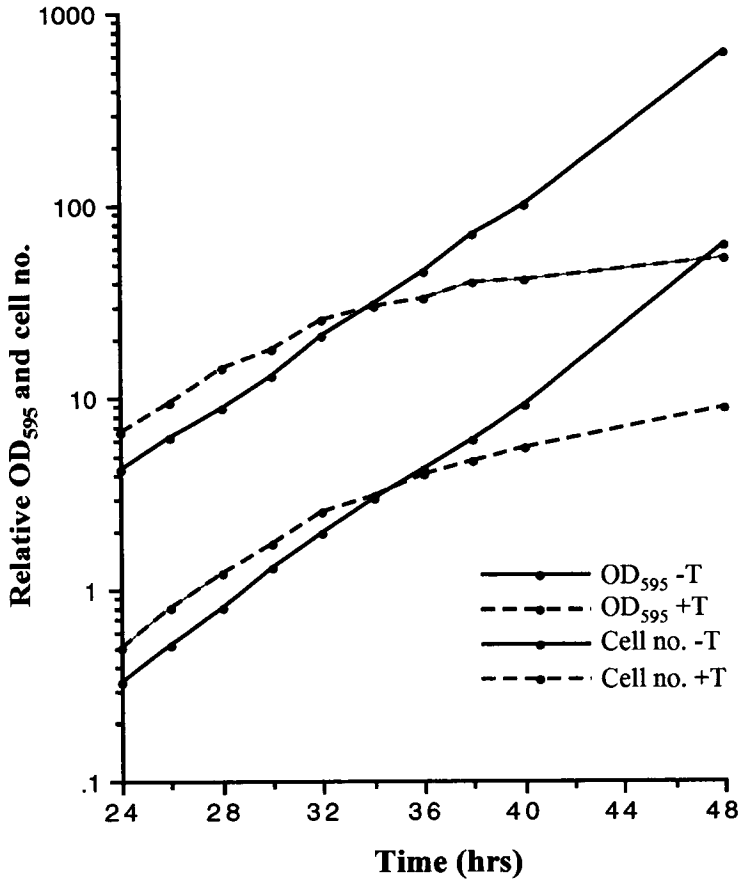


Fig 5.1 Measurement of culture growth and cell number of the *cdc37::ura4*[pREP81.*cdc37*$^+$ORF] strain grown in the presence or absence of thiamine

Samples to measure the culture growth and cell number of the *cdc37::ura4*[pREP81.*cdc37*$^+$ORF] strain grown in the presence or absence of thiamine were taken every 2 hrs between 24 and 48 hrs after the addition of thiamine. Culture growth was estimated by measurement of the OD at 595 nm and cell division was monitored by a cell number count using a Coulter Counter. A y-axis value of 1 on the graph represents an OD_{595} of 1 for the relative OD_{595} plot and a value of 10 represents 10^7 cells/ml for the relative cell no. plot.

the OD at 595 nm (section 2.5.4a) and cell division was monitored by a cell number count using a Coulter Counter (section 2.5.4b). Samples for both culture growth and cell number were taken every 2 hr.

Agreeing well with the observations described in the last chapter (section 4.3.2), between 0 and 24 hr after the addition of thiamine, the culture growth is exponential and has grown slightly faster than a culture grown in the absence of thiamine (Fig 5.1). However, approximately 28 hr after the addition of thiamine, cell division begins to slow down, followed shortly after by a decrease in the culture growth rate. Forty hours after the addition of thiamine, both culture growth and cell division has almost stopped, whilst the cells grown in the absence of thiamine continued to grow and divide exponentially throughout the duration of the experiment. It is not understood why the cells in the presence of thiamine should grow faster than those grown without for the first 24 hr and no obvious explanation exists for this phenomenon.

5.3 Cell length studies on Cdc37 depleted cells

In order to confirm the observation (section 4.3.2) that one of the primary phenotypes of repressing *cdc37*$^{+}$ expression was elongation of the cells, thus suggesting a defect in cell cycle progress, cell length analysis was carried out (Fig 5.2, Fig 5.3). Initially, as with all of the rest of the analyses carried out in this chapter, samples were taken every 4 hr between 24 hr and 40 hr after the addition of thiamine and cell length measurements were taken of several hundred cells in each sample using the IPLab software (section 2.5.4c). However, it was noticed that after 24 hr, the cells grown in the presence of thiamine were significantly shorter than those grown in the absence of thiamine and therefore cells from earlier time-points at 16 hr and 20 hr were examined.

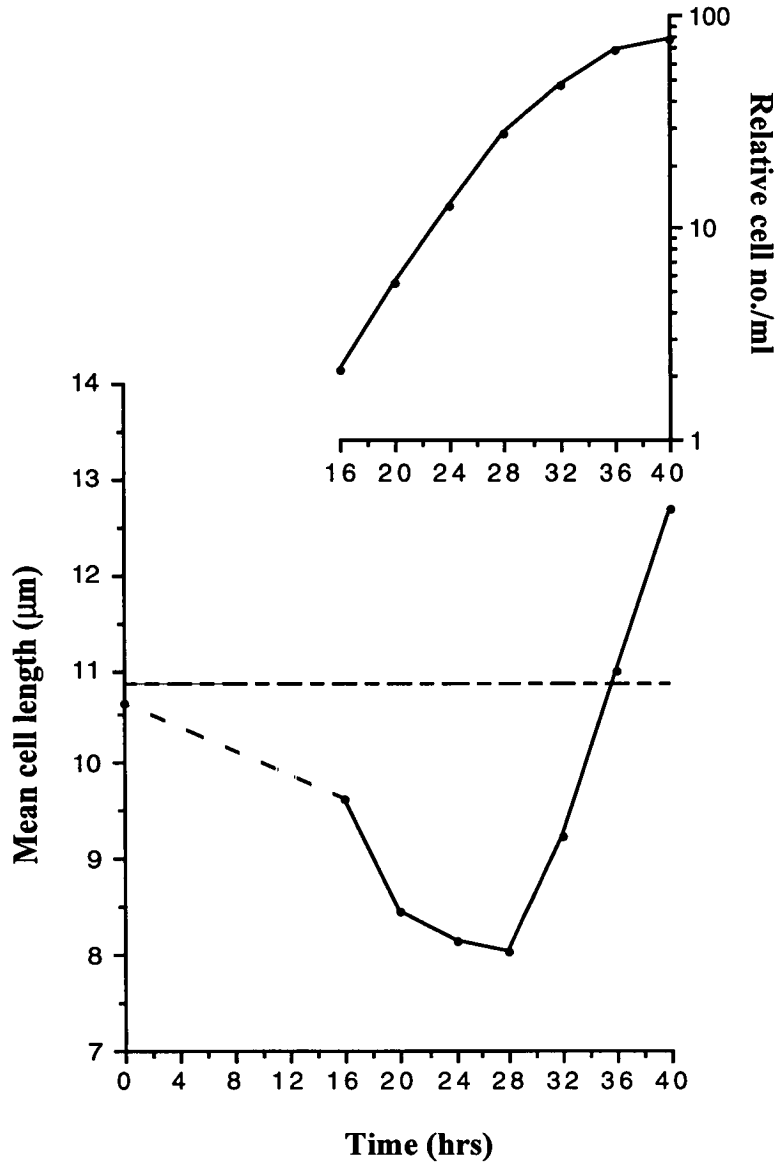


Fig 5.2 Mean cell length of *cdc37::ura4*[pREP81.*cdc37*$^+$ORF] cells grown in the presence of thiamine

The lengths of *cdc37::ura4*[pREP81.*cdc37*$^+$ORF] cells were measured from cultures grown in the presence of thiamine. Shown is a graph of mean cell length for each sample, taken every 4 hr between 16 hr and 40 hr after the addition of thiamine. The dashed line represents the mean cell length of cells grown in the absence of thiamine. Above the main graph is a plot of cell number for this experiment. A y-axis value of 10 on the graph represents 10^7 cells/ml for the relative cell no. plot.

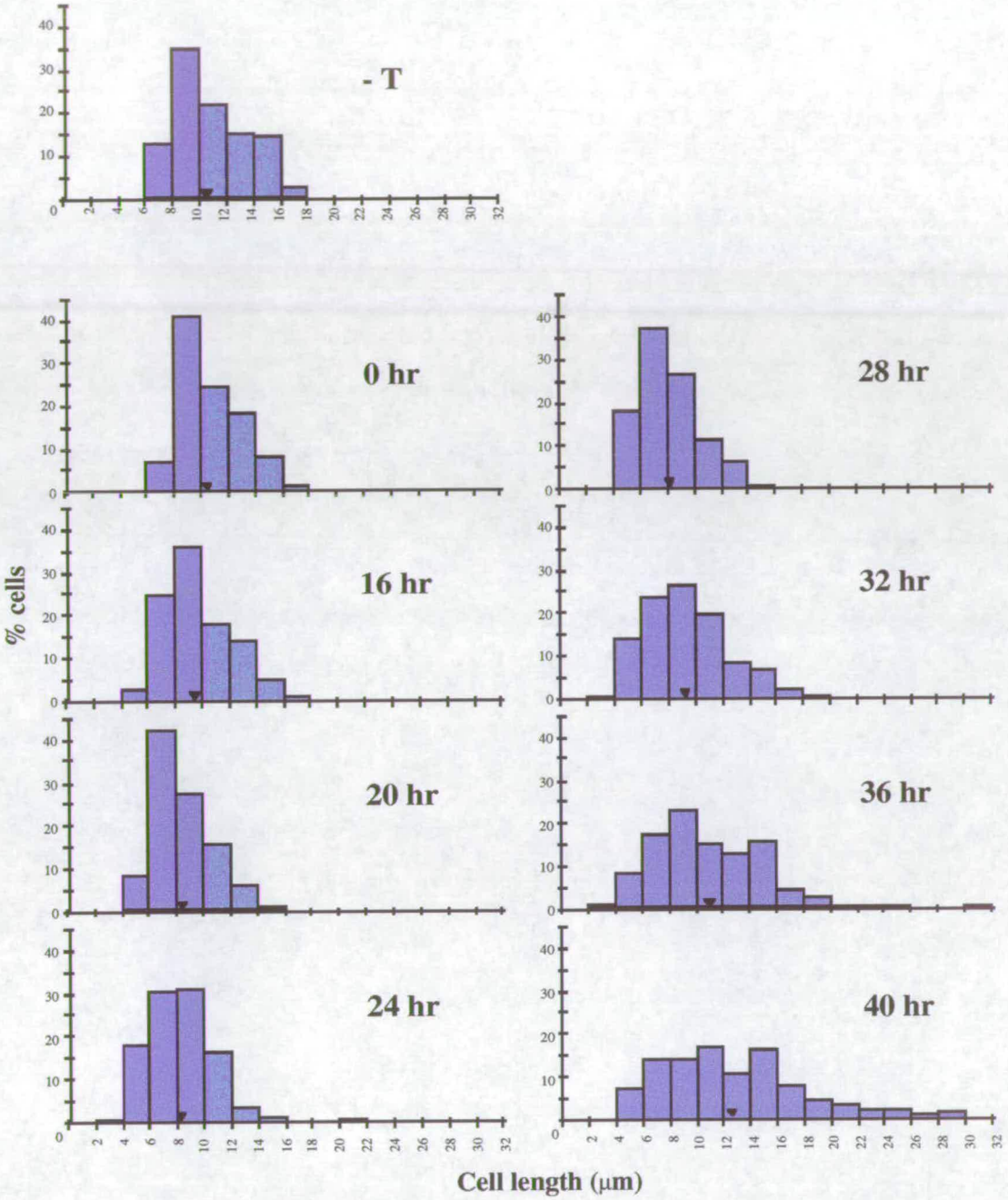


Fig 5.3 Cell length distribution of *cdc37::ura4*[pREP81.*cdc37*$^+$] cells grown in the presence of thiamine

The lengths of ~200 *cdc37::ura4*[pREP81.*cdc37*$^+$] cells were measured from cultures grown in the presence of thiamine. Shown is a histogram of the cell length data for each sample, taken every 4 hr between 16 hr and 40 hr after the addition of thiamine. The mean cell length for each sample is represented by a black triangle on the X-axis. A typical sample from a culture grown in the absence of thiamine is also shown as a control.

Sixteen hours after the addition of thiamine, the mean cell length is significantly shorter than the control cells grown in the absence of thiamine and continues to drop for another ~12 hr (Fig 5.2). This is due to the presence of a population of cells that are much smaller than wild-type and their presence is difficult to explain (Fig 5.3). It is unknown if this is a direct effect of Cdc37 depletion or whether it is the cells adapting in some way to the loss of Cdc37 protein. After ~28 hr after the addition of thiamine the mean cell length begins to increase dramatically and this continues throughout the remainder of the experiment (Fig 5.2). This increase in mean cell length is due to the appearance of a significant population of elongated cells (Fig 5.3). However, despite the fact that a significant proportion of the cells become elongated after ~28 hr after the addition of thiamine, a population of small cells remain throughout the experiment (Fig 5.3).

It seems highly significant that cell division appears to slow down at a similar time to when this increase in mean cell length begins to occur (Fig 5.2). However, although there is definite and significant elongation after ~28 hr after repressing *cdc37*$^{+}$ expression, this elongation phenotype alone does not appear to be severe enough for proliferation to cease. This suggests further biological roles for *cdc37*$^{+}$.

5.4 Measuring DNA content of Cdc37 depleted cells

The cell length analysis (section 5.3) confirmed that elongation is a primary phenotype of cells where *cdc37*$^{+}$ expression is repressed, indicative of a cell cycle defect. Significant elongation in *S.pombe* usually reveals that a cell cycle defect is occurring during interphase (G1, G2 or S phase) as these are the stages during which most growth occurs. Defects in mitosis do not cause significant elongation as very little growth occurs during these stages. To gain more insight into the stages of the cell cycle at which this visible defect occurs, the DNA content of the

cdc37::ura4[pREP81.*cdc37*$^+$ORF] cells grown in the presence or absence of thiamine were subjected to flow cytometric analysis (Fig 5.4). Samples were processed every 4 hr between 24 hr and 40 hr after the addition of thiamine (section 2.5.4f).

In the culture growing in the absence of thiamine, a sharp 2C peak is seen in all samples, which is typical of an exponentially growing *S.pombe* culture (Nurse *et al.*, 1976). This 2C peak is seen because *S.pombe* replicate their DNA almost simultaneously with septation. In the culture growing in the presence of thiamine, only 2C peaks are seen in all samples as well; however, the peaks become less sharp after ~24 hr. This is probably due to effects caused by other roles of *cdc37*$^+$. Unfortunately, due to the way *S.pombe* cells grow and divide it is impossible to distinguish on this basis alone between a G2 arrest and exponentially growing cells. However, since there is no significant 1C peak, indicative of a G1 arrest, or a 4 or 8C peak, indicative of a septation defect, it can be concluded with some confidence that the population of elongated cells caused by Cdc37 depletion is due to a defect in the G2 and/or the mitotic phase of the cell cycle, where the cells have a 2C DNA content.

5.5 Nuclear and cell wall staining of Cdc37 depleted cells

In order to gain further insight into the mechanism of cell cycle arrest caused by Cdc37 depletion, *cdc37::ura4*[pREP81.*cdc37*$^+$ORF] cells were stained with either DAPI, which stains the nucleus (Toda *et al.*, 1981), or calcofluor, which stains the cell wall and particularly the septum (Mitchison and Nurse, 1985), after growth in the presence or absence of thiamine (Fig 5.5-Fig 5.6). Samples were taken every 4 hr between 24 hr and 40 hr after the addition of thiamine, stained and visualised immediately (sections 2.5.4d and 2.5.4e).

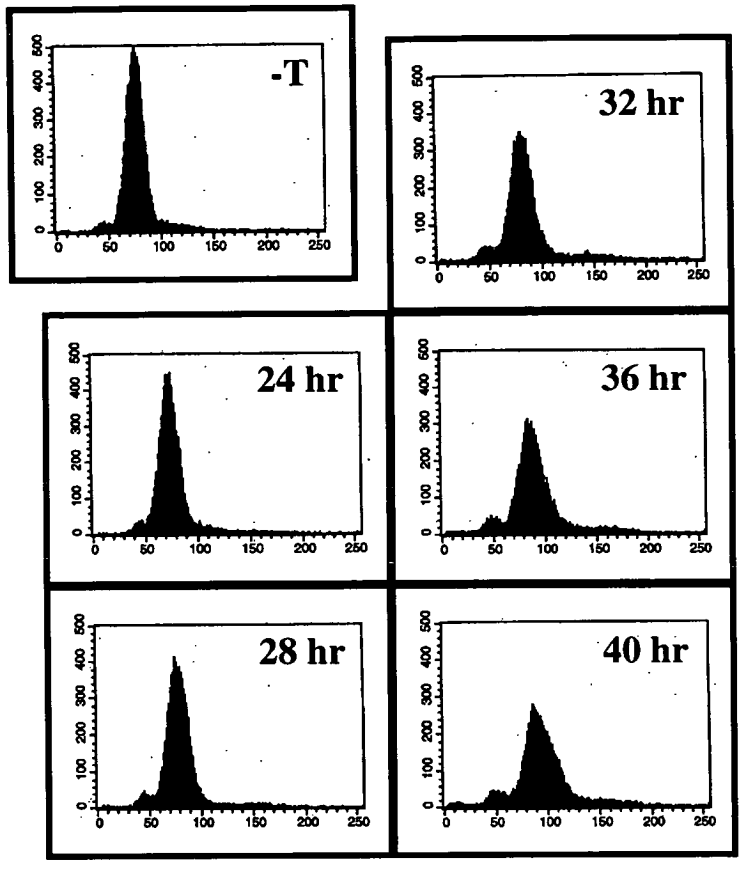


Fig 5.4 FACS analysis of *cdc37::ura4*[pREP81.*cdc37*$^+$] cells in the presence of thiamine

The DNA content of *cdc37::ura4*[pREP81.*cdc37*$^+$] cells stained with propidium iodide was measured by flow cytometry from cultures grown in the presence of thiamine. The linear fluoresence diagrams show relative DNA content (arbitrary units) on the x-axis and cell number on the y-axis for each sample, taken every 4 hr between 24 hr and 40 hr after the addition of thiamine. A typical sample from a culture grown in the absence of thiamine (-T) is also shown as a control.

At ~24 hr after the addition of thiamine, the calcofluor staining shows that most of the *cdc37::ura4*[pREP81.*cdc37*$^{+}$ORF] cells are shorter than the control cells grown in the absence of thiamine (Fig 5.5). At ~32 hr, some of these cells have begun to elongate and some are beginning to look swollen and/or misshapen whereas at ~40 hr most of the cells have ceased proliferation, showing a variety of phenotypes previously described (section 4.3.2). These include cells that are small and round, some that are swollen and/or misshapen and some that are elongated, whilst some are a combination of these phenotypes. Also, there is a significant number of cells that are clearly defective in septation and cytokinesis, containing multiple septa and have also become elongated.

The DAPI staining also reveals a variety of phenotypes (Fig 5.6). At ~24hr after the addition of thiamine, the majority of cells still appear to be dividing normally. However, at ~32 hr and even more so at ~40 hr, cell division ceases to continue normally. The most noticable cells are those that are elongated: some contain what appear to be normal interphase nuclei, perhaps arrested in G2; others contain nuclei that have failed to migrate properly; a smaller number of elongated cells appear to contain multiple nuclei (3 or 4) with or without a septum that have failed to undergo proper division at the septum. There are also a significant proportion of fairly normal length cells that appear to have undergone a defective mitosis and have arrested with hypercondensed chromosomes. As already described with the calcofluor staining, however, there is considerable heterogeneity within the population in this experiment which makes the observations more difficult to interpret.

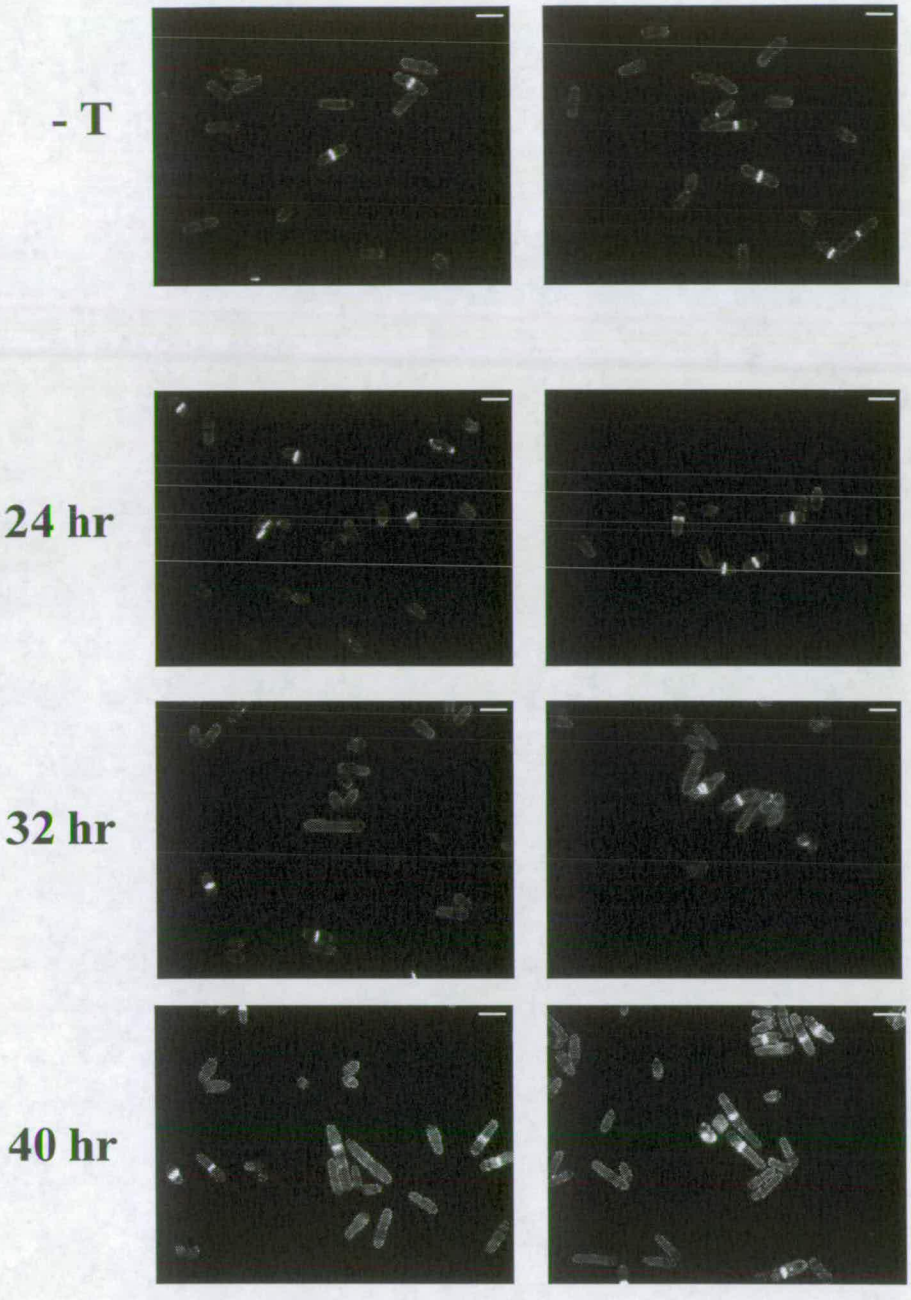


Fig 5.5 Calcofluor staining of *cdc37::ura4*[pREP81.*cdc37*$^+$] cells grown in the presence of thiamine

cdc37::ura4[pREP81.*cdc37*$^+$] cells were grown in the presence of 4 μg/mlthiamine and stained with Calcofluor. Samples were taken at 24 hr, 32 hr and 40 hr after the addition of thiamine. The cells were processed by fixation with 2.5% glutaraldehyde, stained with 0.5 mg/ml Calcofluor and visualised immediately. Typical samples from a culture grown in the absence of thiamine (-T) are also shown as a control. The white bar in the top right hand corner represents 10 μm.

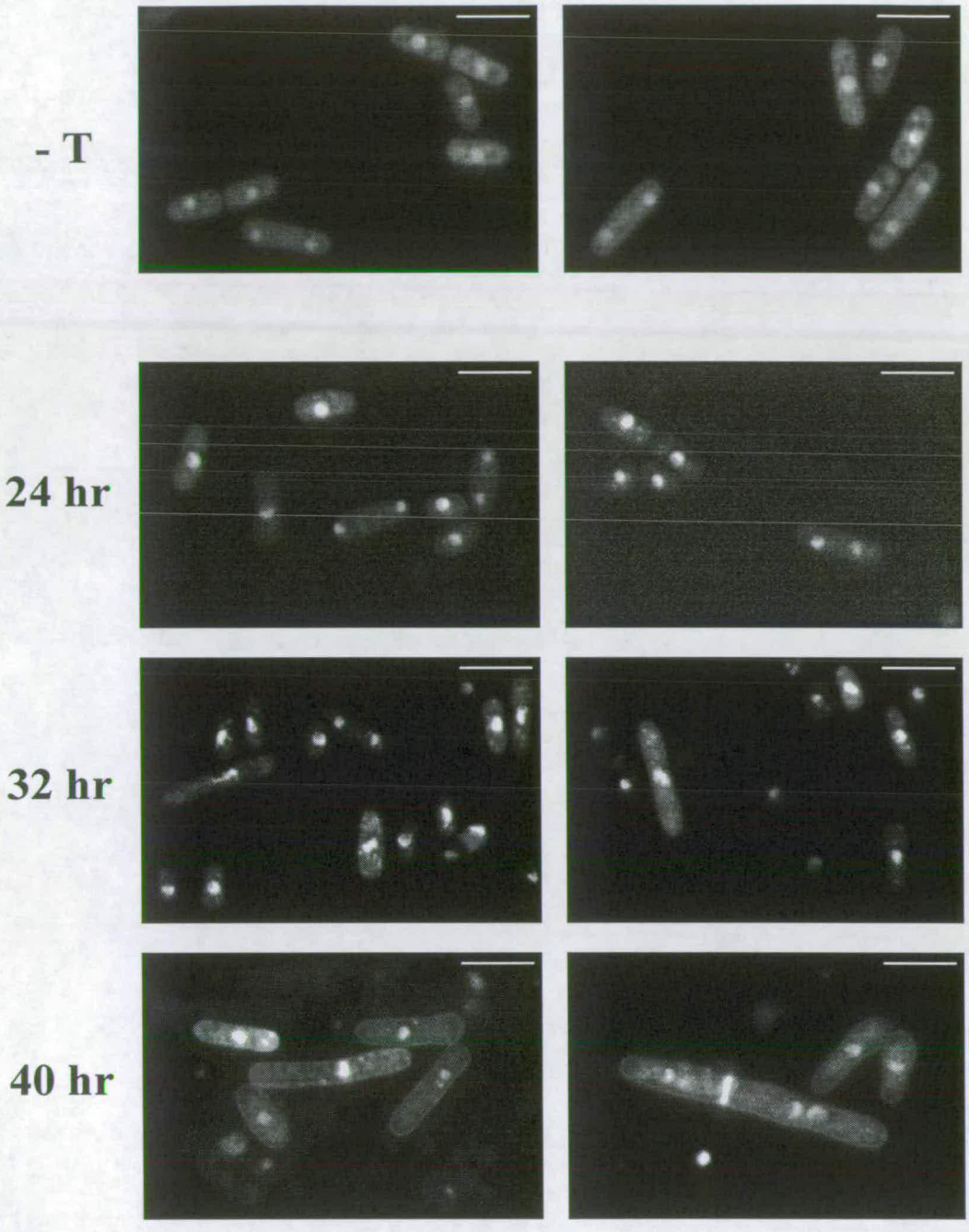


Fig 5.6 DAPI staining of *cdc37::ura4*[*cdc37*$^+$/pREP81] cells grown in the presence of thiamine

cdc37::ura4[*cdc37*$^+$/pREP81] cells were grown in the presence of thiamine and stained with DAPI. Samples were taken at 24 hr, 32 hr and 40 hr after the addition of thiamine. The cells were processed by fixation with 2.5% glutaraldehyde, stained with 10 mg/ml DAPI and visualised immediately. Typical samples from a culture grown in the absence of thiamine (-T) are also shown as a control. The white bar in the top right hand corner represents 10 μm.

5.6 Summary

The examination of the *cdc37::ura4*[pREP81.*cdc37*$^+$ORF] strain described in this chapter provides further information on the role of Cdc37 in the *S.pombe* cell cycle. Cell length analysis confirms that elongation is a primary phenotype of cells where *cdc37*$^+$ expression has been repressed. This is strong evidence that *cdc37*$^+$ plays an important role in the cell cycle.

Interestingly, the FACS analysis and cell staining data suggest that, unlike in *S.cerevisiae* where *CDC37* is required at START in G1, the primary cell cycle defects in *S.pombe* occur during both G2 and mitosis. However, it appears that Cdc37 is not only involved in the cell cycle in *S.pombe*. Depletion of Cdc37 results in a very heterogenous population, suggesting other biological roles. Obvious phenotypes which appear to be unconnected with the cell cycle include swollen and/or misshapen cells. Also, a significant number of cells showed defects in septation; many cells have formed septa but have not undergone cytokinesis, resulting in multiseptated cells. It would be very interesting to examine these other phenotypes in more detail and to investigate the mechanisms involved.

The variety of observations described in this chapter indicates that the biological role of Cdc37 in *S.pombe* is complex and it will take some time to separate the functions and understand them completely. However, there does appear to be a considerable number of cells present with specific cell cycle defects caused by Cdc37 depletion which appear to affect cells in the G2 and mitotic stages of the cell cycle. As explained in Chapter 1, the complex which regulates the G2/M transition in *S.pombe* is the Cdc2/Cdc13 complex. This complex seemed to be a good target for preliminary investigation of interactions with Cdc37 and these experiments are described in Chapter 6.

Chapter 6

Towards determining the cell cycle role of Cdc37

6.1 Introduction

Observations from the previous chapter indicate that *cdc37*$^{+}$ is required both during the G2 phase of the cell cycle and during mitosis. As described in Chapter 1, the G2/M transition is regulated by the Cdc2/Cdc13 complex. Therefore, both of these proteins provided obvious targets for interaction with Cdc37. Also, as described in Chapter 1, Cdc37 has previously been shown to physically interact with the mammalian cell cycle regulator Cdk4 (Dai *et al.*, 1996), though no interaction with Cdc28 has yet been shown in *S.cerevisiae*. This chapter describes preliminary experiments designed to examine the potential interactions of Cdc37 with Cdc2 and Cdc13 using both genetic and biochemical approaches.

The expression constructs described in Chapter 4 (section 4.3.1) allowed examination of the effects of overexpression of *cdc37*$^{+}$ in a wild type strain and various *ts* mutants. This type of analysis is often used in *S.pombe* to detect potential interactions and thus suggest possible biological roles of the gene of interest. Several alleles of *cdc2*^{ts} and *cdc13*^{ts} mutants were available for this investigation and overexpression of *cdc37*$^{+}$ was examined thoroughly in all of these strains.

In order to determine whether any physical interactions of Cdc37 could be detected, a strain expressing a functional His6-tagged version of the protein was constructed. Since we had available a previously successful commercial anti-His antibody (Santa Cruz), anti-Cdc2 and anti-Cdc13 antibodies, it was decided that this was the best approach to use due to the time constraints which were existent.

6.2 Levels of Cdc2 protein in Cdc37 depleted cells

Since the observations from the FACS and cytological analysis suggest that the primary cell cycle defect in Cdc37 depleted cells is during G2 and mitosis, the Cdc2 levels were analysed by Western blotting (section 2.3.4) in another depletion experiment identical to that described in the last chapter (Fig 6.1). Every 4 hr between 24 hr and 40 hr after the addition of thiamine, samples were removed from the culture and denatured protein was extracted as previously described (section 2.5.9).

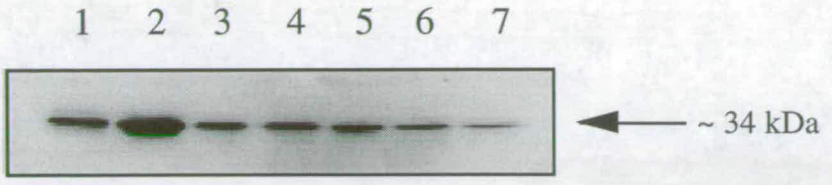
To monitor the Cdc2 levels, the blot was probed with an antibody raised against a peptide with the amino acid sequence PSTAIR (Hamaguchi *et al.*, 1992), a motif conserved in all $p34^{cdc2}$ kinases which include Cdc2 in *S.pombe*. This showed that the level of Cdc2 is significantly reduced in the *cdc37::ura4*[pREP81.*cdc37*$^{+}$ORF] cells 24 hr after the addition of thiamine, when the cells still appear to be dividing normally. The expression level appears to diminish from 24 hr onwards in this experiment, until eventually, the Cdc2 level 40 hr after the addition of thiamine is significantly reduced. This appears to be a significant finding, as the levels of *cdc2*$^{+}$ are normally very stable in *S.pombe*.

An attempt was also made at monitoring the *cdc13*$^{+}$ expression levels in this depletion experiment. However, neither of the available antibodies was found to detect Cdc13 in this lab, even in wild type extracts.

6.3 Genetic interactions of *cdc37*$^{+}$

Another strategy designed to find out more about the interactions of Cdc37 in *S.pombe* was to overexpress the *cdc37*$^{+}$ ORF in various *ts* mutant strains. Since the

A. anti-PSTAIR



B. anti-tubulin

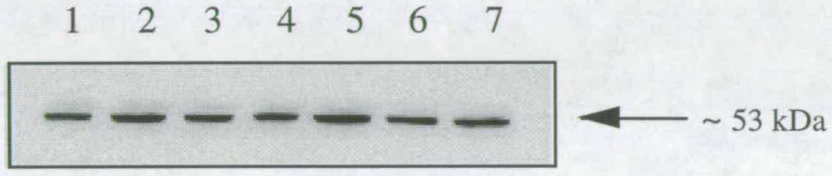


Fig 6.1 Western blot showing Cdc2 levels in *cdc37::ura4*[pREP81.*cdc37*$^{+}$ORF] cells grown in the presence of thiamine

Protein was extracted from *cdc37::ura4*[pREP81.*cdc37*$^{+}$ORF] cells grown in the presence of thiamine. The protein extracts were boiled immediately before electrophoresis on a 12.5% polyacrylamide gel and Western blotting on to a nylon filter. Panel A shows the blot probed with anti-PSTAIR for analysing Cdc2 protein levels whilst panel B shows the blot probed with anti-α-tubulin as a loading control. Lane 1 contains the protein extract from a wild-type strain and lane 2 contains the extract from *cdc37::ura4*[pREP81.*cdc37*$^{+}$ORF] cells grown in the absence of thiamine, and lanes 3, 4, 5, 6 and 7 contain the extract from cells grown in the presence of thiamine 24, 28, 32, 36 and 40 hr after the addition of thiamine, respectively.

observations described in Chapter 5 had suggested the interaction of Cdc37 with the Cdc2/Cdc13 complex, it seemed a sensible step to carry out further analysis with various *cdc2*^{ts} and *cdc13*^{ts} mutants that were available. As discussed in Chapters 1 and 7, other potential targets for interaction included Swo1 (Hsp90) and Wee1, for which *ts* mutants were available. Also, the effect of overexpression in a wild-type strain was tested.

Overexpression of *cdc37*$^{+}$ from pREP41 and pREP81 appeared to have no effect in any of the strains tested and overexpression from any of the pREP plasmids had no effect on the *swo1-26* and *wee1-50* mutant strains. However, overexpression of *cdc37*$^{+}$ from pREP1 had various effects on the *cdc2*^{ts} and *cdc13*^{ts} mutants (Table 6.1, Table 6.2).

In wild-type cells overexpression of *cdc37*$^{+}$ from pREP1 has little or no effect (Fig 6.2, Fig 6.3). At all temperatures between 28°C and 35°C, wild-type cells form normal colonies with or without overexpression of *cdc37*$^{+}$ (Fig 6.2). The cells also appear as normal, though those overexpressing *cdc37*$^{+}$ may be slightly longer (Fig 6.3).

The effect of overexpression of *cdc37*$^{+}$ from pREP1 was then tested in strains containing *cdc2*^{ts} and *cdc13*^{ts} alleles (Fig 6.4 - Fig. 6.7). In two strains containing different *cdc13*^{ts} alleles, *cdc13-117* and *cdc13-9*, overexpression of *cdc37*$^{+}$ had a drastic effect (Fig 6.4, Fig 6.5). The restrictive temperature of the two mutants was severely reduced to such an extent that both of the strains failed to form normal colonies even at 25°C, normally a permissive temperature (Fig 6.4). When examined under the microscope, the *cdc13*^{ts} cells overexpressing *cdc37*$^{+}$ were greatly elongated (Fig 6.5).

Table 6.1 Colony formation of strains carrying various *cdc2ts* and *cdc13 ts* alleles

wild-type	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	***	***	***	***
REP1 -T	***	***	***	***
cdc37$^+$/REP1 +T	***	***	***	***
cdc37$^+$/REP1 -T	***	***	***	***
cdc13-117	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	***	***	**	-
REP1 -T	***	***	**	-
cdc37$^+$/REP1 +T	***	***	**	-
cdc37$^+$/REP1 -T	*	*	-	-
cdc13-9	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	***	***	**	-
REP1 -T	***	***	**	-
cdc37$^+$/REP1 +T	***	***	**	-
cdc37$^+$/REP1 -T	*	*	-	-
cdc2-33	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	***	***	*	-
REP1 -T	***	***	*	-
cdc37$^+$/REP1 +T	***	***	*	-
cdc37$^+$/REP1 -T	**	**	-	-
cdc2-L7	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	***	***	*	-
REP1 -T	***	***	*	-
cdc37$^+$/REP1 +T	***	***	*	-
cdc37$^+$/REP1 -T	**	**	-	-
cdc2-45	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	***	***	*	-
REP1 -T	***	***	*	-
cdc37$^+$/REP1 +T	***	***	*	-
cdc37$^+$/REP1 -T	***	**	-	-
cdc2-17	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	***	***	***	**
REP1 -T	***	***	***	**
cdc37$^+$/REP1 +T	***	***	***	**
cdc37$^+$/REP1 -T	***	***	**	*

cdc2-48	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	***	*	-	-
REP1 -T	***	*	-	-
cdc37$^+$/REP1 +T	***	*	-	-
cdc37$^+$/REP1 -T	**	*	-	-

cdc2-18	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	***	***	***	***
REP1 -T	***	***	***	***
cdc37$^+$/REP1 +T	***	***	***	***
cdc37$^+$/REP1 -T	***	***	***	**

cdc2-130	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	***	**	**	**
REP1 -T	***	**	**	**
cdc37$^+$/REP1 +T	***	**	**	**
cdc37$^+$/REP1 -T	**	**	**	*

cdc2-M26	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	***	*	-	-
REP1 -T	***	*	-	-
cdc37$^+$/REP1 +T	***	*	-	-
cdc37$^+$/REP1 -T	**	*	-	-

Strains containing various *cdc2* and *cdc13 ts* alleles were transformed with the pREP1.*cdc37*$^+$ORF plasmid and empty pREP1 as a control. The strains were then grown at various temperatures in the presence (promoter repressed) or absence (promoter derepressed) of 4 μg/ml thiamine. The transformants were patched on to appropriately supplemented minimal media at 28°C, 32°C, 33.5°C or 35°C for 24 hr, streaked to single colonies and incubated for a further 3 days when visible single colonies were formed. The formation of colonies was scored as follows: - no colony formation, * microcolonies formed, ** small colonies formed, *** normal colonies formed.

Table 6.2 Morphology of cells carrying various *cdc2* and *cdc13 ts* alleles

wild-type	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	3	3	3	3
REP1 -T	3	3	3	3
cdc37$^+$/REP1 +T	3	3	3	3
cdc37$^+$/REP1 -T	3	3	3	3

cdc13-117	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	3	3/4	4	5/6
REP1 -T	3	3/4	4	5/6
cdc37$^+$/REP1 +T	3	3/4	4	5/6
cdc37$^+$/REP1 -T	4	5/6	6	6

cdc13-9	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	3	3/4	4	5/6
REP1 -T	3	3/4	4	5/6
cdc37$^+$/REP1 +T	3	3/4	4	5/6
cdc37$^+$/REP1 -T	4	5/6	6	6

cdc2-33	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	3	3/4	4/5	6
REP1 -T	3	3/4	4/5	6
cdc37$^+$/REP1 +T	3	3/4	4/5	6
cdc37$^+$/REP1 -T	4	4/5	6	6

cdc2-L7	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	3	3/4	4/5	6
REP1 -T	3	3/4	4/5	6
cdc37$^+$/REP1 +T	3	3/4	4/5	6
cdc37$^+$/REP1 -T	4	4/5	6	6

cdc2-45	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	3/4	4	5/6	6
REP1 -T	3/4	4	5/6	6
cdc37$^+$/REP1 +T	3/4	4	5/6	6
cdc37$^+$/REP1 -T	4	4/5	6	6

cdc2-17	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	3	3	3	4/5
REP1 -T	3	3	3	4/5
cdc37$^+$/REP1 +T	3	3	3	4/5
cdc37$^+$/REP1 -T	3/4	4/5	2/6	3/6

cdc2-48	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	3/4	5/6	6	6
REP1 -T	3/4	5/6	6	6
cdc37^{+}/REP1 +T	3/4	5/6	6	6
cdc37^{+}/REP1 -T	4	5/6	5/6	6

cdc2-18	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	3	3/4	3/4	4
REP1 -T	3	3/4	3/4	4
cdc37^{+}/REP1 +T	3	3/4	3/4	4
cdc37^{+}/REP1 -T	4	4/5	4/5	4/5

cdc2-130	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	2/3	2/4	2/4	4/5
REP1 -T	2/3	2/4	2/4	4/5
cdc37^{+}/REP1 +T	2/3	2/4	2/4	4/5
cdc37^{+}/REP1 -T	2/3	2/4	2/4	3/5

cdc2-M26	**28°C**	**32°C**	**33.5°C**	**35°C**
REP1 +T	3	4/5	6	6
REP1 -T	3	4/5	6	6
cdc37^{+}/REP1 +T	3	4/5	6	6
cdc37^{+}/REP1 -T	4	5/6	6	6

Table 6.2 Morphology of cells carrying various *cdc2* and *cdc13 ts* alleles

Strains containing various *cdc2* and *cdc13 ts* alleles were transformed with the pREP1.*cdc37*$^{+}$ORF plasmid and empty pREP1 as a control. The strains were then grown at various temperatures in the presence (promoter repressed) or absence (promoter derepressed) of 4 μg/ml thiamine. The transformants were patched on to appropriately supplemented minimal media at 28°C, 32°C, 33.5°C or 35°C for 24 hr, streaked to single colonies, used to inoculate a liquid culture and incubated for a further 24 hr. The cell morphology was scored as follows: 1, very short, 2, short, 3, wild-type length, 4, long, 5, very long (still proliferating), 6, very long (causing proliferation to cease).

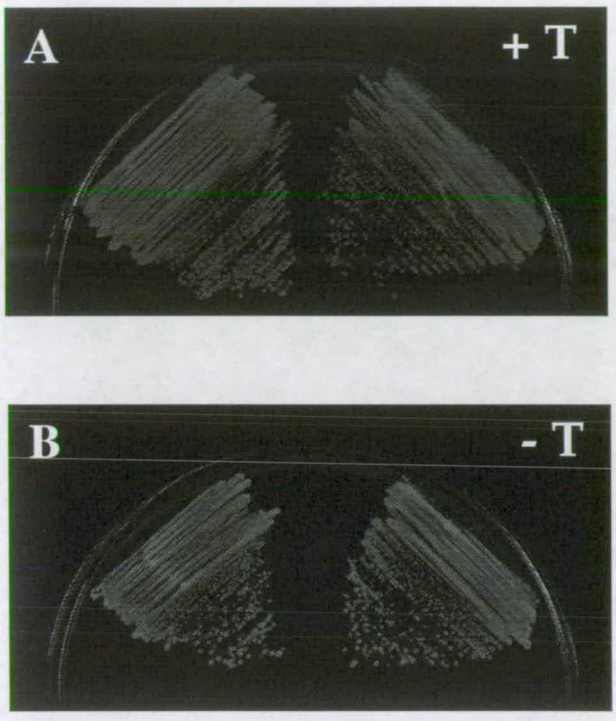


Fig 6.2 Effects of *cdc37*$^{+}$ overexpression in a wild-type *S.pombe* strain

A wild-type strain was transformed with the pREP1.*cdc37*$^{+}$ORF plasmid and empty pREP1 as a control. The strain was then grown at various temperatures in the presence (promoter repressed) or absence (promoter derepressed) of 4 μg/ml thiamine. The transformants were patched on to appropriately supplemented minimal media at 28°C, 32°C, 33.5°C or 35°C for 24 hr, streaked to single colonies and incubated for a further 3 days when visible single colonies were formed. Plate A shows the colony formation of the strain grown at 32°C in the presence of thiamine (+T) and plate B in the absence of thiamine (-T). On both plates, the strain on the left contains pREP1 and the strain on the right contains pREP1.*cdc37*$^{+}$ORF.

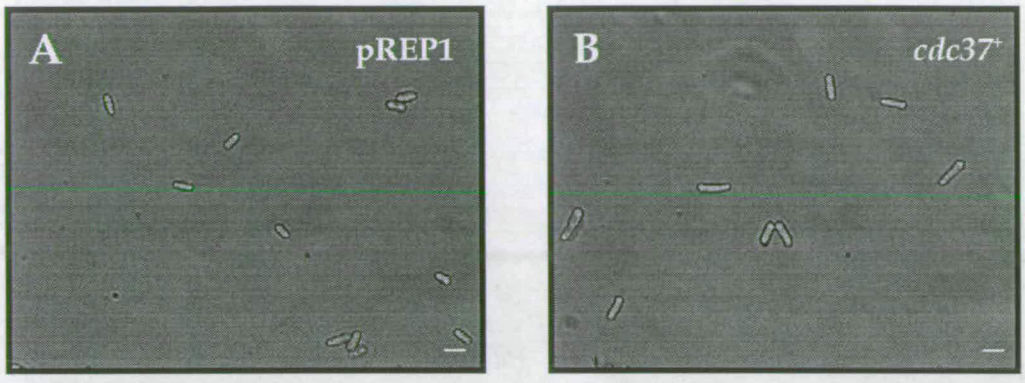


Fig 6.3 Effects of *cdc37*$^+$ overexpression in a wild-type *S.pombe* strain

A wild-type strain was transformed with the pREP1.*cdc37*$^+$ORF plasmid and empty pREP1 as a control. The strain was then grown at various temperatures in the presence (promoter repressed) or absence (promoter derepressed) of 4 μg/ml thiamine. The transformants were patched on to appropriately supplemented minimal media at 28°C, 32°C, 33.5°C or 35°C for 24 hr, used to inoculate a liquid culture and incubated for a further 24 hr. Panel A shows the morphology of cells grown at 32°C containing pREP1 and panel B shows cells containing pREP1.*cdc37*$^+$ORF. The white bar in the bottom right hand corner represents 10 μm.

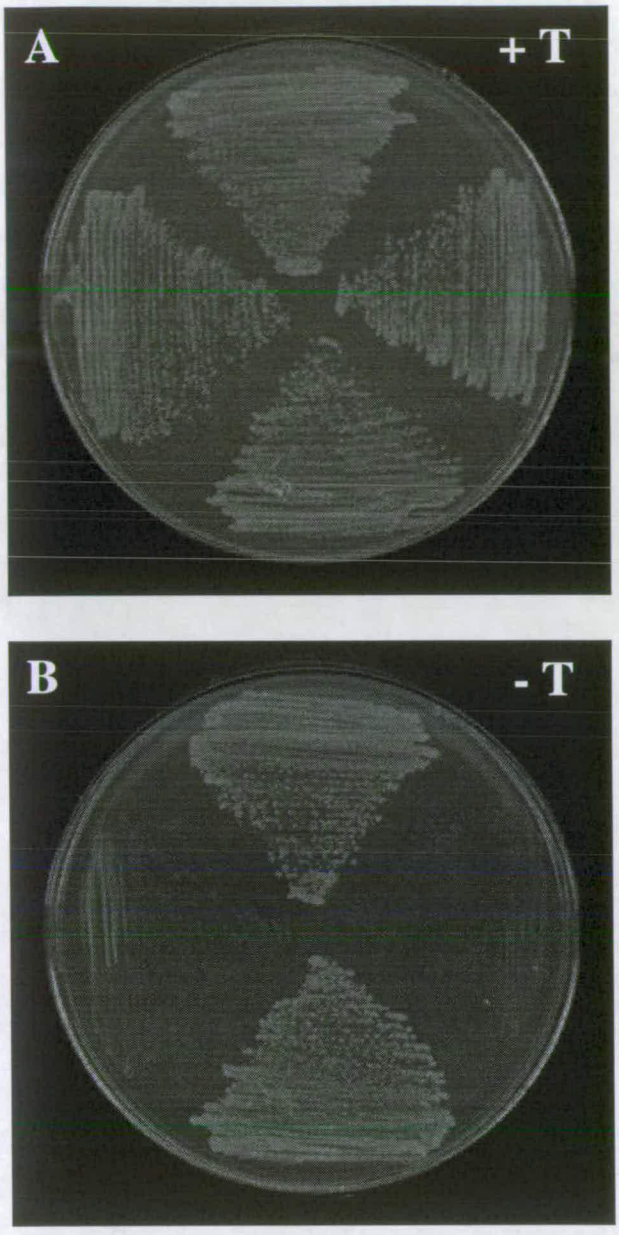


Fig 6.4 Effects of *cdc37*$^{+}$ overexpression in two *cdc13 ts S.pombe* strains

Two *cdc13 ts* strains (*cdc13-117* and *cdc13-9*) were transformed with the pREP1.*cdc37*$^{+}$ORF plasmid and empty pREP1 as a control. The strains were then grown at various temperatures in the presence (promoter repressed) or absence (promoter derepressed) of 4 μg/ml thiamine. The transformants were patched on to appropriately supplemented minimal media at 28°C, 32°C, 33.5°C or 35°C for 24 hr, streaked to single colonies and incubated for a further 3 days when visible single colonies were formed. Plate A shows the colony formation of the strain grown at 32°C in the presence of thiamine and plate B in the absence of thiamine. On both plates, the strains are, clockwise from top: *cdc13-117* (pREP1), *cdc13-117* (pREP1.*cdc37*$^{+}$ORF), *cdc13-9* (pREP1), *cdc13-9* (pREP1.*cdc37*$^{+}$ORF).

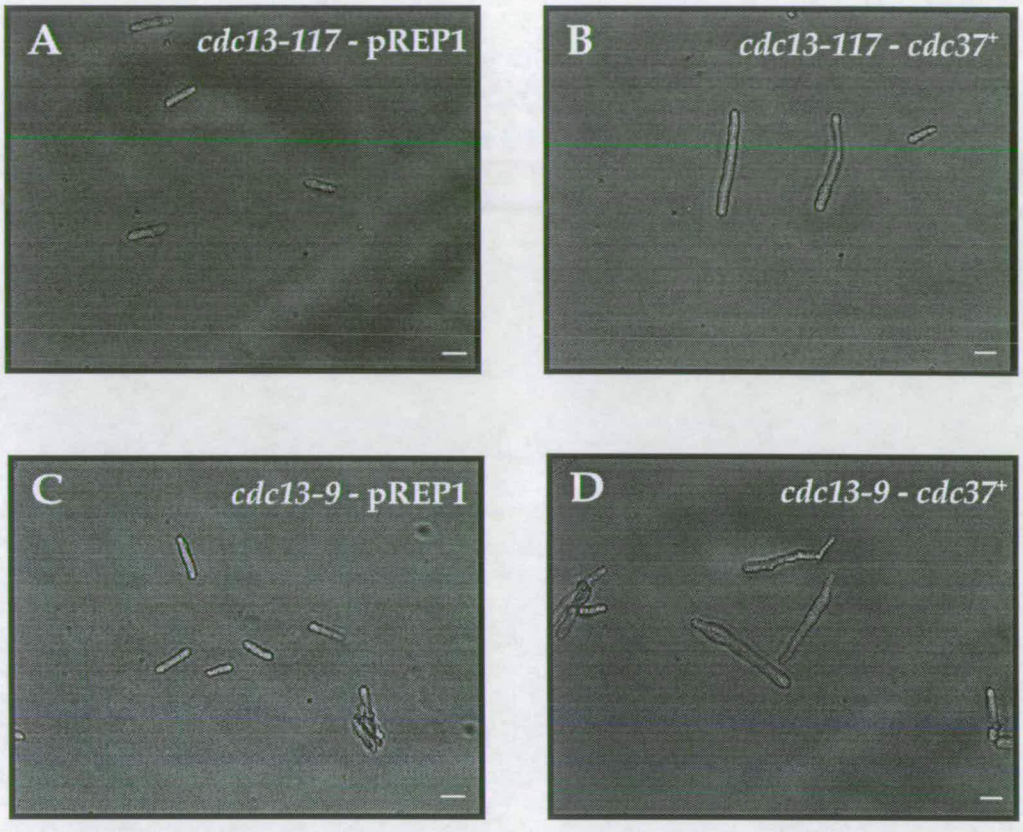


Fig 6.5 Effects of *cdc37*$^+$ overexpression in two *cdc13 ts S.pombe* strains

Two *cdc13 ts* strains (*cdc13-117* and *cdc13-9*) were transformed with the pREP1.*cdc37*$^+$ORF plasmid and empty pREP1 as a control. The strains were then grown at various temperatures in the presence (promoter repressed) and absence (promoter derepressed) of 4 μg/ml thiamine. The transformants were patched on to appropriately supplemented minimal media at 28°C, 32°C, 33.5°C or 35°C for 24 hr, used to inoculate a liquid culture and incubated for a further 24 hr. The panels above show the morphology of cells grown at 32°C in the absence of thiamine. Panel A shows *cdc13-117* (pREP1) cells, panel B *cdc13-117* (pREP1.*cdc37*$^+$ORF), panel C *cdc13-9* (pREP1) and panel D *cdc13-9* (pREP1.*cdc37*$^+$ORF) cells. The white bar in the bottom right hand corner represents 10 μm.

Most of the 8 strains containing *cdc2*^{ts} alleles were also affected by overexpression of *cdc37*$^{+}$ but to a lesser degree than with the *cdc13*^{ts} mutants (Table 6.1, Table 6.2). The two *cdc2*^{ts} alleles where the overexpression of *cdc37*$^{+}$ had most effect were *cdc2-33* and *cdc2-L7* (Fig 6.6, Fig 6.7). In these mutants, at all temperatures the colony formation of the strains was affected by overexpression of *cdc37*$^{+}$, though to a lesser degree than the *cdc13*^{ts} mutants (Fig 6.6). When examined under the microscope, the *cdc2*^{ts} cells overexpressing *cdc37*$^{+}$ were much more elongated and branched than those without overexpression, similar to the *cdc13*^{ts} mutants (Fig 6.7). This is an interesting observation; although the elongation of both the *cdc13*^{ts} and the two *cdc2*^{ts} mutants were both made considerably and comparably worse by *cdc37*$^{+}$ overexpression, the ability to form colonies was only slightly affected in the *cdc2*^{ts} mutants, whilst being drastically affected in the *cdc13*^{ts} mutants. For clarity, a diagram showing the location of mutations of the various *cdc2*^{ts} alleles tested is also included (Fig 6.8).

6.4. Creation of a *Δcdc37* strain expressing a C-terminally His-tagged Cdc37 protein

In order to find out whether there is any physical interaction of Cdc37 with the Cdc2/Cdc13 complex, reagents were prepared for attempting immunoprecipitation of these proteins. Due to the time constraints, a His-tagged version of the Cdc37 protein was constructed. As described in Chapter 3, the N-terminus of the Cdc37 proteins is highly conserved in all species to date, so the His6-tag was engineered on to the C-terminus.

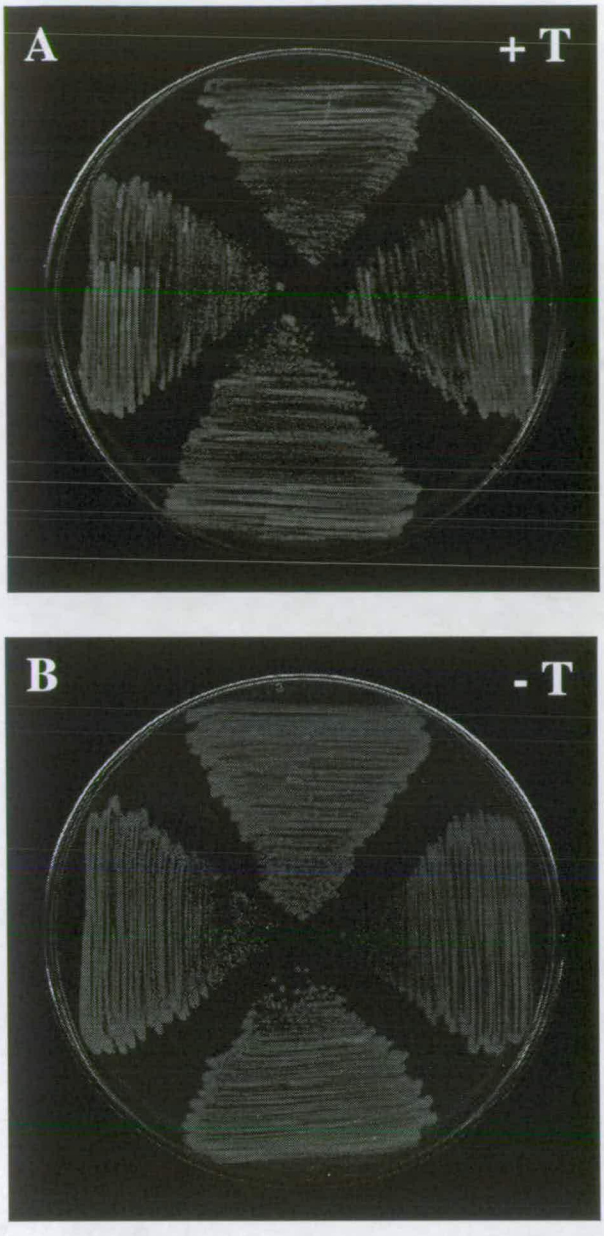


Fig 6.6 Effects of *cdc37*$^{+}$ overexpression in two *cdc2 ts S.pombe* strains

Two *cdc2 ts* strains (*cdc2-33* and *cdc2-L7*) were transformed with the pREP1.*cdc37*$^{+}$ORF plasmid and empty pREP1 as a control. The strains were then grown at various temperatures in the presence (promoter repressed) and absence (promoter derepressed) of 4 μg/ml thiamine. The transformants were patched on to appropriately supplemented minimal media at 28°C, 32°C, 33.5°C or 35°C for 24 hr, streaked to single colonies and incubated for a further 3 days when visible single colonies were formed. Plate A shows the colony formation of the strain grown at 32°C in the presence of thiamine and plate B in the absence of thiamine. On both plates, the strains are, clockwise from top: *cdc2-33* (pREP1), *cdc2-33* (pREP1.*cdc37*$^{+}$ORF), *cdc2-L7* (pREP1), *cdc2-L7* (pREP1.*cdc37*$^{+}$ORF).

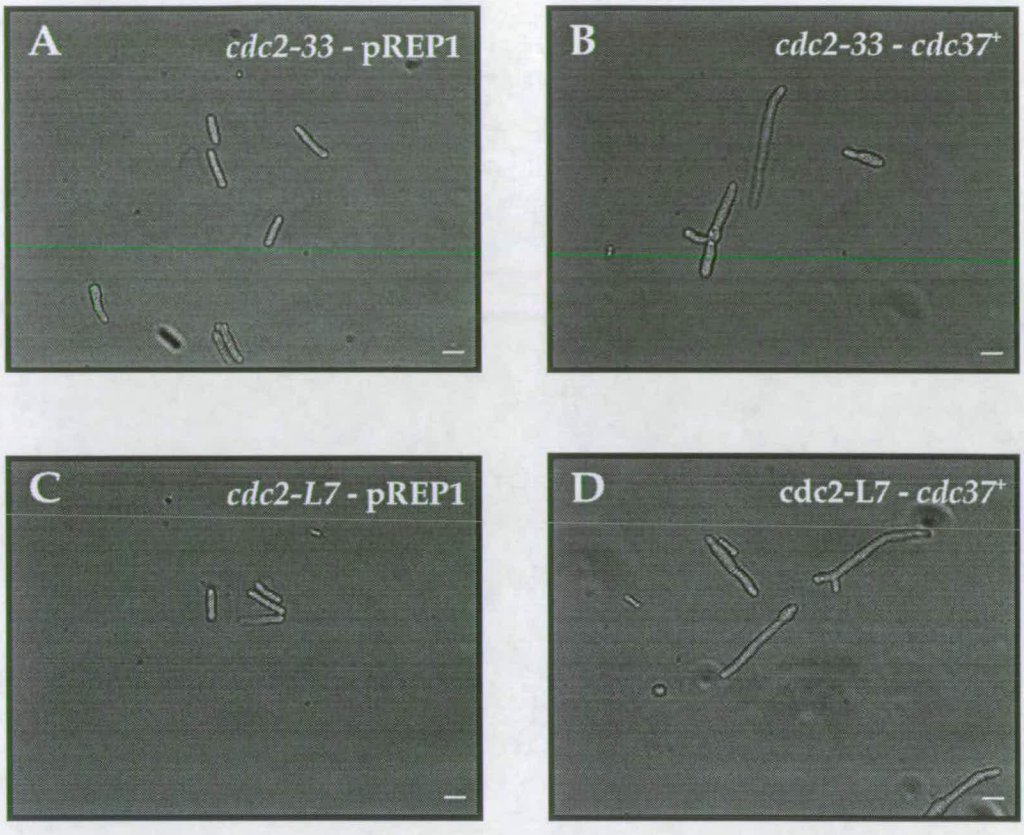
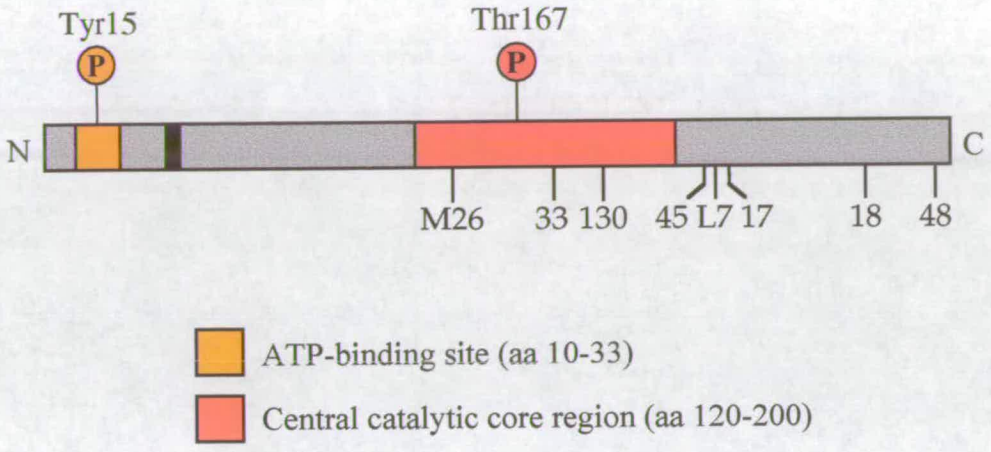


Fig 6.7 Effects of *cdc37*$^+$ overexpression in two *cdc2 ts S.pombe* strains

Two *cdc2 ts* strains (*cdc2-33* and *cdc2-L7*) were transformed with the pREP1.*cdc37*$^+$ORF plasmid and empty pREP1 as a control. The strains were then grown at various temperatures in the presence (promoter repressed) and absence (promoter derepressed) of 4 μg/ml thiamine. The transformants were patched on to appropriately supplemented minimal media at 28°C, 32°C, 33.5°C or 35°C for 24 hr, used to inoculate a liquid culture and incubated for a further 24 hr. The panels above show the morphology of cells grown at 32°C in the absence of thiamine. Panel A shows *cdc2-33* (pREP1) cells, panel B *cdc2-33* (pREP1.*cdc37*$^+$ORF), panel C *cdc2-L7* (pREP1) and panel D *cdc2-L7* (pREP1.*cdc37*$^+$ORF) cells. The white bar in the bottom right hand corner represents 10 μm.



Allele	Position (aa)	Mutation	Defect
cdc2-33	177	Ala-Thr	G1/G2 arrest
cdc2-L7	210	Phe-Leu	G1/G2 arrest
cdc2-45	208	Pro-Ser	G1/G2 arrest
cdc2-17	212	Gly-Ser	G2 arrest
cdc2-48	309	Tyr-His	G1/G2 arrest
cdc2-18	269	Leu-Ser	G2 arrest
cdc2-130	183	Gly-Glu	G1/G2 arrest
cdc2-M26	137	Pro-Ser	G1/G2 arrest

Fig 6.8 Location of mutations and phosphorylation sites of *S.pombe* Cdc2

8 strains containing independent mutant alleles of *cdc2* were investigated in this study. The schematic representation of Cdc2 shows the location of the mutations and the two phosphorylation sites, tyrosine 15 and threonine 167. The ATP-binding site is shown in orange and the catalytic region in red. Also shown in black is the region containing the PSTAIR motif. This sequence is present in all CDKs and is the antigenic region recognisable by the antibody used to detect Cdc2 in this study.

6.4.1 Construction of a *cdc37*$^+$(His6) expression construct

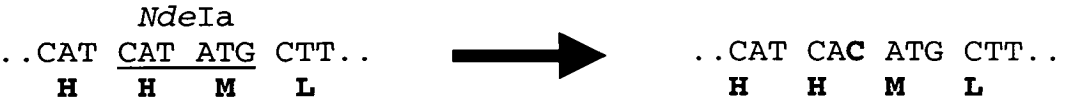
To create a Cdc37 protein with a C-terminal His6-tag, the *cdc37*$^+$ ORF was cloned into pREP1(His6), a plasmid derived from pREP1. The pREP1(His6) plasmid was created by replacing the *Sma*I site of pREP1 (Appendix 1E) with a *Not*I site followed by the sequence that encodes 6 histidine residues and a stop codon (I.Samejima, pers. comm.). To create a His-tagged protein, an ORF with the stop codon removed can be inserted into the *Nde*I-*Not*I sites in-frame with the His6-stop sequence, resulting in the expression of a C-terminal His-tagged protein.

In order to modify the 3' end of the *cdc37*$^+$ ORF for cloning into the pREP1(His6) plasmid, site-directed mutagenesis was carried out. At the same time time as carrying out this 3' end modification, the two internal *Nde*I (*a* and *b*, Fig 3.4) sites were also removed to ease further cloning in the future. To carry out the mutagenesis, the 1167 bp *Bam*HI-*Sma*I fragment of the *cdc37*$^+$ ORF (Appendix 1B) was cloned into the corresponding sites in the plasmid pTZ18u (Appendix 1F), which allows production of single-stranded DNA (section 2.4.5). The two *Nde*I sites were removed by single-stranded site-directed mutagenesis (section 2.2.9a) by a single base pair change in each case, mutating the recognition site whilst at the same time conserving the amino acid sequence (Table 6.3). This mutagenesis proved extremely troublesome and for modification of the 3' end a different mutagenesis technique was used.

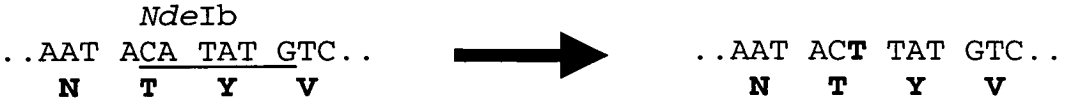
The 3' end of the *cdc37*$^+$ ORF was modified to allow cloning into the pREP1(His6) plasmid. The plasmid pTZ18u.3'*cdc37*$^+$ORF (Appendix 1F) containing the modified ORF (lacking the two internal *Nde*I sites) was modified at the 3' end by double-stranded site-directed mutagenesis (section 2.2.9b). The stop codon and 3' *Sma*I site were replaced with a *Not*I site (Table 6.3). When the resulting 1164 bp *Bam*HI-*Not*I modified fragment was cloned into the corresponding sites in the pREP1(His6) plasmid, this resulted in a sequence encoding 6 histidines and a stop

Table 6.3 Sequence changes created by site-directed mutagenesis

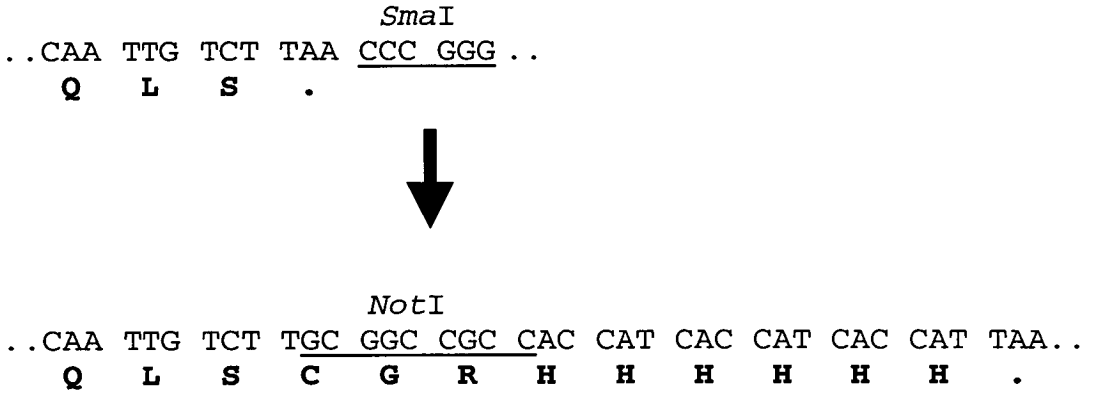
***Nde*Ia site (PW19)**



***Nde*Ib site (PW20)**



3' end (PW27+28)



The two internal *Nde*I sites (*a* and *b*) were removed from the sequence for future ease of cloning (see Fig 3.4 for sequence). This was carried out by single-stranded site-directed mutagenesis. The single base pair change in each case removed the *Nde*I recognition site but the amino acid sequence was preserved. The 3' end of the ORF was modified to allow cloning into the His-tag pREP1 plasmid. This was carried out by double-stranded mutagenesis. This resulted in the stop codon and *Sma*I site being replaced with a *Not*I site followed by a run of sequence encoding 6 histidine residues and a stop codon.

codon being added to the end of the modified *cdc37*$^+$ ORF. Finally, the remaining 238 bp 5' *Nde*I-*Bam*HI fragment of the *cdc37*$^+$ ORF (Appendix 1C) was then cloned into the pREP1(His6) vector to create the pREP1.*cdc37*(His6) construct (Appendix 1G).

6.4.2 Creation of a *Δcdc37* strain expressing a C-terminally His-tagged Cdc37 protein

A *Δcdc37* strain expressing pREP1.*cdc37*(His6) was created exactly the same way as the *Δcdc37* strains expressing the *cdc37*$^+$ ORF from the pREP vectors described in Chapter 4 (section 4.3). The *Δcdc37/cdc37*$^+$ heterozygous diploid 1 was transformed with the pREP1.*cdc37*(His6) construct (section 2.5.5). Diploid transformants were selected on minimal plates with no supplements. The resultant diploids were then allowed to sporulate by incubating on ME plates at 25°C for 48 hr. Random spore analysis was then carried out (section 2.5.3b) and *Δcdc37* haploids (containing *ura4*$^+$) were selected for on minimal plates containing adenine and leucine. These haploids were then tested on minimal plates supplemented only with adenine for the presence of the leucine marker carried on the plasmid. Again, every haploid isolated that carried the *ura4*$^+$ marker for *cdc37*$^+$ deletion also contained the leucine marker from the pREP1 plasmid expressing *cdc37*(His6). Thus, the *Δcdc37* haploids can only survive when maintained by the pREP1.*cdc37*(His6) construct; this indicates that the Cdc37(His6) protein is functional. Furthermore, growing several *Δcdc37* haploids expressing *cdc37*(His6) in the presence or absence of thiamine revealed that this strain behaves in exactly the same manner as the *Δcdc37* strain expressing *cdc37*$^+$ from pREP1 (Fig 4.7).

6.4.3 Testing for expression of the C-terminally His-tagged Cdc37 protein

To confirm that the *Δcdc37* strain containing the pREP1.*cdc37*(His6) plasmid was expressing a His-tagged Cdc37 protein, a Western blot (section 2.3.4) was carried out. The strain was grown in appropriately supplemented minimal medium (in the presence or absence of thiamine) and denatured protein extracted as previously described (section 2.5.9). The blot was probed with an anti-His6 antibody (Santa Cruz) to detect expression (Fig 6.8). A ~60 kDa His-tag protein is expressed in the *Δcdc37* strain containing the pREP1.*cdc37*(His6) plasmid and its expression is thiamine-regulatable. The protein is not expressed in either the *Δcdc37* strain expressing pREP1.*cdc37*$^{+}$ORF or in wild-type extracts. Therefore, this confirmed that the *Δcdc37* strain is expressing a thiamine-regulatable His-tagged Cdc37 protein.

6.5 Immunoprecipitation using Cdc37(His6)

The *Δcdc37*[pREP1.cdc37(His6)] strain was used for immunoprecipitation to investigate any physical interaction of Cdc37 with either Cdc2 and Cdc13. Although the mammalian Cdc37 protein has been shown to associate with Cdk4 (Dai *et al.*, 1996), in *S.cerevisiae*, no physical interaction has been observed between Cdc37 and Cdc28. Therefore, it seemed a very interesting question to find out whether Cdc37 and Cdc2 physically interact in *S.pombe*.

Physical interaction between a His-tagged Cdc37 protein and Cdc2 was investigated by immunoprecipitation using protein extracts from the *Δcdc37* strain expressing cdc37(His6) from pREP1. The strain was grown in appropriately supplemented minimal medium (in the presence or absence of thiamine) and protein extracted as previously described (section 2.5.10). Immunoprecipitation was carried out (section 2.3.1) using the anti-His6 antibody. The immunopreciptated extracts were then

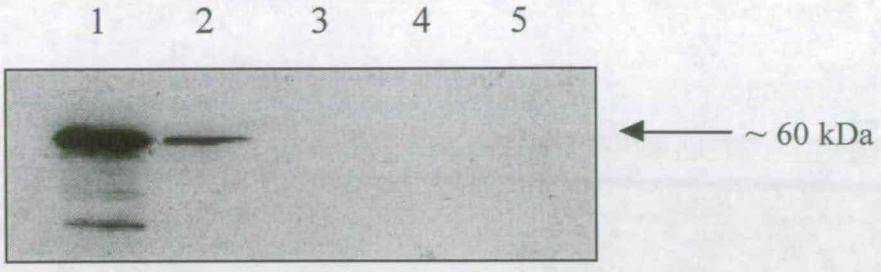


Fig 6.9 Western blot of *Δcdc37* strain expressing *cdc37(His6)* from pREP1

Protein was extracted from *cdc37::ura4*[pREP1.*cdc37(His6)*] and *cdc37::ura4*[pREP1.*cdc37*$^{+}$] cells grown for 24 hr in the presence or absence of thiamine. The extracts were boiled immediately before electrophoresis on a 12.5% polyacrylamide gel and Western blotting on to a nylon filter. The blot shown was probed with anti-His6 (1:1000). Lane 1 contains the extract from the *cdc37::ura4*[pREP1.*cdc37(His6)*] cells grown in the absence of thiamine and lane 2 the extract from the cells grown in the presence of thiamine. Lane 3 contains the extract from the *cdc37::ura4*[pREP1.*cdc37*$^{+}$] cells grown in the absence of thiamine and lane 4 the extract from the cells grown in the presence of thiamine. Lane 5 contains the protein extract from a wild-type strain as a further control.

anti-His6

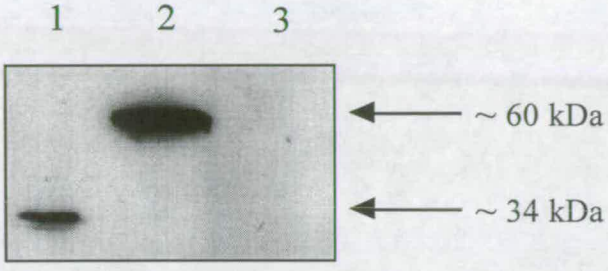


Fig 6.10 Western blot of extracts from immunoprecipitation with anti-HIS6 antibody

Protein complexes were extracted from *cdc37::ura4*[pREP1.*cdc37*(His6)] cells grown in the absence of thiamine. Immunoprecipitation was then carried out using a 1:100 dilution of the anti-His6 antibody. The blot shown was probed with anti-His6 (1:1000) and anti-PSTAIR (1:50) simultaneously. Lane 1 contains the total protein extract from a wild-type strain as a control for the anti-PSTAIR antibody. Lane 2 contains the immunoprecipitated extract from the *cdc37::ura4*[pREP1.*cdc37*(His6)] cells grown in the absence of thiamine, and lane 3 contains the no antibody immunoprecipitation control.

analysed by Western blot (section 2.3.4) and probed with the anti-PSTAIR antibody (Fig 6.9). However, despite numerous attempts with varying conditions, no binding of Cdc2 to Cdc37 was seen.

Though the physical interaction may be less likely since no interaction between Cdc37 and a cyclin has been observed in any other system, investigation of the binding of Cdc13 with Cdc37 would also have been a worthwhile experiment. However, as previously mentioned, neither of the available anti-Cdc13 antibodies was found to detect Cdc13 in this lab.

6.6 Summary

As a preliminary investigation into the role of *cdc37*$^{+}$ in the *S.pombe* cell cycle, Cdc2 protein levels were monitored in a Cdc37 depletion experiment. Cdc2 levels were found to be significantly reduced in a *Δcdc37* strain expressing the *cdc37*$^{+}$ ORF from the pREP81 promoter grown in conditions where *cdc37*$^{+}$ expression is repressed.

When *cdc37*$^{+}$ is strongly overexpressed (from pREP1 promoter) in *S.pombe* strains containing *ts* alleles of *cdc2* or, more strikingly, *cdc13*, the phenotypes of the cells become more exaggerated. In some *cdc2*^{ts} alleles, there is a moderate effect, in others there is none, suggesting that *cdc37*$^{+}$ does interact with *cdc2* in an allele-specific manner to some degree. However, the locations of the mutations in the *cdc2*^{ts} alleles give no clue to the reason why *cdc37*$^{+}$ overexpression is detrimental to the *cdc2*^{ts} mutants. Also, in two independent *cdc13*^{ts} alleles, overexpression of *cdc37*$^{+}$ drastically reduces the restrictive temperature of the strains so even at the normally permissive temperature, the cells are unable to divide and proliferate normally.

These observations appear to show real interactions since overexpression of *cdc37*$^{+}$ in a wild-type strain has little or no detectable effect.

These preliminary analyses, together with the data from Chapter 5, seem to indicate that Cdc37 is involved in some way in the Cdc2/Cdc13 complex which regulates the cell cycle through G2 and mitosis in *S.pombe*. However, preliminary experiments failed to show any physical interactions of a His-tagged Cdc37 protein with Cdc2.

Chapter 7

Discussion

7.1 Introduction

The work in this thesis has identified a gene encoding a *S.pombe* Cdc37 protein which has been cloned and has undergone preliminary characterisation. The *cdc37*$^{+}$ gene has been shown to be essential for viability, and appears to have several roles *in vivo*. This work has begun to investigate these various functions in *S.pombe*, with particular emphasis on those functions involving the key mitotic regulator, Cdc2 and its G2 cyclin partner, Cdc13 (see Chapter 1).

7.2 Cdc37 sequence and relation to its function

The homologues of *S.cerevisiae* Cdc37p from various species, including *Drosophila*, human, rat, mouse and chicken, all share only moderate sequence similarity across the whole length of the proteins. However, all of the protein sequences are highly conserved at the extreme N-terminus. This project has revealed another structural homologue of Cdc37p, from *S.pombe*, which shares the same sequence conservation and is presently the most closely related to *S.cerevisiae* Cdc37p (Fig 3.6). Analysis of the Cdc37 protein sequences from the variety of species already mentioned shows that not only are the *S.pombe* and *S.cerevisiae* proteins most similar to each other but also that the *Drosophila* and vertebrate are more similar to each other than they are to the two yeast proteins (Fig 3.7). This becomes more interesting when taken together with the observation that the *D.melanogaster cdc37* gene has been shown to rescue the *S.cerevisiae ts* mutant *cdc37-1* (Cutforth and Rubin, 1994), whilst there is no evidence of rescue from the vertebrate homologues. It is not particularly clear whether this is because experiments to test complementation of the *cdc37-1* mutant by mammalian homologues have been unsuccessful or whether the question has not been investigated thoroughly. However, the only publication that appears to directly address this question (Ozaki *et al.*, 1995), reports that a clone containing the rat

cdc37 gene has been tested and does not rescue the temperature sensitive phenotype of the *cdc37-1* mutant. The ability of the *S.pombe cdc37*$^+$ gene to rescue the *S.cerevisiae cdc37-1* mutant has not been tested (see section 7.6.2). Due to the higher degree of similarity between the yeast proteins compared to that between the *S.cerevisiae* and *D.melanogaster* proteins, it might be expected that the *cdc37*$^+$ gene will rescue the restrictive phenotype of the *cdc37-1* mutant.

As mentioned above, the N-terminus of the Cdc37 proteins is the most highly conserved region. Over the first 40 amino acids the *S.cerevisiae* and *S.pombe* Cdc37 proteins are ~85% identical (Fig 3.6). The Cdc37 proteins in multicellular organisms are also highly similar to each other in this region and slightly less similar to the yeast proteins. Since the N-terminus is highly conserved among all of the species, it seems probable that it plays an essential role. Although little is known about the structure of the Cdc37 proteins in relation to its function, it has been shown that the N-terminus of mammalian Cdc37 binds the kinase Raf-1 *in vitro*, whilst the C-terminus binds Hsp90 (Grammatikakis *et al.*, 1999). Given that the N-terminus is so highly conserved, this suggests that in all species the N-terminus may bind the target kinases, although there is no other evidence to support this theory to date.

7.3 The effects of loss of *cdc37*$^+$ function

The work in this thesis has shown that loss of *cdc37*$^+$ gene function in *S.pombe* is lethal, consistent with the observation that *CDC37* is an essential gene in *S.cerevisiae* (Gerber *et al.*, 1995). Furthermore, the depletion experiments described in Chapter 5 show that some of the primary effects of reduced Cdc37 protein levels affect cell cycle progress. However, there is evidence that indicates that *S.pombe* can survive even with only very low levels of Cdc37. For example, the analysis of *Δcdc37* haploid strains carrying pREP plasmids in Chapter 4 shows that *cdc37*$^+$ mRNA

levels well below that of wild-type are sufficient to maintain normal proliferation (Fig 4.9), suggesting that wild-type *S.pombe* express far more *cdc37*$^{+}$ than is necessary for survival. It may be that some of the functions of Cdc37 are not crucial and when levels are low, the cells are able to do without or find replacement proteins to carry out the necessary roles. Also, further evidence indicating that only low levels of Cdc37 protein are required is provided by the depletion experiments described in Chapter 5, where the *Δcdc37* haploid strain is able to survive and grow/divide for several generations once *cdc37*$^{+}$ expression from pREP81 has been repressed (Fig 5.1). Assuming that repression of the promoter inhibits almost all *cdc37*$^{+}$ expression and even allowing for plasmid copy number heterogeneity, it is likely that the Cdc37 protein is stable and probably only required at low levels.

When the expression of *cdc37*$^{+}$ from the pREP81 promoter is repressed in a *Δcdc37* *S.pombe* strain, a sub-population of short cells appear and after 16 hours, some are extremely short (Fig 5.2, Fig 5.3). Cells with this phenotype are present throughout the duration of the experiment. Despite the reduction in the mean cell length caused by the presence of these cells, the rate of proliferation appears to be similar, if not slightly higher, than control cells expressing *cdc37*$^{+}$ from pREP81 (Fig 5.1). It is difficult to conclude whether the appearance of short cells is a direct effect of Cdc37 depletion or whether there are secondary effects that cause this phenotype. Two explanations are that depletion of Cdc37 affects target kinase(s) that cause the cell length to shorten, either by slowing down growth relative to cell cycle progression, or by affecting kinase(s) involved in the cell cycle. A potential target could be the inhibitory kinase Wee1, which has already been shown to require Swo1 (Hsp90) for function (Aligue *et al.*, 1994). If Wee1 protein function is only moderately affected by a depletion in Cdc37 levels, or even a complete lack of Cdc37, then the cells would become shorter due to premature entry into mitosis (see Chapter 1) but would continue to proliferate normally.

After expression of *cdc37*$^+$ from pREP81 has been repressed for around 30 hours in the *Δcdc37* strain, a significant population of the cells begin to elongate noticeably (Fig 5.2, Fig 5.3). It is shortly after this that overall cell proliferation begins to decline (Fig 5.2). It therefore seems a reasonable conclusion that these two events are directly related. The data from the depletion experiments described in Chapter 5 suggest that the cell elongation is due to a defect in the G2 or M phase of the cell cycle. From work carried out on other systems (see Chapter 1), one explanation for the observations described is that the depletion of Cdc37 begins to affect the function of Cdc2. If Cdc2 stability and/or kinase activity was to drop due to a lack of association with Cdc37 then it would seem likely that cell elongation would occur due to a delay in the onset of mitosis (see Chapter 1).

The exact mechanisms behind the all of the observations in the Cdc37 depletion experiment are unclear and are very difficult to explain with the data to date. The heterogeneity within the population suggests that Cdc37 plays several important roles and therefore models that attempt to explain the experimental observations are probably over-simplified. A possible scenario is a combination of that described above. When the expression of *cdc37*$^+$ is repressed, the first target may be a kinase that performs less well when Cdc37 protein levels drop, but retains some sort of functionality. As mentioned above, a possible target could be Wee1. Thus, Wee1 may require high levels of Cdc37 to perform properly and when the levels drop, Wee1 may still be able to play its important role but is less efficient in doing so, leading to shorter cells. If Cdc37 preferentially binds to Cdc2, then when the levels start to drop, the remaining Cdc37 could remain involved with Cdc2, hence the reason why Wee1 may be affected first. Then, when the Cdc37 levels drop even further, Cdc2 becomes affected as well, which would result in delayed cell cycle progression, elongation of the cells and cessation of proliferation. This would also explain some of the heterogeneity in cell length that is observed. The cells will have different levels of Cdc37, thus varying the time at which Cdc2 begins to be affected

and elongation becomes visible. This is a tentative and over-simplified attempt at finding an explanation for the observations seen in this experiment. Of course, the heterogeneity observed is also probably due in part to other factors such as plasmid copy number.

Previously, it was shown in *S.cerevisiae* that Cdc28p (≡ Cdc2 in *S.pombe*) levels are three to five times lower in the *cdc37-1* mutant (Gerber *et al.*, 1995). Recent work has suggested that the low Cdc28p levels are due to instability of the protein caused directly by of a lack of Cdc37p (Farrell and Morgan, 2000). This, together with the idea that cell elongation described in Chapter 5 may be due to a lack of interaction between Cdc37 and Cdc2 in *S.pombe*, led to the Cdc2 protein levels being analysed in the depletion experiment. The Cdc2 levels were found to decrease as Cdc37 is depleted (Fig 6.1), consistent with the idea that an interaction between Cdc37 and Cdc2 is necessary for the stability of Cdc2. This observation agrees well with that seen in *S.cerevisiae* as mentioned above. A lack of stability of Cdc2 when Cdc37 is depleted would result in a gradual decrease in Cdc2 protein levels as Cdc37 is depleted from the cell. Therefore, it may be that once the Cdc2 protein levels drop to a point at which the cell can no longer maintain viability, cell cycle arrest occurs either in G2 or mitosis and thus proliferation ceases. However, it is very difficult to confirm or disprove that the drop of Cdc2 protein levels seen in the depletion experiment is enough to account for the arrest. Also, if cell cycle arrest was solely due to a lack of Cdc2p, a more uniform G2 arrest would be expected rather than a mixture of cells arrested at G2 or mitosis as seen in these experiments (Fig 5.5 - 5.6).

7.4 Proteins that associate with Cdc37

From work already described in other systems (Chapter 1), together with the cell cycle observations gathered in Chapter 5 of this thesis, it seems likely that *S.pombe*

Cdc37 is involved in the association between Cdc2 and Cdc13 (see Chapter 1). As discussed above, it appears that Cdc37 has several biological roles and is likely to be involved in mediating the association of several kinase complexes other than that of Cdc2 with Cdc13, including other Cdc2 complexes. The major role of the Cdc2/Cdc13 complex is entry into and during mitosis (see Chapter 1) and therefore this is potentially the most interesting interaction of Cdc37. For this reason, the work described in Chapter 6 investigates the relationship specifically between Cdc37 and the Cdc2/Cdc13 complex.

It has been shown in *S.cerevisiae* that Cdc37p appears to assist the folding of Cdc28p and may not directly interact with the associated cyclins at all (Farrell and Morgan, 2000). The conclusions by the authors of this work were that Cdc37p binds only to Cdc28p - not the cyclins Cln2p or Clb2p - thus holding it in a conformation that is accessible to the cyclins and aiding the formation of the CDK/cyclin complexes. These conclusions are supported by work carried out in mammalian systems which appears to suggest that mammalian Cdc37 specifically interacts with Cdk4 and Cdk6 but not with their associated cyclins (Stepanova *et al.*, 1996). Also, in *S.pombe*, genetic interactions between Swo1 and Cdc2 have previously been documented (Munoz and Jimenez, 1999). With these observations in mind, it seems more likely that in *S.pombe*, Cdc37 will interact with Cdc2 but not with Cdc13. Therefore, it is an interesting observation that overexpression of $cdc37^{+}$ appears to have a more marked effect on $cdc13^{ts}$ mutants than it does on $cdc2^{ts}$ mutants (Fig 6.5 - 6.8). It is also interesting that this overexpression enhances the mutant phenotypes, rather than partial or complete rescue as might be expected.

One model which appears to explain the overexpression observations described in section 6.3, would be if the function of Cdc37 is to bind and hold Cdc2 in an activatable conformation as is believed for *S.cerevisiae* (Farrell and Morgan, 2000). If this were true, then in the case of both the $cdc13^{ts}$ and $cdc2^{ts}$ mutants, the excess

Cdc37 around may be binding excessively to Cdc2 and preventing the Cdc2/Cdc13 interaction. The *cdc13^{ts}* mutants might be affected because the mutant Cdc13 proteins may not be able to bind the wild-type Cdc2 as strongly as wild-type Cdc13. The *cdc2^{ts}* mutants may be less affected because Cdc37 cannot bind as well to the affected mutant forms of Cdc2, therefore interfering less with the Cdc2/Cdc13 interaction. If this was the case, then the phenotypes of *cdc37^{+}* overexpression in *cdc2^{ts}* mutants would be expected to be allele-specific and this is true to a certain extent, where the mutant strains *cdc2-33* and *cdc2-L7* are most affected (Tables 6.1 and 6.2). The model described here is extremely tentative and there are other ways to explain the *cdc37^{+}* overexpression observations described in section 6.3. The model described above may well oversimplify the underlying mechanisms. It is interesting to note, however, that one of the *cdc2^{ts}* alleles (*cdc2-33*) most affected by *cdc37^{+}* overexpression (section 6.3) has also been shown to be synthetically lethal with a mutant allele of *swo1^{+}* (encoding Hsp90, Munoz and Jimenez, 1999) and is severely affected by overexpression of *wos2^{+}*, a homologue encoding a cochaperone of Hsp90 (Munoz *et al.*, 1999).

Also described in Chapter 6 is the observation that a his-tagged version of the Cdc37 protein does not appear to immunoprecipitate with Cdc2 (Fig 6.9). As has been previously mentioned, it is thought that the conserved N-terminus of Cdc37 is responsible for the interaction with the target protein kinase (Grammatikakis *et al.*, 1999), although, Cdc37 proteins with an N-terminal tag have previously been used successfully (Grammatikakis *et al.*, 1999). For this reason, the his-tag for the *S.pombe* Cdc37 protein used in this work was placed at the C-terminus to avoid any disruption of this interaction. The lack of physical evidence in *S.pombe* is disappointing, since a direct interaction between Cdc2 and Cdc37 would have been an important discovery and naturally there remains a question of the clarity of this negative result. Although to date, there is significant physical evidence of mammalian Cdc37 directly interacting with CDKs (Dai *et al.*, 1996; Stepanova *et al.*, 1996;

Mahony *et al.*, 1998) there has been no evidence of a physical interaction between Cdc28 and Cdc37 in *S.cerevisiae*. Maybe this is because in yeast the interaction is more transient. This could also be the reason why it was not possible to show a physical interaction between the his-tagged Cdc37 protein and wild-type Cdc2 in *S.pombe*.

As mentioned in Chapter 1, Hsp90 has usually been found in mammalian Cdc37-Cdk4/Cdk6 complexes (Hunter and Poon, 1997). For this reason, it seems reasonable to predict that Cdc37 will interact with Swo1 (Hsp90) in *S.pombe*. The only Swo1 reagent that was available during this project was the *swo1-26^{ts}* mutant and overexpression of *cdc37^{+}* appeared to have no effect on the phenotype of this mutant (section 6.3). Also, the cell cycle protein Wee1 has been shown to require Swo1 interaction for function (Aligue *et al.*, 1994). Therefore, we also tested the *wee1-50^{ts}* mutant by overexpression of *cdc37^{+}* and again there was no obvious effect (section 6.3). If normal function of *wee1^{+}* requires the formation of a Wee1/Hsp90/Cdc37 complex, then it might have been expected that overexpression of *cdc37^{+}* might partially rescue a *wee1^{ts}* mutant unless the mutant is completely without function at the restrictive temperature. Obviously, the observations described when *cdc37^{+}* is overexpressed in the *swo1^{ts}* and *wee1^{ts}* mutants do not rule out possible interactions of Cdc37 with either of these two proteins, but additional reagents such as tagged proteins and antibodies are required for further investigation.

7.5 The biological roles of Cdc37

The work in this thesis suggests that in *S.pombe*, Cdc37 has several biological roles. To separate and identify these roles will take a great deal of careful investigation and is the next logical step in this project. This work does strongly suggest however, that one of the most important functions of Cdc37 is its involvement with the interaction

between Cdc2 and Cdc13, and thus entry into and during mitosis. The most likely model is that Cdc37 acts as a molecular chaperone, binding and stabilising Cdc2, preventing aggregation or misfolding of the kinase and thus allowing association with Cdc13 to form the active complex when it is required (see Chapter 1). Other potential targets of Cdc37 are fairly broad but evidence from other systems suggests strongly that the majority of these will be specific protein kinases, some of which may also be involved in the cell cycle (see Chapter 1). It will be interesting to find out whether Cdc37 performs some or all of these functions as part of a complex with Hsp90 or whether it has some functions that it carries out alone (Kimura *et al.*, 1997).

7.6 Further research for this project

7.6.1 Immediate experiments

This project has reached an exciting stage where many paths can be followed to investigate further the biochemical and cellular role of Cdc37 in *S.pombe*. One experiment that could be carried out without too much preparation would be to carry out a spore germination experiment similar to the depletion experiment described in Chapter 5. This may result in a less heterogenous population and the phenotypes may be easier to identify. In the depletion experiment factors such as plasmid number and stability may affect the resultant phenotypes and these would be eliminated in a spore germination experiment. Also, further staining including that of the microtubules and spindle pole bodies could also help to identify the phenotypes more specifically. From the DAPI staining described in section 5.5, it appears that a population of the Cdc37 depleted cells arrest with condensed chromosomes, suggesting a mitotic defect. Staining the microtubules and spindle pole bodies may help to confirm this observation and narrow down the exact role of Cdc37. However,

to carry out most of these next stages, more reagents are required. This section describes the questions that are of most interest in the future.

7.6.2 Testing the complementation between the *S.pombe* and *S.cerevisiae* homologues

It is an interesting question to find out whether the *S.pombe cdc37*$^{+}$ gene rescues the defects of the previously described *S.cerevisiae* mutant *cdc37-1*. Obviously, the reciprocal of this experiment could be carried out, whether a *S.pombe cdc37*^{ts} mutant could be rescued by the *S.cerevisiae CDC37* gene. The results of this experiment would probably not be hugely informative since the *D.melanogaster cdc37* gene rescues the *cdc37-1* mutant but the vertebrate homologues do not appear to, even though the *Drosophila* gene is more similar in sequence to the mammalian genes than the *S.cerevisiae* gene.

7.6.3 Construction and examination of a *S.pombe cdc37*^{ts} mutant

So far, the approaches described in this thesis have been informative about the effects of gradual removal of Cdc37 from the cell. A better insight into the roles of a gene such as *cdc37*$^{+}$ could be obtained by examining the behaviour of a *ts* mutants. The advantage of this is that the gene product is rapidly inactivated and the observed phenotype reflects the immediate consequences of this, without allowing the cells time to adapt to the lack of Cdc37 as might happen in depletion experiments. The generation of a *cdc37*^{ts} mutant is a high priority for the next stages of this project.

7.6.4 The cell cycle targets of Cdc37 and investigation of its activity throughout the cell cycle

The work carried out in this thesis has shown that in *S.pombe*, Cdc2 is a likely target of Cdc37. This could be tested by examining the levels of Cdc2, Cdc13 and the G1 cyclin Cig2, in cells with compromised *cdc37* function. Also, the extent of association between Cdc2 and these two cyclins could be determined by co-immunoprecipitation, and the kinase activities of these complexes could also be analysed. Cdc37 immunoprecipitation experiments in $cdc2^{+}$ and $cdc2^{ts}$ strains could also be carried out to determine whether Cdc37 can be shown to associate directly with Cdc2. It is possible that any interaction of Cdc37 with wild-type Cdc2 may be transient and difficult to detect, whilst mutant proteins may bind more stably as discussed in Hunter and Poon, 1997. Other potential cell cycle targets of Cdc37 include Wee1, which has been previously shown to bind Swo1 (Hsp90), requiring it for normal function (Aligue *et al.*, 1994). This and other interactions could be investigated in a similar manner.

Since $cdc37^{+}$ has been shown to genetically interact with genes involved in the cell cycle and loss of Cdc37 in *S.pombe* affects cell cycle progress, it is possible that Cdc37 abundance or activity may vary periodically during the cell cycle. Therefore, it would be interesting to determine the abundance of Cdc37 mRNA and protein in synchronous cultures generated by elutriation. Also, it would be interesting to investigate whether Cdc37 is subject to control by phosphorylation as this does not appear to have been investigated in other systems. If Cdc37 is phosphorylated and this results in a gel mobility shift, it will be straightforward to ask whether the phosphorylation pattern varies during the cell cycle which may give clues to its possible roles within the cell cycle.

7.6.5 Other cellular targets of Cdc37 and proteins it interacts with

Due to the heterogeneity of the population in the depletion experiments described in this thesis, it seems likely that there are several targets of Cdc37. A genetic screen could be carried out for mutants showing synthetic lethality with a *cdc37* defect, as some of these mutations will probably lie in genes whose products require Cdc37 for function. Following the isolation of synthetically lethal mutations, the corresponding genes could be identified by genetic analysis (such as crossing to candidate genes) and library screening by complementation. For this approach, a $cdc37^{ts}$ mutant would probably be most useful. Other genetic screens include using a $cdc37^{ts}$ strain to screen for genes whose overexpression rescues the lethality at the restrictive temperature of the mutant, thus leading to isolation of Cdc37 target genes directly, or screening for mutants whose growth depends on high level expression of $cdc37^{+}$, which has also been shown to be an effective approach to identifying functionally interacting genes (Cullen *et al.*, 2000).

Direct interactions of Cdc37 could be investigated by two approaches: firstly, the yeast two-hybrid approach using Cdc37 as bait. The second is a more direct biochemical method, where recombinant Cdc37 is attached to a column and extracts from *S.pombe* are passed over it. Proteins that bind could then be subjected to mass spectrometric procedures in order to identify them. Since Cdc37 has been shown to interact directly with Hsp90 and with specific protein kinases, Hsp90 and specific target protein kinases may be isolated by this approach, along with other proteins that may be involved in these complexes.

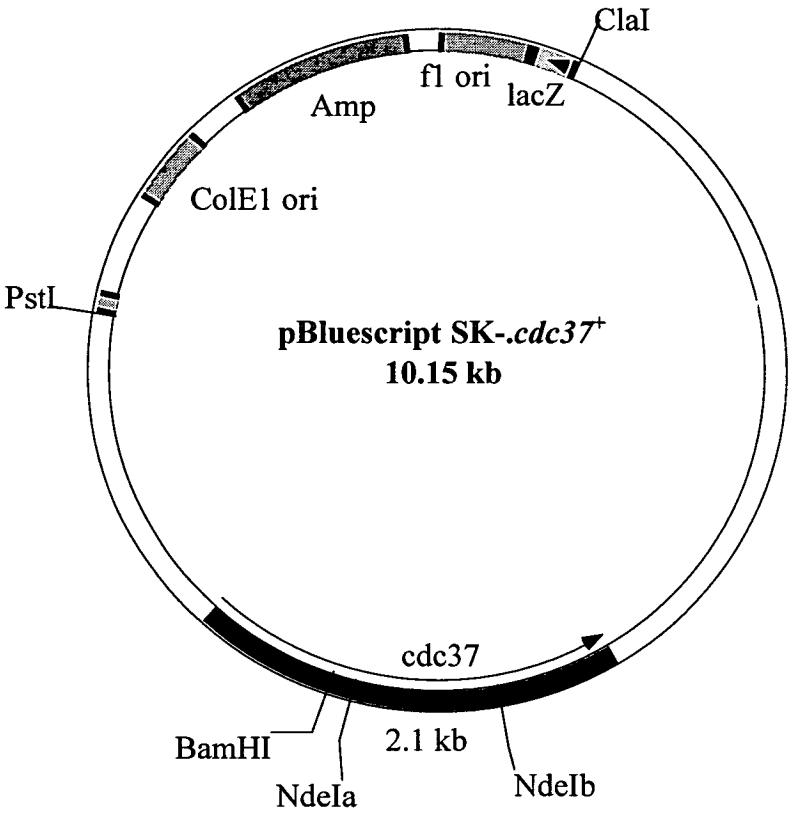
7.6.6 The structure of Cdc37 and relation to its function

Since few structure-function studies have been carried out on any Cdc37 protein, this would also be an interesting point of investigation in *S.pombe*. In at least one system, the N-terminal region of the Cdc37 protein binds to a protein kinase domain and the C-terminal region binds to Hsp90 (Grammatikakis *et al.*, 1999). This could be investigated in *S.pombe* in detail. The regions and residues that are required for interaction with Swo1 (Hsp90) and various protein kinases could be identified if they occur in *S.pombe*. This would then allow further genetic analysis of $cdc37^{+}$ in *S.pombe*, giving further clues to how its structure affects the various biological functions.

Appendix 1

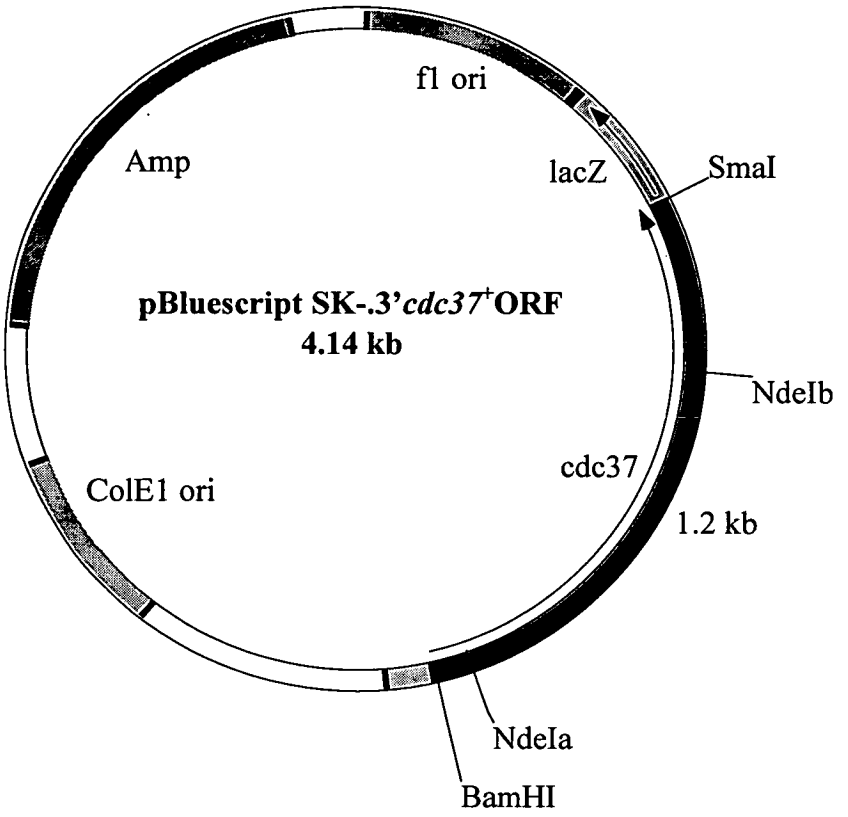
Plasmids and Constructs

A



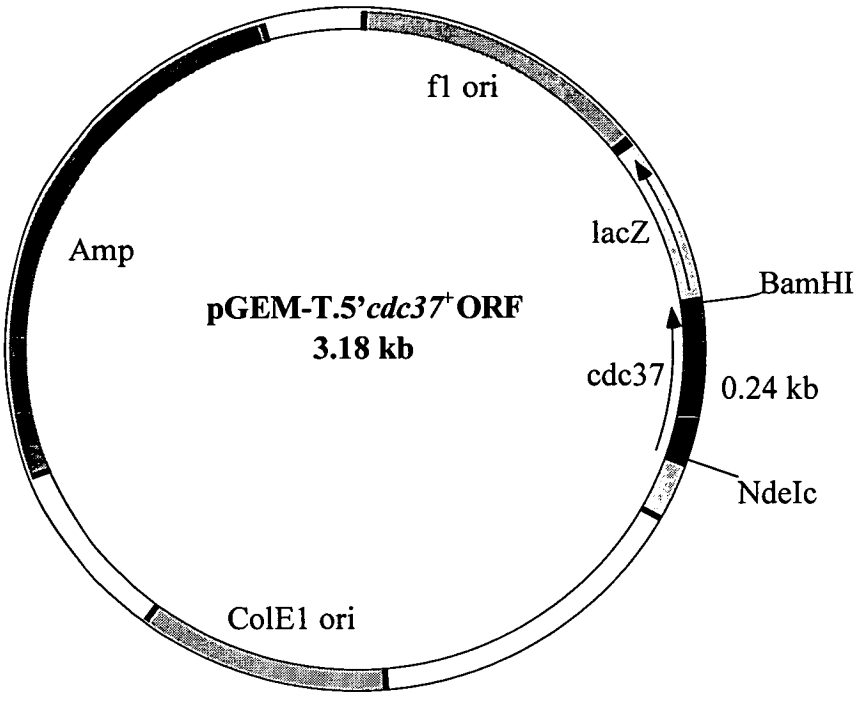
Plasmid pBluescript SK-.*cdc37*$^+$. Described in section 3.3.

B



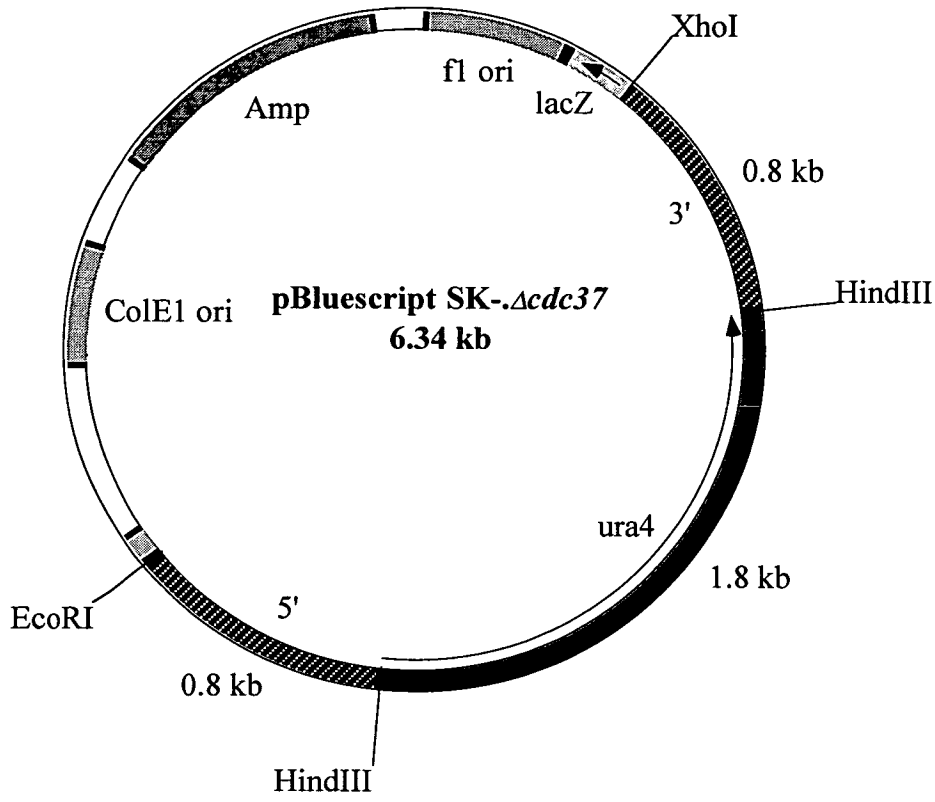
Plasmid pBluescript SK-.3'*cdc37*$^{+}$ORF. Described in section 3.4.3.

C



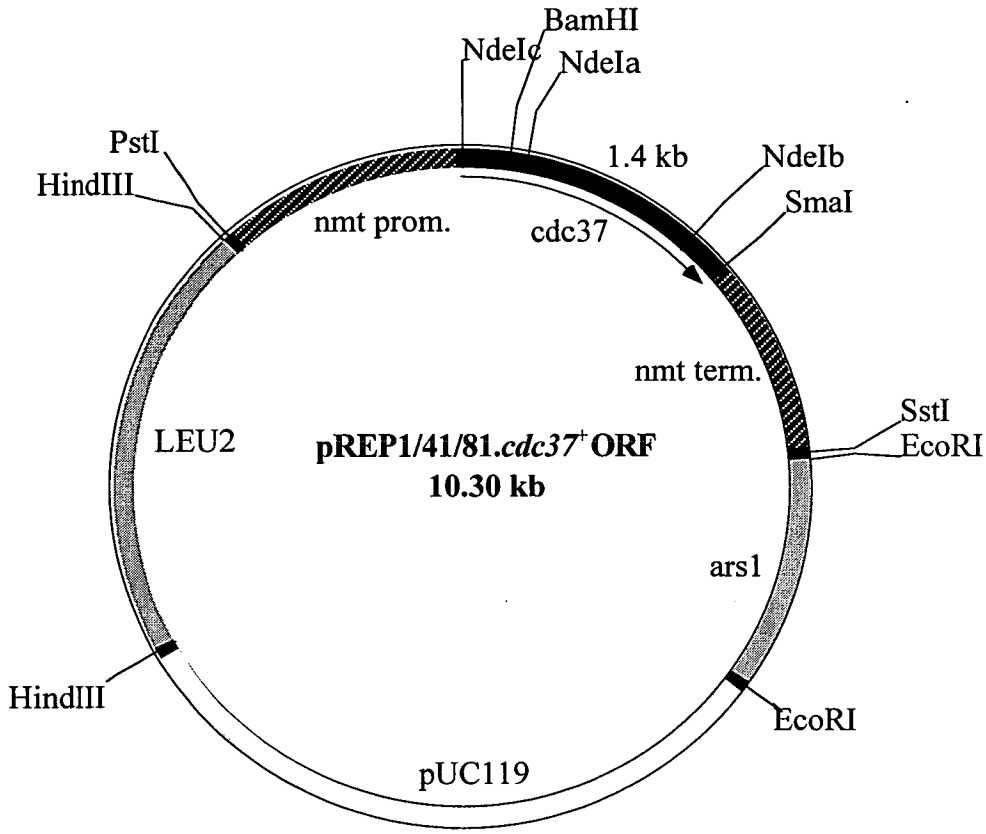
Plasmid pGEM-T.5'*cdc37*$^{+}$ORF. Described in section 3.4.3.

D



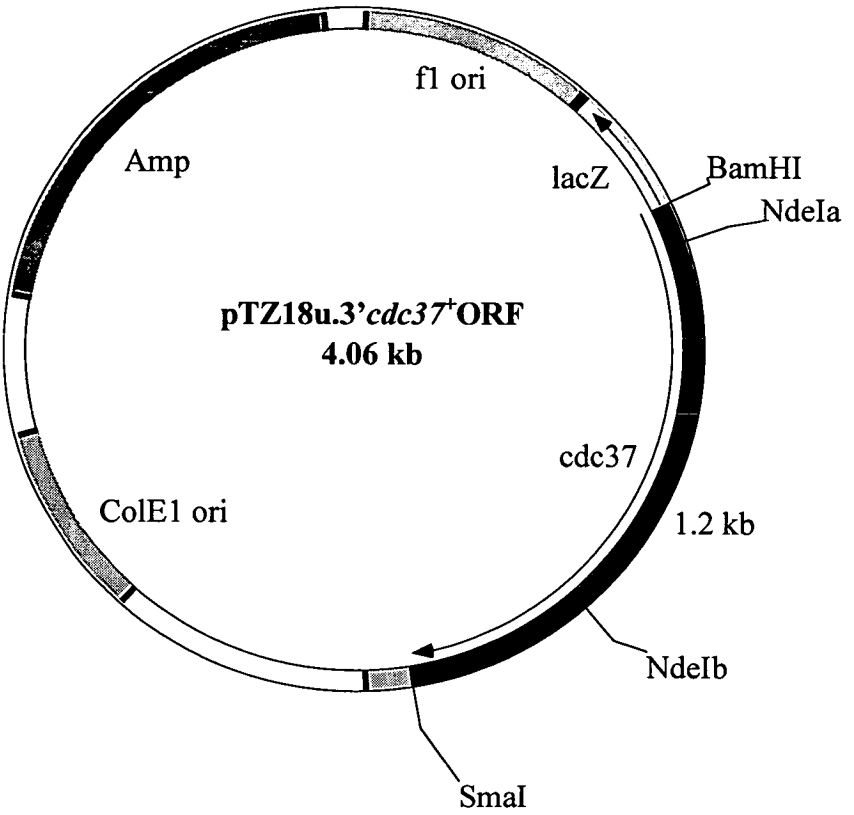
Plasmid pBluescript SK-.*Δcdc37*. Described in section 4.2.1.

E



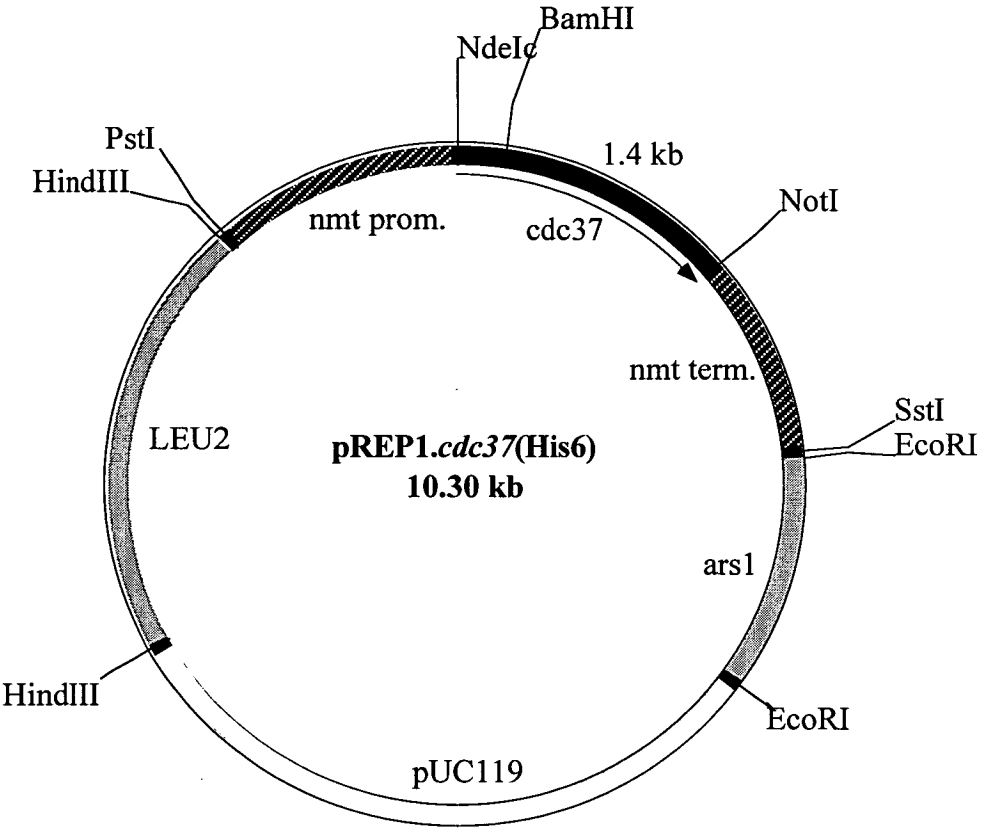
Plasmid pREP1/41/81.*cdc37*$^{+}$ORF. Described in section 4.3.1.

F



Plasmid pTZ18u.3'*cdc37*$^{+}$. Described in section 6.4.1.

G



Plasmid pREP1.*cdc37*(His6). Described in section 6.4.1.

Bibliography

Adkins, B., Hunter, T. and Sefton, B.M. (1982) The transforming proteins of PRCII-virus and Rous-sarcoma virus form a complex with the same 2 cellular phosphoproteins. *J. Virol.*, **43**, 448-455.

Ahn, N.G. (1993) The map kinase cascade - discovery of a new signal transduction pathway. *Mol. Cell. Biochem.*, **128**, 201-209.

Alfa, C., Fantes, P., Hyams, J., McLeod, M. and Warbrick, E. (1993) *Experiments with fission yeast: A laboratory manual.* Cold Spring Harbour Press, Cold Spring Harbour, New York.

Aligue, R., Akhaven-Niak, H. and Russell, P. (1994) A role for Hsp90 in cell cycle control: Wee1 tyrosine kinase activity requires interaction with Hsp90. *EMBO J.*, **13**, 6099-6106.

Basi, G., Schmid, E. and Maundrell, K. (1993) TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. *Gene*, **123**, 131-136.

Boddy, M.N., Furnari, B., Mondesert, O. and Russell, P. (1998) Replication checkpoint enforced by kinases Cds1 and Chk1. *Science*, **280**, 909-912.

Bohen, S.P. and Yamamoto, K.R. (1994) *The biology of heat shock proteins and molecular chaperones.* Cold Spring Harbor Laboratory Press.

Bolton, E.T. and McCarthy, B.J. (1962) A general method for the isolation of RNA complementary to DNA. *Proc. Nat. Acad. Sci. USA*, **48**, 1390.

Booher, R. and Beach, D. (1987) Interaction between *cdc13*$^+$ and *cdc2*$^+$ in the control of mitosis in fission yeast; dissociation of the G1 and G2 roles of the *cdc2*$^+$ protein kinase. *EMBO J.*, **6**, 3441-3447.

Booher, R. and Beach, D. (1988) Involvement of *cdc13*$^+$ in mitotic control in *Schizosaccharomyces pombe*: possible interaction of the gene product with microtubules. *EMBO J.*, **7**, 2321-2327.

Borkovich, K.A. (1989) Hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. *Mol. Cell. Biol.*, **9**, 3919-3930.

Boschelli, F., Uptain, S.M. and Lightbody, J.L. (1993) The lethality of p60(v-src) in *Saccharomyces cerevisiae* and the activation of p34(cdc28) kinase are dependent on the integrity of the SH2 domain. *J. Cell Sci.*, **105**, 519-528.

Breter, H.J., Ferguson, J., Peterson, T.A. and Reed, S.I. (1983) Isolation and transcriptional characterization of 3 genes which function at start, the controlling event of the *Saccharomyces cerevisiae* cell division cycle - *CDC36*, *CDC37*, and *CDC39*. *Mol. Cell. Biol.*, **3**, 881-891.

Broek, D., Bartlett, R., Crawford, K. and Nurse, P. (1991) Involvement of $p34^{cdc2}$ in establishing the dependency of S phase on mitosis. *Nature*, **349**, 388-393.

Brugge, J., Yonemoto, W. and Darrow, D. (1983) Interaction between the Rous sarcoma virus transforming protein and two cellular phosphoproteins: analysis of the turnover and distribution of the complex. *Mol. Cell. Biol.*, **3**, 9-19.

Brugge, J.S. (1986) Interaction of the Rous sarcoma virus protein $pp60^{src}$ with the cellular proteins pp50 and pp90. *Curr. Top. Microbiol. Immunol.*, **123**, 1-23.

Brugge, J.S., Erikson, E. and Erikson, R.L. (1981) The specific interaction of the Rous sarcoma virus transforming protein, $pp60^{src}$, with 2 cellular proteins. *Cell*, **25**, 363-372.

Buchberger, A., Schroder, H., Hesterkamp, T., Schonfeld, H.J. and Bukau, B. (1996) Substrate shuttling between the DnaK and GroEL systems indicates a chaperone network promoting protein folding. *J. Mol. Biol.*, **261**, 328-333.

Bukau, B. and Horwich, A.L. (1998) The Hsp70 and Hsp60 chaperone machines. *Cell*, **92**, 351-366.

Caplan, A.J. (1999) Hsp90's secrets unfold: new insights from structural and functional studies. *Trends Cell Biol.*, **9**, 262-268.

Carr, A.M., MacNeill, S.A., Hayles, J. and Nurse, P. (1989) Molecular cloning and sequence analysis of mutant alleles of the fission yeast *cdc2* protein kinase gene: implications for $cdc2^{+}$ protein structure and function. *Mol. Gen. Genet.*, **218**, 41-49.

Carrello, A. (1999) The common tetratricopeptide repeat acceptor site for steroid receptor-associated immunophilins and Hop is located in the dimerization domain of Hsp90. *J. Biol. Chem.*, **274**, 2682-2689.

Chen, T. and Zhang, M.Q. (1998) Pombe: A gene-finding and exon-intron structure prediction system for fission yeast. *Yeast*, **14**, 701-710.

Clackson, T., Gussow, D. and Jones, P.T. (1994) General applications of PCR to gene cloning and manipulation. In McPherson, M.J., Quirke, P. and Taylr, G.R. (eds.), *PCR: A practical approach*. Oxford University Press, Oxford, pp. 187-214.

Coleman, T.R., Tang, Z. and Dunphy, W.G. (1993) Negative regulation of the Wee1 protein kinase by direct action of the Nim1/Cdr1 mitotic inducer. *Cell*, **72**, 919-929.

Courtneidge, S.A. and Bishop, J.M. (1982) Transit of pp60$^{v\text{-}src}$ to the plasma membrane. *Proc. Natl. Acad. Sci. USA*, **79**, 7117-7121.

Cullen, C.F., May, K.M., Hagan, I.M., Glover, D.M. and Ohkura, H. (2000) A new genetic method for isolating functionally interacting genes: High *plo1*$^{+}$-dependent mutants and their suppressors define genes. *Genetics*, **155**, 1521-1534.

Cutforth, T. and Rubin, G.M. (1994) Mutations in *hsp83* and *cdc37* impair signalling by the sevenless receptor tyrosine kinase in *drosophila*. *Cell*, **77**, 1027-1036.

Dai, K., Kobayashi, R. and Beach, D. (1996) Physical interaction of mammalian Cdc37 with Cdk4. *J. Biol. Chem.*, **271**, 22030-22034.

Dey, B., Lightbody, J.J. and Boschelli, F. (1996) *CDC37* is required for p60$^{v\text{-}src}$ activity in yeast. *Mol. Biol. Cell*, **7**, 1405-1417.

Draetta, G. and Beach, D. (1988) Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. *Cell*, **54**, 17-26.

Ducommun, B., Draetta, G., Young, P. and Beach, D. (1990) Fission yeast Cdc25 is a cell-cycle regulated protein. *Bichem. Biophys. Res. Commun.*, **167**, 301-309.

Durkacz, B., Beach, D., Hayles, J. and Nurse, P. (1985) The fission yeast cell cycle control gene *cdc2* - structure of the *cdc2* region. *Mol. Gen. Genet.*, **201**, 543-545.

Egel, R. and Egel-Mitani, M. (1974) Premeiotic DNA synthesis in fission yeast. *Exp. Cell Res.*, **88**, 127-134.

Ellis, R.J. (ed.) (1996) *The chaperonins*. Academic Press, San Diego, CA.

Enoch. (1990) Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. *Cell*, **60**, 665-673.

Evans, T., Rosenthal, E.T., Youngblom, J., Distel, D. and Hunt, T. (1983) Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell*, **33**, 389-396.

Fantes, P. (1979) Epistatic gene interactions in the control of division in fission yeast. *Nature*, **279**, 428.

Fantes, P. (1989) Yeast cell cycle. *Curr. Opin. Cell Biol.*, **1**, 250-255.

Fantes, P.A. (1981) Isolation of cell size mutants of a fission yeast by a new selective method: characterisation of mutants and implications for division control mechanisms. *J. Bacteriol.*, **146**, 746-754.

Farrell, A. and Morgan, D.O. (2000) Cdc37 promotes the stability of protein kinases Cdc28 and Cak1. *Mol. Cell. Biol.*, **20**, 749-754.

Featherstone, C. and Russell, P. (1991) Fission yeast p107^{wee1} mitotic inhibitor is a tyrosine/serine kinase. *Nature*, **349**, 808-811.

Feder, M.E. and Hofmann, G.E. (1999) Heat shock proteins, molecular chaperones and the stress response. *Annu. Rev. Physiol.*, **61**, 243-282.

Fenton, W.A., Kashi, Y., Furtak, K. and Horwich, A.L. (1997) GroEL-mediated protein folding. *Protein Sci.*, **6**, 743-760.

Ferguson, J., Ho, J.Y., Peterson, T.A. and Reed, S.I. (1986) Nucleotide-sequence of the yeast-cell division cycle start genes *CDC28*, *CDC36*, *CDC37*, and *CDC39*, and a structural-analysis of the predicted products. *Nucleic Acids Res.*, **14**, 6681-6697.

Fikes, J.D., Becker, D.M., Winston, F. and Guarante, L. (1990) Striking conservation of TFIID in *Schizosacccharomyces pombe* and *Saccharomyces cerevisiae*. *Nature*, **346**.

Fink, G.R. (1987) Pseudogenes in yeast? *Cell*, **49**, 5-6.

Fliss, A.E., Fang, Y.F., Boschelli, F. and Caplan, A.J. (1997) Differential *in vivo* regulation of steroid hormone receptor activation by Cdc37p. *Mol. Biol. Cell*, **8**, 2501-2509.

Forsburg, S.L. and Nurse, P. (1991) Cell-cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Annu. Rev. Cell Biol.*, **7**, 227-256.

Fujimura, H.A. (1994) Yeast homolog of mammalian mitogen-activated protein-kinase, *FUS3/DAC2* kinase, is required both for cell-fusion and for G1 arrest of the cell cycle and morphological-changes by the *cdc37* mutation. *J. Cell Science*, **107**, 2617-2622.

Furnari, B., Blasina, A., Boddy, M.N., McGowan, C.H. and Russell, P. (1999) Cdc25 inhibited *in vivo* and *in vitro* by checkpoint kinases Cds1 and Chk1. *Mol. Biol. Cell*, **10**, 833-845.

Gerber, M.R., Farrell, A., Deshaies, R.J., Herskowitz, I. and Morgan, D.O. (1995) Cdc37 is required for association of the protein-kinase Cdc28 with G1 and mitotic cyclins. *Proc. Nat. Acad. Sci. USA*, **92**, 4651-4655.

Gething, M.J. (ed.) (1997) *Guidebook to molecular chaperones and protein folding catalysts*. Oxford Univ. Press, Oxford, UK.

Gething, M.J. and Sambrook, J. (1992) Protein folding in the cell. *Nature*, **355**, 33-45.

Gietz, D., St. Jean, A., Woods, R.A. and Schiestl, R.H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.*, **20**, 1425.

Gould, K.L., Moreno, S., Owen, D.J., Sazer, S. and Nurse, P. (1991) Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* $p34^{cdc2}$ function. *EMBO J.*, **10**, 3297-3309.

Gould, K.L., Moreno, S., Tonks, N.K. and Nurse, P. (1990) Complementation of the mitotic activator, $p80^{cdc25}$, by a human protein-tyrosine phosphatase. *Science*, **250**, 1573-1576.

Gould, K.L. and Nurse, P. (1989) Tyrosine phosphorylation of the fission yeast $cdc2^{+}$ protein kinase regulates entry into mitosis. *Nature*, **342**, 39-45.

Grammatikakis, N., Lin, J.-H., Grammatikakis, A., Tsichlis, P.N. and Cochran, B.H. (1999) $p50^{cdc37}$ acting in concert with Hsp90 is required for Raf-1 function. *Mol. Cell. Biol.*, **19**, 1661-1672.

Gussow, D. and Clackson, T. (1989) Direct clone characterisation from plaques and colonies by the polymerase chain reaction. *Nucleic Acids Res.*, **17**, 4000.

Gutz, H., Heslot, H., Leupold, U. and Loprieno, N. (1974) *Schizosaccharomyces pombe. In: Handbook of Genetics, Vol. 1 (R.C. King, ed.)*. Plenum Publishing Corp., New York.

Hall, M. and Peters, G. (1996) Genetic alterations of cyclins, cyclin-dependent kinases, and CDK inhibitors in human cancer. *Adv. Cancer Res.*, **68**, 67-108.

Hamaguchi, J.R., Tobey, R.A., Pines, J., Crissman, H.A., Hunter, T. and Bradbury, E.M. (1992) Requirement for $p34^{cdc2}$ kinase is restricted to mitosis in the mammalian *cdc2* mutant FT210. *J. Cell Biol.*, **117**, 1041-1053.

Hanahan, D. (1983) Studies on transformation of *Eschericia coli* with plasmids. *J. Mol. Biol.*, **166**, 557-580.

Hartl, F.U. (1996) Molecular chaperones in cellular protein folding. *Nature*, **381**, 571-580.

Hartwell, L.H. (1974) *Saccharomyces cerevisiae* cell cycle. *Bacteriol. Rev.*, **38**, 164-198.

Hartwell, L.H. and Unger, M.W. (1977) Unequal division in *Saccharomyces cerevisiae* and its implication for the control of cell division. *J. Cell Biol.*, **75**, 422-435.

Hereford, L.M. and Hartwell, L.H. (1974) Sequential gene function in the initiataion of *Saccharomyces cerevisiae* DNA synthesis. *J. Mol. Biol.*, **84**, 445-461.

Hill, J., Donald, K.A. and Griffiths, D.E. (1991) DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res.*, **19**, 5791.

Huang, L., Grammatikakis, N. and Toole, B.P. (1998) Organization of the chick *cdc37* gene. *J. Biol. Chem.*, **273**, 3598-3603.

Hunter, T. and Poon, R.Y.C. (1997) Cdc37: A protein kinase chaperone? *Trends Cell Biol.*, **7**, 157-161.

Ito, H., Fukada, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.*, **153**, 163-168.

Jakob, U. and Buchner, J. (1994) Assisting spontaneity - the role of Hsp90 and small Hsps as molecular chaperones. *Trends Biochem. Sci.*, **19**, 205-211.

Johnston, G.C., Pringle, J.R. and Hartwell, L.H. (1977) Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. *Exp. Cell Res.*, **105**, 79-98.

Kato, J.Y., Matsuoka, M., Strom, D.K. and Sherr, C.J. (1994) Regulation of cyclin D dependent kinase 4 (Cdk4) by Cdk4 activating kinase. *Mol. Cell. Biol.*, **14**, 2713-2721.

Kaufer, N., Simanis, V. and Nurse, P. (1985) Fission yeast *Schizosaccharomyces pombe* correctly excises a mammalian RNA transcript interveneing sequence. *Nature*, **318**, 78-80.

Kimura, Y., Rutherford, S.L., Miyata, Y., Yahara, I., Freeman, B.C., Yue, L., Morimoto, R.I. and Lindquist, S. (1997) Cdc37 is a molecular chaperone with specific functions in signal transduction. *Genes Develop.*, **11**, 1775-1785.

Kinoshita, N., Yamano, H., Niwa, H., Yoshida, T. and Yanagida, M. (1993) Negative regulation of mitosis by the fission yeast protein phosphatase Ppa2. *Genes & Dev.*, **7**, 1059-1065.

Kohli, J., Hottinger, H., Munz, P., Strauss, A. and Thuriaux, P. (1977) Genetic mapping in *Schizosaccharomyces pombe* by mititic and meiotic analysis and induced haploidisation. *Genetics*, **87**, 423-471.

Krek, W. and Nigg, E.A. (1991) Mutations of $p34^{cdc2}$ phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to $p34^{cdc2}$ kinase activation in vertebrates. *EMBO J.*, **10**, 3331-3341.

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

Lamphere, L., Fiore, F., Xu, X., Brizuela, L., Keezer, S., Sardet, C., Draetta, G.F. and Gyuris, J. (1997) Interaction between Cdc37 and Cdk4 in human cells. *Oncogene*, **14**, 1999-2004.

Lee, K.M., Saiz, J.E., Barton, W.A. and Fisher, R.P. (1999) Cdc2 activation in fission yeast depends on Mcs6 and Csk1, two partially redundant CDK-activating kinases (CAKs). *Curr. Biol.*, **9**, 441-444.

Lee, M.G. and Nurse, P. (1987) Complementation used to clone a human homolog of the fission yeast cell cycle control gene *cdc2*. *Nature*, **327**, 31-35.
Lindquist, S. (1986) The heat-shock response. *Annu. Rev. Biochem.*, **55**, 1151-1191.

Lipsich, L.A., Cutt, J.R. and Brugge, J.S. (1982) Association of the transforming proteins of Rous, Fujinami, and Y73 avian-sarcoma viruses with the same 2 cellular proteins. *Mol. Cell. Biol.*, **2**, 875-880.

Lollier, M., Jaquet, L., Nedeva, T., Lacroute, F., Potier, S. and Souciet, J.-L. (1995) As in *Saccharomyces cerevisiae*, aspartate transcarbamyolase is asssembled on a multifuctional protein including a dihydroorotase-like cryptic domain in *Schizosaccharomyces pombe*. *Curr. Genet.*, **28**, 138-149.

Louvion, J.F., Warth, R. and Picard, D. (1996) Two eukaryote-specific regions of Hsp82 are dispensable for its viability and signal transduction functions in yeast. *Proc. Natl. Acad. Sci. USA*, **93**, 13937-13942.

Lundgren, K., Walworth, N., Booher, R., Dembski, M., Kirschner, M. and Beach, D. (1991) Mik1 and Wee1 cooperate in the inhibitory tyrosine phosphorylation of Cdc2. *Cell*, **64**, 1111-1122.

Macneill, S.A. and Fantes, P. (1993) Methods for analysis of the fission yeast cell cycle. In Fantes, P. and R., B. (eds.), *The Cell Cycle: A Practical Approach*. IRL Press.

Macneill, S.A. and Fantes, P.A. (1994) Controlling entry into mitosis in fission yeast. *Frontiers in molecular biology: The cell cycle*. IRL Press.

Mahony, D., Parry, D.A. and Lees, E. (1998) Active Cdk6 complexes are predominantly nuclear and represent only a minority of the Cdk6 in T cells. *Oncogene*, **16**, 603-611.

Matsuoka, M., Kato, J.Y., Fisher, R.P., Morgan, D.O. and Sherr, C.J. (1994) Activation of cyclin dependent kinase 4 (Cdk4) by mouse Mo15-associated kinase. *Mol. Cell. Biol.*, **14**, 7265-7275.

Matsushime, H., Quelle, D.E., Shurtleff, S.A., Shibuya, M., Sherr, C.J. and Kata, J. (1994) D-type cyclin dependent kinase activity in mammalian cells. *Mol. Cell. Biol.*, **14**, 2066-2076.

Maundrell, K. (1990) *nmt1* of fission yeast. *J. Biol. Chem.*, **265**, 10857-10864.

Millar, J.B., Lenaers, G. and Russell, P. (1992) Pyp3 PTPase acts as a mitotic inducer in fission yeast. *EMBO J.*, **11**, 4933-4941.

Millar, J.B., McGowan, C.H., Lenaers, G., Jones, R. and Russell, P. (1991) $p80^{cdc25}$ mitotic inducer is the tyrosine phosphatase that activates $p34^{cdc2}$ kinase in fission yeast. *EMBO J.*, **10**, 4301-4309.

Minami, Y., Kimura, Y., Kawasaki, H., Suzuki, K. and Yahara, I. (1994) The carboxy terminal region of mammalain Hsp90 is required for its dimerisation and function *in vivo*. *Mol. Cell. Biol.*, **14**, 1459-1464.

Mitchison, J.M. (1957) The growth of single cells. I. *Schizosaccharomyces pombe*. *Exp. Cell Res.*, **13**, 244.

Mitchison, J.M. (1970) Physiological and cytological methods for *Schizosaccharomyces pombe*. *Meth. Cell Physiol.*, **4**, 131-165.

Mitchison, J.M. (1990) The fission yeast *Schizosaccharomyces pombe*. *BioEssays*, **12**, 189-191.

Mitchison, J.M. and Nurse, P. (1985) Growth in cell length in the fission yeast Schizosaccharomyces pombe. *J. Cell Sci.*, **75**, 357-376.

Moreno, S., Klar, A. and Nurse, P. (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Meth. Enzym.*, **94**, 795-823.

Moreno, S. and Nurse, P. (1994) Regulation of progression through the G1 phase of the cell cycle by the *rum1*$^{+}$ gene. *Nature*, **367**, 236-242.

Moreno, S., Nurse, P. and Russell, P. (1990) Regulation of mitosis by cyclic accumulation of p80^{cdc25} mitotic inducer in fission yeast. *Nature*, **344**, 549-552.

Morgan, D.O. (1995) Principles of CDK regulation. *Nature*, **374**, 131-134.

Morimoto, R.I., Tissieres, A. and Georgopoulos, C. (eds.) (1994) *The biology of heat-shock proteins and molecular chaperones*. Cold Spring Harbor Lab. Press, Cold Spring Harbor, N.Y.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich, H. (1986) Specific enzymatic ampilification of DNA *in vitro* - the polymerase chain reaction. *Cold Spring Harbor Sympos. Quant. Biol.*, **51**, 263-273.

Munoz, M.J., Bejarano, E.R., Daga, R.R. and Jimenez, J. (1999) The identification of Wos2, a p23 homologue that interacts with Wee1. *Genetics*.

Munoz, M.J. and Jimenez, J. (1999) Genetic interactions between Hsp90 and the Cdc2 mitotic machinery in the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Genet.*, **261**, 242-250.

Murray, A.W. (1992) Creative blocks: cell-cycle checkpoints and feedback controls. *Nature*, **359**, 599-604.

Murray, A.W. and Kirschner, M.W. (1991) What controls the cell cycle? *Sci. Amer.*, **264**, 34-41.

Nasmyth, K. (1993) Control of the yeast cell cycle by the Cdc28 protein kinase. *Curr. Opin. Cell Biol.*, **5**, 166-179.

Nasmyth, K. and Nurse, P. (1979) A control acting over the initiation of DNA replication in the yeast *Schizosaccharomyces pombe*. *J. Cell Sci.*, **36**, 155-168.

Neiman, A.M., Chang, F., Komachi, K. and Herskowitz, I. (1990) *CDC36* and *CDC39* are negative elements in the signal transduction pathway of yeast. *Cell Regul.*, **1**, 391-401.

Nigg, E.A. (1995) Cyclin dependent protein kinases - key regulators of the cell cycle. *BioEssays*, **17**, 471-480.

Norbury, C., Blow, J. and Nurse, P. (1991) Regulatory phosphorylation of the $p34^{cdc2}$ protein kinase in vertebrates. *EMBO J.*, **10**, 3321-3329.

Nurse, P. (1975) Genetic control of cell size at cell division in yeast. *Nature*, **256**, 547-551.

Nurse, P. (1985) Cell cycle control in yeast. *Trends Genet.*, **1**, 51-55.

Nurse, P. (1990) Universal control mechanism regulating onset of M-phase. *Nature*, **344**, 503-508.

Nurse, P. and Fantes, P. (1981) Cell cycle controls in fission yeast: a genetic analysis. In John, P.C.L. (ed.) *The cell cycle*. Cambridge University press, pp. 85-98.

Nurse, P. and Thuriaux, P. (1980) Regulatory genes controlling mitosis in the fission yeast *Schizosaccharomyces pombe*. *Genetics*, **96**, 627-637.

Nurse, P., Thuriaux, P. and Nasmyth, K. (1976) Genetic control of the cell division cycle in the the fission yeast *Schizosaccharomyces pombe*. *Mol. Gen. Gen.*, **146**, 167-178.

Oppermann, H., Levison, W. and Bishop, J.M. (1981) A cellular protein that associates with the transforming protein of Rous-sarcoma virus is also a heat-shock protein. *Proc. Natl. Acad. Sci. USA*, **78**, 1067-1071.

OwensGrillo, J.K., Czar, M.J., Hutchison, K.A., Hoffman, K., Perdew, G.H. and Pratt, W.B. (1996) A model of protein targeting mediated by immunophilins and other proteins that bind to Hsp90 via tetratricopeptide repeat domains. *J. Biol. Chem.*, **271**, 13468-13475.

Ozaki, T., Irie, K. and Sakiyama, S. (1995) Molecular cloning and cell cycle-dependent expression of a novel gene that is homologous to *cdc37*. *DNA Cell Biol.*, **14**, 1017-1023.

Pardee, A.B. (1974) A restriction point for control of normal animal cell proliferation. *Proc. Natl. Acad. Sci. USA*, **71**, 1286-1290.

Parker, L.L., Walter, S.A., Young, P.G. and Piwnica-Worms, H. (1993) Phosphorylation and inactivation of the mitotic inhibitor Wee1 by the Nim1/Cdr1 kinase. *Nature*, **363**, 736-738.

Parker, L.L.A.-F., S. and Piwnica-Worms, H. (1992) $p107^{wee1}$ is a dual-specificity kinase that phosphorylates $p34^{cdc2}$ on tyrosine 15. *Proc. Natl. Acad. Sci. USA*, **89**, 2917-2921.

Parsell, D.A. and Lindquist, S. (1993) The function of heat shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.*, **27**, 437-496.

Patra, D., Wang, S.X., Kumagai, A. and Dunphy, W.G. (1999) The Xenopus Suc1/Cks protein promotes the phosphorylation of G2-M regulators. *J. Biol. Chem.*, **274**, 36839-36842.

Pauli, D. and Mahowald, A.P. (1990) Germ-line sex determination in *Drosophila melanogaster*. *Trends Genet.*, **6**, 259-264.

Perdew, G.H. and Whitelaw, M.L. (1991) Evidence that the 90 kDa heat-shock protein (Hsp90) exists in cytosol in heteromeric complexes containing Hsp70 and 3 other proteins with Mr of 63,000, 56,000 and 50,000. *J. Biol. Chem.*, **266**, 6708-6713.

Perdew, G.H., Wiegand, H., VandenHeuvel, J.P., Mitchell, C. and Singh, S.S. (1997) A 50 kilodalton protein associated with raf and $pp60^{v\text{-}src}$ protein kinases is a mammalian homolog of the cell cycle control protein Cdc37. *Biochemistry*, **36**, 3600-3607.

Peter, M., Gartner, A., Horecka, J., Ammerer, G. and Herskowitz, I. (1993) *FAR1* links the signal transduction pathway to the cell cycle machinery in yeast. *Cell*, **73**, 747-760.

Pratt, W.B. and Toft, D.O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.*, **18**, 306-360.

Prentice, H.L. (1991) High efficiency transformation of *Schizosaccharomyces pombe* by electroporation. *Nucleic Acids Res.*, **20**, 621.

Reed, S.I. (1980a) The selection of amber mutations in genes required for completion of start, the controlling event of the cell division cycle of *S.cerevisiae*. *Genetics*, **95**, 579-588.

Reed, S.I. (1980b) The selection of *S.cerevisiae* mutants defective in the start event of cell division. *Genetics*, **95**, 561-577.

Reed, S.I. (1992) The role of p34 kinases in the G1 to S phase transition. *Annu. Rev. Cell Biol.*, **8**, 529-661.

Ritossa, F. (1996) Discovery of the heat shock response. *Cell Stress Chaperones*, **1**, 97-98.

Rodriguez, C.R., Cho, E.J., Keogh, M.C., Moore, C.L., Greenleaf, A.L. and Buratowski, S. (2000) Kin28, the TFIIH-associated carboxy-terminal domain kinase, facilitates the recruitment of mRNA processing machinery to RNA polymerase II. *Mol. Cell. Biol.*, **20**, 104-112.

Rothstein, R.J. (1983) One step gene disruption in yeast. In Wu, R., Grossman, L. and Moldave, K. (eds.), *Meth. Enzym.* Academic Press, Vol. 101, pp. 202-211.

Russell, P. (1989) Gene cloning and expression. In Nasim, A., Young, P. and Johnston, B.F. (eds.), *Mol. Biol. Fission Yeast.* Academic Press, pp. 224-267.
Russell, P. (1998) Checkpoints on the road to mitosis. *Trends Biochem. Sci.*, **23**, 399-402.

Russell, P. and Nurse, P. (1986) *cdc25*$^{+}$ functions as an inducer in the mitotic control of fission yeast. *Cell*, **45**, 145-153.

Russell, P. and Nurse, P. (1987a) The mitotic inducer *nim1*$^{+}$ functions in a regulatory network of protein kinase homologs controlling the initiation of mitosis. *Cell*, **49**, 569-576.

Russell, P. and Nurse, P. (1987b) Negative regulation of mitosis by *wee1*, a gene encoding a protein kinase homolog. *Cell*, **49**, 559-567.

Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A. and Arnheim, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle-cell anaemia. *Science*, **230**, 1350-1354.

Sambrook, J., Fritch, E.F. and Maniatis, T. (1989) *Molecular cloning: a laboratory handbook.* Cold Spring Harbour Press, Cold Spring Harbour, New York.

Schiestl, R.H. and Gietz, R.D. (1989) High efficiency transformation of intact cells using single-stranded nucleic acids as a carrier. *Curr. Genet.*, **16**, 339-346.

Schutz, A.R., Giddings, T.H., Steiner, E. and Winey, M. (1997) The yeast *CDC37* gene interacts with *MPS1* and is required for proper execution of spindle pole body duplication. *J. Cell Biol.*, **136**, 969-982.

Sheldrick, K.S. and Carr, A.M. (1993) Feedback controls and G2 checkpoints: fission yeast as a model system. *BioEssays*, **15**, 775-782.

Shiozaki and Russell, P. (1996) Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. *Genes Dev.*, **10**, 2276-2288.

Silverstein, A.M., Grammatikakis, N., Cochran, B.H., Chinkers, M. and Pratt, W.B. (1998) $p50^{cdc37}$ binds directly to the catalytic domain of Raf as well as to a site on Hsp90 that is topologically adjacent to the tetratricopeptide repeat binding site. *J. Biol. Chem.*, **273**, 20090-20095.

Somero, G.N. (1995) Proteins and temperature. *Annu. Rev. Physiol.*, **57**, 43-68.

Stancato, L.F., Chow, Y.H., Hutchison, K.A., Perdew, G.H., Jove, R. and Pratt, W.B. (1993) Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. *J. Biol. Chem.*, **268**, 21711-21716.

Stepanova, L., Leng, X.H. and Harper, J.W. (1997) Analysis of mammalian Cdc37, a protein kinase targeting subunit of heat shock protein 90. *Meth. Enzym.*, **283**, 220-229.

Stepanova, L., Leng, X.H., Parker, S.B. and Harper, J.W. (1996) Mammalian $p50^{cdc37}$ is a protein kinase targeting subunit of Hsp90 that binds and stabilizes Cdk4. *Genes & Dev.*, **10**, 1491-1502.

Szabo, A., Langer, T., Schroder, H., Flanagan, J., Bukau, B. and Hartl, F.U. (1994) The ATP hydrolysis-dependent reaction cycle of the *Eschericia coli* Hsp70 system-DnaK, DnaJ and GrpE. *Proc. Natl. Acad. Sci. USA*, **91**, 10345-10349.

Th'ng, J.P., Wright, P.S., Hamaguchi, J., Lee, M.G., Norbury, C.J., Nurse, P. and Bradbury, E.M. (1990) The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive *CDC2* gene product. *Cell*, **63**, 313-324.

Timmerman, S., Hinds, P.W. and Munger, K. (1997) Elevated activity of cyclin dependent kinase 6 in human squamous cell carcinoma lines. *Cell Growth Diff.*, **8**, 361-370.

Toda, T., Yamamoto, M. and Yanagida, M. (1981) Sequential alteration in the nuclear chromatin region during mitosis of the fission yeast *Schizosaccharomyces pombe*: Video fluoresence microscopy of synchronously growing wild-type and cold-sensitive *cdc* mutants by using a DNA-binding fluorescent probe. *J. Cell Sci.*, **52**, 271-287.

Towbin, H., Steahelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA*, **76**, 4350-4354.

Tyers, M. and Futcher, B. (1993) Far1 and Fus3 link the mating pheromone signal transduction pathway to 3 G1-phase Cdc28 kinase complexes. *Mol. Cell. Biol.*, **13**, 5659-5669.

Valay, J.G., Simon, M., Dubois, M.F., Bensaude, O., Facca, C. and Faye, G. (1995) The *KIN28* gene is required both for RNA-polymerase II mediated transcription and phosphorylation of the Rpb1p ctd. *J. Mol. Biol.*, **249**, 535-544.

Wartmann, M. and Davis, R.J. (1994) The native structure of the activated Raf protein-kinase is a membrane-bound multisubunit complex. *J. Biol. Chem.*, **269**, 6695-6701.

Welch, W.J. and Feramisco, J.R. (1982) Purification of the major mammalian heat shock proteins. *J. Biol. Chem.*, **257**, 14949-14959.

Whitelaw, M.L., Hutchison, K. and Perdew, G.H. (1991) A 50-kda cytosolic protein complexed with the 90-kda heat-shock protein (Hsp90) is the same protein complexed with pp60$^{v\text{-}src}$Hsp90 in cells transformed by the Rous-sarcoma virus. *J. Biol. Chem.*, **266**, 16436-16440.

Woollard, A. and Nurse, P. (1995) G1 regulation and checkpoints operating around START in fission yeast. *Bioessays*, **17**, 481-490.

Wu, L. and Russell, P. (1993) Nim1 kinase promotes mitosis by inactivating Wee1 tyrosine kinase. *Nature*, **363**, 738-741.

Xu, Y. and Lindquist, S. (1993) Heat-shock protein Hsp90 governs the activity of pp60$^{v\text{-}src}$ kinase. *Proc. Natl. Acad. Sci. USA*, **90**, 7074-7078.

Yanagida, M. (1998) Fission yeast cut mutations revisited: control of anaphase. *Trends Cell Biol.*, **8**, 144-149.

Young, J.C., Obermann, W.M.J. and Hartl, F.U. (1998) Specific binding of tetratricopeptide repeat proteins to the C-terminal 12 kDa domain of Hsp90. *J. Biol. Chem.*, **273**, 18007-18010.

Young, P.G. and Fantes, P.A. (1987) *Schizosaccharomyces pombe* mutants affected in their division response to starvation. *J. Cell Sci.*, **88**, 295-304.

Zhang, M.Q. and Marr, T.G. (1994) Fission yeast gene structure and recognition. *Nucleic Acids Res.*, **22**, 1750-1759.

Ziemiecki, A. (1986) Characterization of the monomeric and complex-associated forms of the gag-onc fusion proteins of 3 isolates of feline sarcoma virus - phosphorylation, kinase-activity, acylation, and kinetics of complex formation. *Virology*, **151**, 265-273.