Functional analysis of the cell cycle control gene cdc18 in Schizosaccharomyces pombe

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Abstract

The *Schizosaccharomyces pombe* gene *cdc18* has a role both in promoting DNA replication and initiating the checkpoint that acts to prevent mitosis until DNA replication has been successfully completed. It therefore plays an essential role in coordinating the cell cycle, thus maintaining genome integrity. I performed a functional analysis of *cdc18* in order to understand its role throughout the cell cycle.

I used different *cdc18p* constructs to discover which domains of the protein are required to carry out its functions. I examined this by assaying the ability of cells to re-replicate and block mitosis when the constructs were overexpressed. I discovered that the C-terminus of *cdc18p* is required for the re-replication phenotype and that this does not require a decrease in the activity of the mitotic kinase. The C-terminus is also required for the checkpoint function of *cdc18p*. The N-terminus of *cdc18p* can also block mitosis indirectly by binding to cdc2p.

I then examined the *Saccharomyces cerevisiae* (ScCDC6), *Xenopus laevis* (XICDC6) and *Homo sapiens* (HsCDC6) *cdc18* homologues to determine their ability to complement the *cdc18-K46* mutant. I also investigated whether they, like *cdc18*, are able to induce re-replication when overexpressed. None of the homologues was able to complement *cdc18-K46*, but overexpression of ScCDC6 was able to induce re-replication.

Finally, I constructed a strain in which *cdc18* was under the control of a repressible promoter. I made several site specific mutants of *cdc18* – the nucleotide triphosphate (NTP) binding and hydrolysis motif mutants, known as the Walker A (WA) and Walker B (WB) mutants, respectively, the putative nuclear localization signal mutants (NLS1, NLS2 and NLS1+NLS2) and the cdc2p phosphorylation site mutant (P1-6) – and introduced these at a different locus, where they were expressed under control of the *cdc18* promoter. I investigated whether the mutants could proliferate in the absence of wildtype *cdc18*. Only the *cdc18* WA and WB mutants failed to proliferate under these conditions. The WA mutant cannot initiate replication but is unable to inhibit mitosis, so undergoes an aberrant mitosis. The WB mutant is able to initiate, but not complete, replication and initiates a checkpoint signal. However, the checkpoint cannot be maintained and cells also enter an aberrant mitosis.
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Chapter 1

Introduction

1.1 The Cell Cycle

The cell cycle is the ordered sequence of events by which one cell grows and divides to produce two viable daughter cells. A complex regulation system has evolved to ensure that a cell first duplicates, then segregates its cellular components. It is particularly important that the chromosomes are segregated equally to both daughter cells. A cell must also be responsive to environmental cues which provide nutrition and differentiation factors to ensure that it does not attempt to grow and divide inappropriately.

Most organelles are amassed throughout the cell cycle during general growth so are present in multiple copies. Mitochondria, the endoplasmic reticulum, the nuclear envelope and the Golgi apparatus all fall into this category. These organelles are segregated randomly which is most effective with high copy number organelles. Organelles are positioned randomly throughout the mitotic cell cytoplasm and nearly equal partitioning occurs as the cytokinetic mechanism ensures that the cell is divided into two equal daughter cells. In the fission yeast Schizosaccharomyces pombe this occurs due to septum placement in the middle of the cell. The Golgi apparatus has a low copy number but undergoes extensive fragmentation prior to mitosis allowing it to be dispersed throughout the cytoplasm (Warren and Wickner, 1996). Other low copy number organelles such as the chromosomes and the centrosome are present in single or low copy and need to be duplicated precisely once during the cell cycle. Centrosomes, or the spindle pole body in yeast, are responsible for organising interphase microtubules and the mitotic spindle and are duplicated in a conserved manner (Adams and Kilmartin, 2000; Byers and Goetsch, 1974; Ding et al., 1997; Winey et al., 1991). Their function in the mitotic spindle is such that they are always placed at opposite cell poles during cell division so each is segregated to a different daughter cell.
Chromosomes are replicated semi-conservatively during S phase (defined as the period during which DNA replication occurs) and are then partitioned during mitosis as the sister chromatids separate. S phase and mitosis are separated by two gap phases, G1 before S phase and G2 following S phase and prior to mitosis (Fig. 1.1 A).

As survival of all organisms depends on the accurate transmission of genetic information from one cell to its daughters, cells have evolved complex regulatory systems to find the solution to two problems:
Completion problem → the initiation of later events is dependent on the completion of earlier events
Alternation problem → that DNA replication is followed by mitosis, not another round of replication (Murray and Hunt, 1993).

The completion problem can be solved in two ways. Fertilized frog eggs use a relative timing mechanism in which the events that lead to mitosis are triggered by an event that occurs on completion of the preceding mitosis. As long as it takes less time to complete DNA replication than it does to initiate mitosis, cells do not enter mitosis before the completion of DNA replication (Murray, 1992). However, this mechanism is not very sophisticated and does not make allowances for mistakes. Many organisms therefore use feedback controls to monitor cell cycle events and arrest the cell cycle at checkpoints until a process is completed (Hartwell and Weinert, 1989). Checkpoints exist to inhibit mitosis if DNA is damaged or replication is incomplete which ensures that chromosome breakage does not occur during anaphase. The metaphase to anaphase transition is also inhibited if the mitotic spindle is defective or chromosomes are incorrectly aligned. Again this protects cells from mis-segregating their chromosomes (Clarke and Giminez-Abian, 2000; Murakami and Nurse, 2000).

The alternation problem concerns the inhibition of either two successive rounds of mitosis, or DNA replication. The former would result in chromosome loss and cell death, the latter in polyploid cells. However, there are situations in which both of these events occur without loss of viability to the cell. The meiotic cell cycle can be thought of as a modified mitotic cell cycle in which DNA replication is followed by
two rounds of nuclear division (Yamamoto et al., 1997). The first is a reductional division as homologues are segregated, the second is equational and results in sister chromatid separation. Endoreduplication occurs in *Drosophila* embryos as many rounds of DNA replication occur without an intervening mitosis (Spradling, 1993). The existence of these modified cell cycles demonstrates that specific controls are required in a mitotic cycle to ensure alternation of chromosome replication and segregation. The nature of these controls will be discussed later.

The environmental signals that govern entry into the mitotic cell cycle are somewhat different in yeast and multicellular organisms. The availability of nutrients tends to be the limiting factor for entry into the yeast cell cycle (Fig. 1.1). Cells must attain a critical size prior to entry into the cell cycle which ensures a tight coupling between cell proliferation and growth (Fantes and Nurse, 1977; Johnston et al., 1977). The only differentiation factors so far identified in yeast are the mating pheromones which initiate a sequence of events culminating in zygote formation (Fig. 1.1B) (Kurjan, 1993; Nielsen and Davey, 1995). Nutrients are rarely limiting in multicellular organisms so cells rely on extracellular signals such as growth and differentiation factors (Norbury and Nurse, 1992; Planas-Silva and Weinberg, 1997). Inability to respond correctly to these signals can occur after genetic alteration and can result in uncontrolled cell growth and cancer (Sherr, 1996).

In discussing the cell cycle in more detail I will mainly refer to work performed in fission yeast. However, I will also mention results achieved from both budding yeast and higher eukaryotes where relevant. Progress through the cell cycle in all organisms is regulated at two major points, Start (in fission yeast) or the Restriction Point (in mammalian cells) and mitosis. Start refers to the point at which fission yeast cells choose whether to enter the mitotic or meiotic cycle. Once the decision has been taken cells become committed to complete the chosen developmental programme. Start has not been molecularly characterized but it can be a useful physiological term. Mitosis is the sequence of events that leads to the equal segregation of chromosomes to two daughter cells. As mentioned previously, checkpoints exist to monitor cell cycle progression. I will refer particularly to the S
phase checkpoint that ensures DNA replication is completed before mitosis is initiated.

The key components required for regulating the cell cycle, initiating both DNA replication and mitosis and ensuring that the completion and alternation problems are solved, are the Cyclin Dependent Kinases (CDKs).

1.2 Cyclin Dependent Kinases-master regulators of the cell cycle

Two approaches have been used to identify the components that control the major cell cycle transitions, a genetical approach and a biochemical approach. The \textit{cdc2} gene was originally identified in a screen for \textit{cdc} mutants (mutants which grow but are unable to complete the cell division cycle) in fission yeast (Nurse et al., 1976). \textit{Cdc2} was originally thought to be required solely for nuclear division but a second function was later identified when it was shown that two mutants, \textit{cdc2} and \textit{cdc10}, were able to conjugate from their block points (Nurse and Bissett, 1981). As cells only conjugate from G1 (Fig. 1.1) \textit{cdc2} was shown to be required in G1 for commitment to the mitotic cell cycle and in G2 for initiation of mitosis. The universal nature of CDKs was first demonstrated when a budding yeast homologue, \textit{CDC28}, was shown to functionally substitute for \textit{cdc2p} in a \textit{cdc2} temperature sensitive mutant (Beach et al., 1982) and later a human homologue was cloned by its ability to complement a \textit{cdc2} temperature sensitive mutant at the restrictive temperature (Lee and Nurse, 1987). Further analysis of the fission yeast \textit{cdc2p} showed it to be a phosphoprotein with protein kinase activity against two substrates, histone H1 and casein (Simanis and Nurse, 1986). \textit{Cdc2p} was therefore thought to act by phosphorylating key substrates to induce DNA replication and mitosis.

The biochemical approach was instigated when an activity was identified in \textit{Xenopus laevis} that could induce entry into mitosis and maturation into an egg (Masui and Markert, 1971). This activity, known as M-phase or Maturation Promoting Factor (MPF), was purified from both \textit{Xenopus} and starfish oocytes and shown to contain two proteins (Labbe et al., 1989a; Labbe et al., 1988; Labbe et al.,
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1989b; Lohka et al., 1988). The first was a 32kDa (*Xenopus*) or 34kDa (starfish) protein that was recognised by fission yeast cdc2p antibodies (Gautier et al., 1988; Labbe et al., 1988) and bound to a suc1p column, resulting in the depletion of MPF activity from the extract (Dunphy et al., 1988). One component of MPF was therefore shown to be cdc2p. The second protein was shown to migrate with the same mobility as starfish cyclin (Labbe et al., 1989b). Cyclins were originally identified in sea urchins as proteins which are required for the 8 rapid divisions occurring during embryo cleavage which are destroyed at the end of each mitosis (Evans et al., 1983). Both sequencing and immunoprecipitation experiments confirmed that the cdc2p partner was a cyclin (Draetta et al., 1989; Labbe et al., 1989a). Cdc2p was hence known as a cyclin dependent kinase (CDK).

Cdc2p activity is controlled by three factors in fission yeast; availability of a cyclin partner, phosphorylation and CDK inhibitors (Fig. 1.2). These factors will be discussed in turn with reference to the cell cycle stage at which they are employed.

1.2.1 Cyclins

In fission yeast, unlike in higher eukaryotes, only one CDK, cdc2p is required during the cell cycle. However, several cyclins have been identified (Fig. 1.2 A). Cdc13p is the mitotic cyclin, first shown to act closely with cdc2p as overexpression of *cdc2* suppressed a *cdc13* temperature sensitive mutant (Booher and Beach, 1987). Cdc13p was cloned by its ability to rescue the *cdc13-117* mutant and sequence analysis showed it to be related to cyclins (Booher and Beach, 1988; Hagan et al., 1988). The null mutant was unable to undergo mitosis, indicating that it was required along with cdc2p for entry into mitosis (Booher and Beach, 1988; Hagan et al., 1988). Cdc2p was shown to form a stable complex with cdc13p, the binding of which was required for the kinase activity of cdc2p (Booher et al., 1989; Moreno et al., 1989). This kinase activity oscillates throughout the cell cycle and decreases after metaphase which occurs concomitantly with cdc13p destruction (Fig. 1.3), providing further proof that cdc13p is a cyclin (Booher et al., 1989).

Cdc13p is required only for mitotic entry indicating that other cyclins present in fission yeast could act as the cyclin partner for DNA replication. Two B-type cyclins and one G1 type cyclin have thus far been identified in fission yeast. *Cig1*
was cloned using degenerate PCR in an attempt to identify other cyclin B homologues (Bueno et al., 1991; Bueno et al., 1993). Cig2/cyc17 is also a B-type cyclin which was isolated independently by three groups (Bueno and Russell, 1993; Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994).

The cig1p associated kinase activity is activated at mitosis with similar kinetics to the cdc2p/cdc13p kinase (Basi and Draetta, 1995). A clear role has yet to be provided for the cdc2p/cig1p kinase. The cig2p transcript (Connolly and Beach, 1994), protein and associated kinase activity (Mondesert et al., 1996) have all been shown to peak at the G1/S transition, indicating that cig2p is the major S phase cyclin (Fig. 1.3). However, both cdc13p and cig1p are able to substitute for cig2p in its absence (Fisher and Nurse, 1996; Martin-Castellanos et al., 1996; Mondesert et al., 1996) indicative of functional redundancy amongst the fission yeast B-type cyclins. This has led to the proposal of the quantitative model, in which the level of kinase activity, not the cyclin partner, regulates cell cycle progression (reviewed in Stern and Nurse, 1996). This will be discussed in relation to the alternation problem later in the chapter.

The G1 type cyclin, puc1, was originally identified as a cDNA that conferred alpha factor resistance to *Saccharomyces cerevisiae* cells deficient in a single G1 cyclin, CLN3 (Forsburg and Nurse, 1991). Puc1p does not play a role in normal S phase progression but appears to affect the timing of sexual development (Forsburg and Nurse, 1994a), a mechanism for which has recently been proposed. The cdc2p/puc1p kinase is able to phosphorylate the CDK inhibitor rum1p (Martin-Castellanos et al., 2000) resulting in its degradation (Benito et al., 1998; Jallepalli et al., 1998; Kominami and Toda, 1997). This role had previously been proposed for the cig1p associated kinase (Benito et al., 1998; Correa-Bordes and Nurse, 1995). The puc1p cyclin therefore affects the length of G1 via its regulation of rum1p levels and is involved in sexual development as rum1p accumulation is essential for the G1 arrest prior to meiotic entry.

### 1.2.2 Phosphorylation

Cdc2p is a phosphoprotein (Simanis and Nurse, 1986), phosphorylated on two residues, Tyrosine-15 (Y15) and Threonine-167 (T167) (Gould et al., 1991; Gould...
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and Nurse, 1989). T167 phosphorylation requires the activity of a CAK (CDK activating kinase) (Lee et al., 1999) early in the cell cycle. Phosphorylation of this residue precedes cyclin binding in both fission and budding yeasts and is therefore required for activation of cdc2p (Fig. 1.2) (Gould et al., 1991; Ross et al., 2000).

The Y15 residue is located within the ATP binding domain of cdc2p and phosphorylation prevents activation of the kinase and entry into mitosis (Gould and Nurse, 1989). The phosphorylation state of Y15, and hence the activity of cdc2p, is therefore determined by the balance between inhibitory kinases and activatory phosphatases (Fig. 1.2 B).

Weel was cloned by its ability to rescue the lethal phenotype of premature mitotic entry that occurred when a mitotic inducer, cdc25, was overproduced in a wee1-50 temperature sensitive mutant. It was shown to be a dose-dependent inhibitor of mitosis with homology to protein kinases (Russell and Nurse, 1987b). Weelp is a dual specificity kinase which phosphorylates both tyrosine and serine residues (Featherstone and Russell, 1991; Parker et al., 1992). The wee1p kinase was found to phosphorylate the Y15 residue of cdc2p only when complexed with cyclin. Furthermore this phosphorylation was shown to directly inhibit the histone H1 kinase activity of cdc2p (Parker et al., 1992). The inhibitory phosphorylation of cdc2p by wee1p occurs only after the cdc10 mutant block point in G1, before which a different mechanism operates to maintain the cdc2p/cdcl3p mitotic kinase in an inactive state (Hayles and Nurse, 1995). The wee1p kinase activity is regulated later in the cell cycle by a complex network of proteins. Niflp negatively regulates the onset of mitosis by inhibiting nimlp/cdr1p (Wu and Russell, 1997). The nimlp/cdr1p and cdr2p protein kinases act to inhibit wee1p kinase activity, probably via direct phosphorylation (Fig. 1.2 B) (Breeding et al., 1998; Coleman et al., 1993; Kanoh and Russell, 1998; Parker et al., 1993; Russell and Nurse, 1987a; Wu and Russell, 1993; Young and Fantes, 1987). Deletion of either gene causes a cell cycle delay in G2 as wee1p is not inhibited, however, cells eventually divide indicating that accumulation of the activatory cdc25p phosphatase is sufficient to drive cells into mitosis.
Another tyrosine kinase, mik1p, has also been shown to contribute to the inhibition of cdc2p (Lee et al., 1994). It was identified by its ability to suppress the mitotic catastrophe phenotype of the cdc2-3w wee1-50 strain at 36°C when overexpressed (Lundgren et al., 1991). A null mutant does not affect cell cycle progression unless wee1p function is also compromised (Lundgren et al., 1991). Mik1p is therefore thought to play a minor role in cdc2p inhibition (Fig. 1.2 B).

The activatory phosphatase in fission yeast is known as cdc25p (Fig. 1.2 B). It was cloned by complementation of the cdc25-22 temperature sensitive mutant (Russell and Nurse, 1986). Initial results indicated that cdc25p acted as an inducer of mitosis and that it competed with wee1p to control cdc2p activation (Russell and Nurse, 1986; Russell and Nurse, 1987b). However, it was not initially known whether cdc25p acted directly on cdc2p or further upstream in the activatory pathway. Tyrosine dephosphorylation of cdc2p was shown to trigger mitosis when a human protein tyrosine phosphatase was expressed in fission yeast. This phosphatase could also substitute for cdc25p function, thereby closely linking tyrosine dephosphorylation to cdc25p activity (Gould et al., 1990). Cdc25p was eventually shown to be the activatory phosphatase using two different systems. Purified human Cdc25p was used to dephosphorylate and activate a cdc2p/cyclin B complex from G2-arrested starfish oocytes (Strausfeld et al., 1991) and Drosophila cdc25p was shown to activate Xenopus MPF and induce premature entry into mitosis after dephosphorylation of cdc2p (Kumagai and Dunphy, 1991). Cdc25p activity does not appear to be regulated during the cell cycle. Instead, both the message and the protein accumulate throughout interphase until a critical level is reached (Moreno et al., 1990). At this stage the activating signals outweigh the inhibitory signals and cells proceed into mitosis.

1.2.3 CDK inhibitors
Activity of CDKs are also controlled by a class of proteins known as CDK inhibitors (CKIs) (Fig. 1.2 C). Only one, rum1p, has thus far been identified in fission yeast. Rum1 was identified as a gene that induced multiple rounds of replication in the absence of mitosis when overexpressed (Moreno and Nurse, 1994). Deletion of the gene eliminated the pre-Start interval normally present to allow cells to grow to achieve the critical size for Start. Rum1 is also required for
cell cycle arrest prior to Start so a *cdc10-129 rum1Δ* double mutant is unable to maintain a G1 arrest when placed at the restrictive temperature and cells proceed into a premature mitosis (Moreno and Nurse, 1994). Rum1p is therefore important in G1 for the regulation of Start.

As *cdc10-129 rum1Δ* cells are unable to arrest properly or inhibit mitosis, the indication was that rum1p was required for the pre-Start checkpoint that acts prior to the inhibitory phosphorylation of cdc2p by wee1p (Hayles and Nurse, 1995). This was demonstrated by the fact that early G1 cells accumulate rum1p which leads to a decrease in the histone H1 kinase activity of cdc2p. This was visualised by measuring the histone H1 kinase activity of cells entering a *cdc10-129* G1 block either in the presence of rum1p or in a *rum1Δ* background. The decrease in kinase activity is not due to Y15 phosphorylation of cdc2p as it also occurs in a *wee1-50 mik1Δ* strain at 36°C when *rum1* is overexpressed (Correa-Bordes and Nurse, 1995).

Bacterially purified rum1p has been shown to act as a specific *in vitro* inhibitor of the cdc2p/cdc13p mitotic kinase. Rum1p associates with both cdc2p and cdc13p in G1 and appears to target cdc13p for degradation in G1, possibly by acting as an adaptor protein, promoting transfer of cdc13p to the proteolytic machinery (Correa-Bordes and Nurse, 1995; Correa-Bordes and Nurse, 1997). Rum1p has also been shown to partially inhibit cdc2p/cig2p kinase activity *in vitro*. This complex could be inhibited transiently *in vivo* until the critical cell size for Start is achieved (Correa-Bordes and Nurse, 1995; Martin-Castellanos et al., 1996).

Rum1p levels are sharply periodic, accumulating at anaphase and decreasing as cells exit G1. The mRNA levels also oscillate throughout the cell cycle but not to the extent suggested by the change in protein, indicating that post-translational controls are also involved (Benito et al., 1998). Rum1p is stabilized when the threonine residues of two cdc2p consensus phosphorylation sites, T58 and T62, are mutated to alanine and poly-ubiquitinated rum1p also accumulates in a proteasome mutant (Benito et al., 1998). Phosphorylation therefore appears to target rum1p for degradation via the 26S proteasome (Jallepalli et al., 1998; Kominami and Toda, 1997). There is some debate as to which cdc2p/cyclin complex phosphorylates rum1p. Early reports suggested that the CDK could be
cdc2p/cig1p as it was insensitive to rum1p inhibition and was shown to phosphorylate rum1p in vitro (Benito et al., 1998; Correa-Bordes and Nurse, 1995). However a more recent report proposes puc1p as the cdc2p cyclin partner for rum1p phosphorylation (Martin-Castellanos et al., 2000). Whether puc1p alone is required for rum1p degradation, or whether different cyclin partners contribute to the phosphorylation of rum1p remains to be conclusively determined.

Sic1p has been shown to play an analagous role in maintaining a low level of Cdc28/cyclinB kinase activity in G1 in S. cerevisiae (Schwob et al., 1994). Sic1p and rum1p can functionally substitute for each other, confirming that they are functionally homologous (Sanchez-Diaz et al., 1998). Interestingly, Sic1p is post-translationally controlled by proteolysis following phosphorylation by a Cdc28/Cln complex, Clns being the G1 cyclins homologous to puc1p (Feldman et al., 1997; Schwob et al., 1994; Skowyra et al., 1997; Verma et al., 1997a; Verma et al., 1997b).

Having described CDKs and their regulation throughout the cell cycle, I will now discuss how they, in combination with other proteins, initiate the major cell cycle events of mitosis and DNA replication.

1.3 Initiation of mitosis

The major mitotic cyclin in Schizosaccharomyces pombe is cdc13p (Booher and Beach, 1988; Hagan et al., 1988). It accumulates following rum1p degradation at the end of G1 (Benito et al., 1998) and forms a stoichiometric complex with cdc2p (Moreno et al., 1989). The cdc2p kinase activity is maintained at a low level throughout S phase and G2 due to the inhibitory phosphorylation of the Y15 residue of cdc2p, located in the ATP binding cleft (Gould and Nurse, 1989). Mitosis is therefore triggered when the balance between the inhibitory kinase and activatory phosphatase shifts in favour of the phosphatase which occurs due to inhibition of the wee1p kinase and accumulation of the cdc25p phosphatase (Breeding et al., 1998; Coleman et al., 1993; Kanoh and Russell, 1998; Moreno et al., 1990; Parker et al., 1993; Russell and Nurse, 1987a; Wu and Russell, 1993; Young and Fantes, 1987).
The events of mitosis are visually dramatic, chromosomes condense and a mitotic spindle is generated. Chromosomes line up along the spindle, attaching via the kinetochore. Sister chromatids are then separated and segregate to opposite poles at anaphase. Formation of a septum and cytokinesis proceed with a short time lag (Fig. 1.1). The substrates for the mitotic kinase have not yet been identified although potential substrates include histone H1 which could be required for chromosome condensation and Lamin B which could be involved in nuclear envelope breakdown in higher organisms (reviewed in Moreno and Nurse, 1990). Fission yeast cells undergo a closed mitosis so the nuclear membrane remains intact.

Cyclins were first identified in sea urchin embryos as proteins that were destroyed at the end of each mitosis (Evans et al., 1983). The same is true for cdc13p which is degraded at the metaphase to anaphase transition. Programmed proteolysis of the cyclin subunit is an important regulatory feature of CDKs. This was first demonstrated in Xenopus egg extracts when a proteolysis-resistant cyclin mutant was shown to prevent inactivation of MPF and exit from mitosis (Murray et al., 1989). This was confirmed by work in fission yeast using an indestructible cdc13 mutant, mutated in its destruction box, as it was shown to be unable to exit mitosis and proceed into the next G1 (Yamano et al., 1996).

Cyclin proteolysis requires the APC (anaphase promoting complex), a ubiquitin ligase which collaborates with a ubiquitin activating and ubiquitin conjugating enzyme to catalyse the transfer of ubiquitin molecules to target proteins (reviewed in Morgan, 1999). APC activity oscillates throughout the cell cycle and is thought to be responsible for targeting proteolysis. The vertebrate APC consists of an 8 subunit core structure that associates with one of another two proteins. These may regulate either the timing of APC activation or the substrate specificity (Morgan, 1999). Homologues of several of these subunits have been identified in Schizosaccharomyces pombe, including cut4, cut9, nuc2, apc10, hcn1, cut20, cut23, slp1 and srw1/ste9 (Hirano et al., 1988; Kim et al., 1998; Kitamura et al., 1998; Kominami et al., 1998b; Samejima and Yanagida, 1994; Yamada et al., 1997; Yamaguchi et al., 1997; Yamashita et al., 1996; Yamashita et al., 1999). The securin, cut2p must also be
destroyed by the APC prior to sister chromatid separation (Funabiki et al., 1996; Funabiki et al., 1997; Yanagida, 2000).

1.4 Initiation of DNA replication

Passage through Start and into S phase requires the function of two genes in fission yeast; cdc2 and cdc10. The mitotic partner for cdc2p, cdc13p, is kept low during G1 due to the function of two proteins, srw1p and rum1p (Correa-Bordes and Nurse, 1995; Kitamura et al., 1998; Yamaguchi et al., 1997). However, the B-type cyclin, cig2p, accumulates in G1 to allow a cdc2p/cig2p complex to promote entry into S phase (Fisher and Nurse, 1996; Martin-Castellanos et al., 1996; Mondesert et al., 1996). The activatory S phase targets for cdc2p/cig2p have yet to be identified, however several proteins have been shown to be phosphorylated after the initiation of DNA replication to inhibit re-replication. These, along with potential activatory substrates, will be discussed later.

CdclO is a transcription factor that acts with res1, res2 and rep2 to allow the periodic transcription of several S phase genes (Aves et al., 1985; Caligiuri and Beach, 1993; Lowndes et al., 1992; Miyamoto et al., 1994; Nakashima et al., 1995; Tanaka et al., 1992; Zhu et al., 1994b). These include cdc18, cdt1, cdc22 and cig2. The functions of some of these genes will be discussed in relation to the initiation of DNA replication. Initial results indicated that cdcl0p formed a complex with res1p to promote transcription during the mitotic cycle and res2p for transcription during the meiotic cycle (Miyamoto et al., 1994; Tanaka et al., 1992; Zhu et al., 1994b). Rep2p was shown to act as a transcriptional activator (Nakashima et al., 1995). However, recent evidence suggests that the situation is more complex. Cdcl0p, res1p and res2p have been shown to exist as a heteromeric complex throughout the mitotic cycle (Whitehall et al., 1999) and both res1p and res2p are required for periodic transcription. Cdcl8 transcript levels are constitutively low in res1Δ cells and constitutively high in res2Δ cells (Baum et al., 1997). Cell cycle regulated transcription cooperates with other mechanisms to ensure that DNA replication occurs only once per cell cycle. This will be discussed later in relation to the alternation problem.
I will now go on to describe the sequence of events leading up to the initiation of DNA replication. These include the formation of a pre-replicative complex at origins preceding initiation, a process that requires the activity of two protein kinases, cdc2p and hsk1p. I will also discuss the involvement of the MCM proteins in DNA replication and the evidence suggesting that they act as the S phase helicase.

Table 1.1
Comparison of gene names between \textit{Schizosaccharomyces pombe} and \textit{Saccharomyces cerevisiae} of genes required for DNA replication

<table>
<thead>
<tr>
<th>\textit{Schizosaccharomyces pombe}</th>
<th>\textit{Saccharomyces cerevisiae}</th>
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<tbody>
<tr>
<td>\textit{cdc18}</td>
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</tr>
<tr>
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<td>ORC1</td>
</tr>
<tr>
<td>\textit{orp2}</td>
<td>ORC2</td>
</tr>
<tr>
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<td>ORC3</td>
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<tr>
<td>\textit{spOrc5}</td>
<td>ORC5</td>
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<tr>
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<td>ORC6</td>
</tr>
<tr>
<td>\textit{cdc19/nda1}</td>
<td>MCM2</td>
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<tr>
<td>\textit{mcm3}</td>
<td>MCM3</td>
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<td>\textit{hsk1}</td>
<td>CDC7</td>
</tr>
<tr>
<td>\textit{dfp1}</td>
<td>DBF4</td>
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</tbody>
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24
1.4.1 Origins

Despite the strong conservation of the basic mechanism of DNA replication amongst eukaryotes, the origins of replication display a surprisingly diverse structure. The most well defined origins are those of *Saccharomyces cerevisiae*. The origins are short, being 100-150 bp in length and consist of several elements. The A domain contains an 11bp consensus sequence, \((A/T)TTTA(T/C)(A/G)TTT(A/T)\), known as the ARS consensus sequence (ACS). This domain is essential but not sufficient for origin activity and several auxiliary elements also exist, B1, B2, B3 and C. Little sequence conservation is observed between these domains but they tend to be A/T rich and are sensitive to alterations in distance and orientation relative to the ACS. A specific role for each element has not been determined, although the B1 element has been shown to cooperate with the ACS in the efficient binding of ORC and the B3 element binds the Abf1 transcription factor (reviewed in Donovan and Diffley, 1996).

Metazoan origins represent the opposite extreme as they tend to be large and heterogeneous. The length of S phase varies greatly during development reflecting a change in the frequency of initiation, not the rate of fork movement. Regulation of replication initiation must therefore alter during animal development. There has been some controversy over how metazoan origin specification occurs. Functional ARS (autonomously replicating sequence) elements have been difficult to determine in mammalian cells and the techniques used to identify origins have also produced different results. 2-d gel electrophoresis identified large initiation zones with no clear preference for initiation at particular sites. However, a different technique using labelled nucleotide precursors identified most initiation events as occurring at specific sites of 0.5-2kb. The current opinion appears to be that initiation zones exist consisting of one or more high frequency initiation sites and several low frequency initiation sites. All sites are detected by the 2-d gel method but only the high frequency sites are detected using biolabelling methods. The DNA sequence is still important in determining an initiation site despite the lack of a defined consensus sequence. Origins tend to contain an ORC binding site and one or more A/T rich elements. Several factors cooperate to determine whether an origin fires including nuclear organization, chromatin structure and in some cases DNA methylation (reviewed in DePamphilis, 1999).
The fission yeast origin lies between that of budding yeast and metazoans in structure, displaying characteristics of both. Several origins have now been analyzed including \textit{ars1}, \textit{ars3001} and \textit{ars3002} and some conclusions can be drawn from these investigations (Clyne and Kelly, 1995; Dubey et al., 1996; Kim and Huberman, 1998). Fission yeast origins tend to be larger than those of budding yeast, generally between 0.5 and 1.5kb. This could be attributed to the fact that they contain many redundant elements as most short deletions barely have an effect on ARS activity and often several domains must be deleted before a significant effect is seen. However one or more essential regions and several important regions have been identified for each origin using deletional analysis. The important regions are A/T rich and asymmetric, having mostly As on one strand and Ts on the other. Some consensus sequences have been identified for the fission yeast origins and these tend to be clustered in the important regions of the ARS elements, however a clear role for these has not yet been established (Clyne and Kelly, 1995; Kim and Huberman, 1998; Maundrell et al., 1988; Zhu et al., 1994a).

1.4.2 ORC

The main function of an origin is to facilitate the binding of a heterohexameric protein complex known as the origin recognition complex (ORC). ORC was first identified in \textit{Saccharomyces cerevisiae} as a DNA binding activity that gave protection to the A and B1 elements of \textit{ARS1} from DNaseI digestion both in vitro and in vivo (Bell and Stillman, 1992; Diffley and Cocker, 1992). This binding is both ATP dependent and origin specific as mutations in the ACS of the A element either reduced or eliminated ORC binding (Bell and Stillman, 1992). Genomic footprinting allowed further characterization of budding yeast origins and identified two states that existed at different stages of the cell cycle (Diffley et al., 1994). The post-replicative footprint was similar to the one produced in vitro by ORC (Fig. 1.4 A) and the pre-replicative footprint, present from the end of mitosis until the beginning of S phase, produced an extended footprint, overlapping that produced by ORC (Diffley et al., 1994).

ORC binds to origins throughout the cell cycle (Liang and Stillman, 1997) indicating that it is not sufficient for replication. ORC is, however, required for
replication as temperature sensitive mutations in two subunits, \textit{orc2-1} and \textit{orc5-1}, are unable to allow initiation of replication at the restrictive temperature as determined by the lack of a bubble arc on a 2-d gel (Liang et al., 1995). \textit{CDC6}, which is homologous to \textit{cdc18} from fission yeast, was identified as a multicopy suppressor of the \textit{orc5-1} mutant and was later shown to be required for the formation of the pre-replicative complex (Cocker et al., 1996; Liang et al., 1995). The function of Cdc6/18p in DNA replication and its involvement with the pre-replicative complex will be discussed later.

\textit{ORC} homologues have been identified in many higher eukaryotes (reviewed in Quintana and Dutta, 1999) as well as \textit{Schizosaccharomyces pombe}. An Orc1p homologue was isolated independently by two groups. Once by virtue of its sequence homology to \textit{cdcl8} (Muzi-Falconi and Kelly, 1995) and also in a mutant screen for new cell cycle genes (Grallert, 1996). Orp1p was shown to be an essential protein required for both DNA replication and the checkpoint that acts to prevent mitosis until S phase has been successfully completed. Both the mRNA (Muzi-Falconi and Kelly, 1995) and the protein were shown to be present at a constant level throughout the cell cycle and the protein also localized to the nucleus (Grallert, 1996). \textit{Orp1} displayed genetic interactions with \textit{cdc18} and \textit{cdc21} and the protein could also immunoprecipitate both \textit{cdc18p} and \textit{cdc21p}, indicating that a complex could exist between \textit{ORC}, \textit{cdc18p} and the MCMs (Grallert, 1996). More recently, \textit{orp1p} has been shown to bind to the origins \textit{ars2004} and \textit{ars3002} throughout the cell cycle (Ogawa et al., 1999).

The second \textit{ORC} subunit identified in fission yeast was \textit{orp2p}, the Orc2p homologue (Leatherwood et al., 1996). It was isolated in a 2-hybrid screen with \textit{cdc2p} and has been shown both to contain \textit{cdc2p} consensus phosphorylation sites (Leatherwood et al., 1996) and to be a phosphoprotein. Orp2p is differentially modified throughout the cell cycle, indicating that \textit{ORC} activity could be regulated by \textit{cdc2p} (Lygerou and Nurse, 1999).

\textit{Orp4p} was isolated using degenerate PCR with budding yeast \textit{Orc4p} primers (Chuang and Kelly, 1999). The C-terminus of \textit{orp4p} displays sequence homology to the human, \textit{Xenopus} and budding yeast counterparts. However the N-terminus
Chapter 1 Introduction

contains a unique sequence consisting of 9 copies of an A-T hook motif known to mediate DNA binding to the minor groove of A-T tracts (Chuang and Kelly, 1999). As fission yeast origins do not possess an ACS, the DNA binding properties of orp4p could facilitate ORC binding to the A/T rich sequence of an origin.

This hypothesis is supported by the fact that ORC purification from fission yeast initially yielded a 5-subunit complex excluding orp4p. The purification of the heterohexamer first required removal of the DNA by DNaseI digestion (Moon et al., 1999). This purification also allowed identification of the less well conserved spOrc3p and spOrc6p homologues.

The final subunit, spOrc5p was isolated after a database search to look for Orc5p homologues (Ishiai et al., 1997). SpOrc5p was shown to be essential as deletion of the gene led to cells which first arrested with a 1C DNA content and then underwent an aberrant mitosis due to a defective checkpoint (Lygerou and Nurse, 1999). Orp1p, orp2p and spOrc5p were shown to exist in a complex as orp1p could immunoprecipitate both orp2p and spOrc5p. The orp1p/spOrc5p complex was shown to exist throughout the cell cycle. The three proteins also remained localized to the nucleus and bound to chromatin at all stages of the cell cycle (Lygerou and Nurse, 1999).

Current evidence therefore suggests that the budding yeast and fission yeast ORC complexes act in a similar manner, as they are both required for DNA replication and remain chromatin bound throughout the cell cycle. I will now discuss some of the proteins that associate and function with ORC to promote DNA replication.

1.4.3 Cdc18

Cdc18 was cloned simultaneously in two ways, as a multicopy suppressor of the cdc10-129 temperature sensitive mutant and by complementation of the cdc18-K46 mutant (Kelly et al., 1993). Cdc18 transcription is cdc10p dependent and accumulates periodically through the cell cycle, increasing in late mitosis and decreasing at S phase (Baum et al., 1998; Kelly et al., 1993). The cdc18 protein mimics this periodicity although it does not accumulate until exit from mitosis (Baum et al., 1998). The rapid decrease in protein seen on entry into S phase is
indicative of an unstable protein, and in fact the half life has been estimated at <8 minutes (Muzi-Falconi et al., 1996). The restriction of cdc18p to G1 and early S phase indicated a role in the promotion of DNA replication. This was confirmed by the analysis of a cdc18Δ strain after spore germination. Cdc18 is essential and its deletion results in cells that are unable to initiate DNA replication but proceed into mitosis, accumulating with a <1C DNA content and a cut phenotype, indicative of a lack of the S phase checkpoint (Kelly et al., 1993). Overexpression of cdc18 results in cells that initiate repeated rounds of DNA replication in the absence of mitosis, supporting the proposal that cdc18p is a key regulator of DNA replication and is important for coordinating alternate rounds of S phase and M phase (Nishitani and Nurse, 1995).

The role that cdc18p plays in initiating S phase was first elucidated in Saccharomyces cerevisiae with the cdc18 homologue, CDC6 (Lisziewicz et al., 1988; Zhou et al., 1989). Identification of two complexes bound to replication origins at different stages of the cell cycle indicated that proteins other than ORC were required at the level of the origin (Diffley et al., 1994). Cdc6p was shown by genomic footprinting to be required for formation of the G1 specific complex, the pre-replicative complex, as described previously (Cocker et al., 1996). The function of Cdc6p was further characterized by the development of two techniques: a cell fractionation protocol that enriches for the chromatin fraction (Donovan et al., 1997) and a chromatin precipitation (CHIP) protocol (Tanaka et al., 1997). The latter uses formaldehyde to cross-link proteins both to each other and to DNA, followed by immunoprecipitation of proteins that may be bound to origin DNA and PCR to selectively amplify the DNA sequence bound to the immunoprecipitated protein. This technique allows the identification of proteins bound to individual origins at different cell cycle stages. Results obtained using these techniques identified Cdc6p as a loading factor for the MCM complex during G1 (Fig. 1.4 B and C) (Aparicio et al., 1997; Donovan et al., 1997; Liang and Stillman, 1997; Tanaka et al., 1997).

Similar results have recently been obtained in fission yeast. Chromatin assays have been used to demonstrate that depletion of cdc18p prevents the loading of cdc21p (mcm4p) onto chromatin (Nishitani et al., 2000). An in situ chromatin assay also
(mcm4p) onto chromatin (Nishitani et al., 2000). An in situ chromatin assay also provides evidence to support this hypothesis. Cdc21-GFPp has been shown to be depleted from the nucleus after cells are treated with enzymes to permeabilize the cell walls and a non-ionic detergent wash to remove non-chromatin bound proteins, when cdc18p is absent from the cell (Kearsey et al., 2000).

1.4.4 MCMs
The minichromosome maintenance family of proteins (mcm2-7p) were first isolated as mutants that showed origin specific defects in the maintenance of minichromosomes in budding yeast (Maine et al., 1984; Sinha et al., 1986). They were later shown to be required for DNA replication and part of the pre-replicative complex, as described above (Fig. 1.4 C). Homologues have been identified in a number of different organisms (reviewed in Tye, 1999) and experiments using Xenopus extracts demonstrated that the MCM complex was a component of "licensing factor", a factor thought to confer on cells the ability to replicate only after passage through mitosis (Chong et al., 1995; Kubota et al., 1997; Kubota et al., 1995; Madine et al., 1995; Thommes et al., 1997).

Several of the Schizosaccharomyces pombe MCM genes were cloned by complementation of either temperature sensitive or cold sensitive mutants deficient in DNA replication. Mcm2 was cloned by two groups, initially by complementation of the cold sensitive mutant, nda1-376 (Miyake et al., 1993) and secondly by complementation of the temperature sensitive mutant, cdc19-P1 (Forsburg and Nurse, 1994b). Mcm4 was cloned by complementation of the temperature sensitive cdc21-M68 mutant (Coxon et al., 1992). Mcm5 was isolated at the same time as nda1/mcm2 by virtue of its ability to complement the nda4-108 cold sensitive mutant (Miyake et al., 1993). Finally mcm6 was cloned by its ability to complement the mis5-268 temperature sensitive mutant (Takahashi et al., 1994). Schizosaccharomyces pombe mcm3 was cloned using a hybridization strategy to identify a gene with homology to a region with high sequence similarity between Saccharomyces cerevisiae, Xenopus, human and mouse MCM3 homologues (Sherman and Forsburg, 1998). The mcm7 gene has not yet been cloned in fission yeast but it has been purified as part of a heterohexameric complex with the other five MCM proteins (Adachi et al., 1997).
The MCM proteins localize to the nucleus throughout the cell cycle in fission yeast (Maiorano et al., 1996; Okishio et al., 1996; Sherman and Forsburg, 1998). Mutation of a single mcm leads to the redistribution of the entire complex from the nucleus to the cytoplasm. This process requires active transport as it does not occur in a crm1 mutant (Pasion and Forsburg, 1999). This is different to the situation in budding yeast in which MCMs localize to the nucleus only during G1 and S phase (Dalton and Whitbread, 1995; Hennessy et al., 1990; Yan et al., 1993). The localization of the MCM complex in budding yeast is one way in which cells can limit S phase to once per cell cycle and this indicates that other controls must exist to prevent re-initiation of DNA replication in fission yeast. These will be discussed later.

The function of the MCM complex has remained elusive. Several lines of evidence indicate that it acts as the S phase helicase. CHIP has been used to demonstrate that the budding yeast MCM complex leaves replication origins after initiation and appears to move with replication forks associating with non-origin DNA with similar kinetics to DNA polymerase ε (Fig. 1.4 E) (Aparicio et al., 1997). A recent report in budding yeast has also shown that the MCMs are required throughout DNA replication, for elongation as well as initiation (Labib et al., 2000). Both of these results would be consistent with the MCMs acting as the S phase helicase. Biochemical evidence from both mouse and humans has shown that a hexameric complex consisting of two Mcm4, 6 and 7 trimers exhibits weak DNA helicase activity having the ability to displace 17-mer but not longer oligonucleotides (Ishimi, 1997; You et al., 1999). The poor processivity of the helicase could reflect the fact that the MCMs are not the true S phase helicase, or could relate to technical difficulties with the in vitro experiment. For example, use of either a different substrate or a different MCM sub-complex could solve the problem. Different sub-complexes of the MCMs have been identified in fission yeast as mcm4p and mcm6p form a core complex with which mcm2p associates more loosely (Sherman et al., 1998). It is possible that a MCM sub-complex possesses the biochemical activity and the other subunits are involved in regulating the activity but the fact that all six subunits co-purify (Adachi et al., 1997) and are required for the correct localization of the complex (Pasion and Forsburg, 1999) indicates that the view of a heterohexameric complex is probably correct (Fig. 1.4 C).
1.4.5 Additional pre-replicative complex proteins

Several proteins other than ORC, cdc18p and MCMs may also be involved in the formation of the pre-replicative complex. *Cdt1* was isolated using a cyclical procedure of immunoprecipitation and amplification to identify genomic DNA sequences bound by the cdc10p transcription factor (Hofmann and Beach, 1994). *Cdt1* was shown to be transcribed periodically in a cdc10p-dependent manner and overexpression of *cdt1* is able to complement a *cdc10-129* mutant at the semi-permissive temperature of 33°C. *Cdt1* is an essential gene and the null mutant has the same phenotype as a *cdc18Δ* in that it is deficient in both DNA replication and the S phase checkpoint (Hofmann and Beach, 1994; Kelly et al., 1993). Recent data suggests that cdc18p and cdt1p act closely together as cdt1p enhances the ability of cdc18p to promote re-replication (Nishitani et al., 2000). The two proteins interact via the C-terminus of cdc18p and both are required for the loading of cdc21p (Mcm4) onto chromatin but not for the chromatin association of each other (Nishitani et al., 2000). The current evidence therefore suggests that cdt1p is also a component of the pre-replicative complex (Fig. 1.4 B). Homologues have recently been isolated in *Xenopus* and *Drosophila* which appear to function in a similar way to fission yeast cdt1p (Maiorano et al., 2000; Whittaker et al., 2000). Database searches have identified homologues in several other organisms including humans, although no budding yeast homologue has so far been discovered. These results indicate that cdt1p provides an essential and conserved function in DNA replication.

*Cut5* was isolated in a screen for mutants that undergo cytokinesis in the absence of normal nuclear division (cell untimely torn) (Hirano et al., 1986). It was later cloned by complementation of the *cut5-T401* temperature sensitive mutant and was shown to be identical to *rad4* (Fenech et al., 1991; Saka and Yanagida, 1993) and homologous to the budding yeast gene *DPB11* (Araki et al., 1995). Cells lacking wildtype cut5p are unable to initiate DNA replication or the S phase checkpoint and undergo an aberrant mitosis and accumulate cells with a <1C DNA content (Saka and Yanagida, 1993). The phenotype is similar to that of both the *cdc18Δ* (Kelly et al., 1993) and *cdt1Δ* (Hofmann and Beach, 1994) strains, and all three proteins are nuclear localized (Nishitani et al., 2000; Nishitani and Nurse, 1995; Saka et al., 1994) indicating that cut5p could also function in the formation of
the pre-replicative complex (Fig. 1.4 B). However, experiments using the \textit{rad4-116} allele have enabled separation of the replication and checkpoint functions as cells are able to initiate replication despite lack of the checkpoint. This indicates that cut5p/rad4p plays a direct role in generating the S phase checkpoint signal and does not merely allow initiation of the signal as a consequence of pre-replicative complex formation (McFarlane et al., 1997).

\textit{Cdc23} was isolated by complementation of the temperature sensitive \textit{cdc23-M36} mutant (Aves et al., 1998). It is an essential gene required for the correct initiation of DNA replication. Both the \textit{cdc23-M36} mutant and a null mutant arrest with a single nucleus and an elongated phenotype. Sequence analysis suggested that \textit{cdc23} was a homologue of the budding yeast gene \textit{DNA43 (MCM10)}. This was confirmed when expression of \textit{cdc23} in budding yeast was shown to rescue the temperature sensitive \textit{dna43-1} mutant at the restrictive temperature (Aves et al., 1998). Dna43p/Mcm10p is thought to form part of the pre-replicative complex as it binds to members of the MCM complex (Merchant et al., 1997) and to chromatin (Fig. 1.4). The chromatin association of Dna43p/Mcm10p is required for the subsequent loading of Mcm2p (Homesley et al., 2000). Interestingly, Dna43p/Mcm10p is constitutively bound to chromatin throughout the cell cycle (Homesley et al., 2000), indicating that post-translational modification could mediate the interaction with the MCM complex. The presence of cdc2p consensus phosphorylation sites in both \textit{cdc23p} and Dna43p/Mcm10p could provide a basis for this modification.

The \textit{sna41} gene was first identified as a suppressor of the \textit{nda4-108 (mcm5)} mutant and was subsequently cloned by complementation of the temperature sensitive \textit{sna41-912} mutant (Miyake and Yamashita, 1998). It is an essential gene required for DNA replication and shows homology to the \textit{CDC45} gene from budding yeast (34\%) (Miyake and Yamashita, 1998), of which a more detailed characterization has been performed. \textit{CDC45} was isolated by complementation of the cold sensitive \textit{cdc45-1} mutant and is required for DNA replication (Hardy, 1997; Hopwood and Dalton, 1996; Zou et al., 1997). The protein levels remain constant throughout the cell cycle (Owens et al., 1997) despite the periodicity of the mRNA (Hardy, 1997). Cdc45p also localizes to the nucleus throughout the cell cycle (Hopwood and
Dalton, 1996). Cdc45p interacts genetically with orc2-1 and either genetically or physically with several members of the MCM complex (Dalton and Hopwood, 1997; Hardy, 1997; Hopwood and Dalton, 1996; Zou et al., 1997). It is recruited to chromatin and the pre-replicative complex late in G1, between Start and the initiation of DNA replication, to form part of a new complex named the pre-initiation complex (Fig. 1.4 D) (Aparicio et al., 1997; Zou and Stillman, 1998). Chromatin association of Cdc45p requires Cdc28p/Clb kinase activity as well as Cdc6p and Mcm2p but Cdc45p does not contain consensus phosphorylation sites indicating that the requirement is indirect (Zou and Stillman, 1998). In both *Saccharomyces cerevisiae* and higher eukaryotes such as *Xenopus* and humans, Cdc45p appears to be involved in the recruitment of DNA polymerase α to replication origins (Aparicio et al., 1999; Kukimoto et al., 1999; Mimura and Takisawa, 1998; Walter and Newport, 2000). Cdc45p and DNA polymerase α are both assembled differentially at early and late origins. Late origin binding of both proteins occurs after DNA replication initiation from early origins and can be inhibited if the S phase checkpoint is activated (Aparicio et al., 1999; Zou and Stillman, 2000). Cdc45p, like the MCMs also appears to move with replication forks (Fig. 1.4 E) (Aparicio et al., 1997).

1.4.6 Cdc2p/cyclin activity

It has long been known that cdc2p/cyclin activity is required for the initiation of DNA replication (Nurse and Bissett, 1981), however substrates that fulfil the requirements for this positive role of CDKs have not yet been identified. It is known that Cdc28p/Clb activity is required for loading of Cdc45p onto chromatin in budding yeast (Fig. 1.4 D and E, and above) however as neither Cdc45p nor the fission yeast homologue sna41p contain CDK consensus phosphorylation sites it is possible that phosphorylation of another component of the pre-replicative complex promotes a conformational change to allow binding of Cdc45p/sna41p. Potential candidates for this could be an ORC subunit, the most likely of which would be Orc6p which is modified to a slower migrating form on entry into S phase in budding yeast. This form is not present when the four Cdc28p consensus phosphorylation sites are mutated (Liang and Stillman, 1997). The fission yeast orp2p has also been shown to be differentially modified throughout the cell cycle (Lygerou and Nurse, 1999). Orp2p is a phosphoprotein and contains cdc2p
consensus phosphorylation sites (Leatherwood et al., 1996; Lygerou and Nurse, 1999); in fact it was first isolated in a 2-hybrid screen for cdc2p interacting proteins (Leatherwood et al., 1996). However the modification occurs in mitosis and the protein becomes converted to a faster migrating form as cells enter G1 (Lygerou and Nurse, 1999). The timing of orc2p phosphorylation is therefore inconsistent with it being a S phase target of CDK activity. An alternative candidate could be cdc23p which contains two cdc2p consensus phosphorylation sites in the N-terminal region of the protein (Aves et al., 1998). I previously suggested that cdc23p phosphorylation could be involved in promoting an interaction with the MCM complex. Either of these options are possible, although as cdc2p kinase activation occurs later in the cell cycle than MCM loading, this hypothesis is less likely.

1.4.7 Hsklp/dfp1p kinase
The second kinase activity required for initiation of DNA replication is that of hsk1/dfp1 in fission yeast. The homologous kinase, Cdc7p/Dbf4p, has been shown to act after Cdc28p/Clb in Saccharomyces cerevisiae, indicative of the sequential phosphorylation of pre-replicative complex components by the two kinases (Fig. 1.4) (Nougarede et al., 2000). Like CDKs, DDKs (Dbf4-dependent kinases) also consist of a kinase subunit, hsklp and a regulatory partner, dfp1p. The hsk1 gene was isolated by degenerate PCR using oligonucleotide primers against budding yeast CDC7 (Masai et al., 1995). Purification of the kinase led to the identification of dfp1p as the regulatory subunit since it was found to be associated with hsk1p. Further analysis of dfp1p has shown it to be an essential gene required for both DNA replication and the S phase checkpoint (Brown and Kelly, 1999; Takeda et al., 1999). In fact dfp1p is phosphorylated in a chk1p kinase dependent manner in response to HU treatment (Brown and Kelly, 1999). Dfp1p is regulated both transcriptionally and post-translationally. This results in an increase in protein level at the G1/S transition which corresponds to activation of the hsk1p kinase (Brown and Kelly, 1999; Takeda et al., 1999).

The MCM complex has been identified as a substrate of the DDK in a number of organisms. Mcm2p has been shown to be a DDK substrate in budding yeast, fission yeast and Xenopus (Brown and Kelly, 1998; Jiang et al., 1999a; Lei et al.,
1997). In fission yeast the hsk1p/dfp1p kinase specifically phosphorylated cdc19p (mcm2p) when presented with the purified MCM heterohexameric complex (Brown and Kelly, 1998). The Cdc7p/Dbf4p kinase was also purified from baculovirus and shown to phosphorylate Pol α-primase and all members of the MCM complex with the exception of Mcm5p (Weinreich and Stillman, 1999).

Another putative target of the DDK is Cdc45p. It has been shown to act at the same time as Cdc7p in reciprocal shift experiments indicating that the two proteins are dependent on each other for function (Owens et al., 1997). Xenopus Cdc7p is also required for the chromatin association of Cdc45p (Jares and Blow, 2000). Finally a recent report has shown that budding yeast Cdc45p can be phosphorylated by the Cdc7p/Dbf4p kinase in vitro (Nougarede et al., 2000).

One hypothesis for the function of the DDK is to aid local unwinding of DNA at origins. The structure of replication origins, determined by genomic footprinting in budding yeast, changes between the CDC7 and CDC8 (a thymidy late kinase required for DNA replication) execution points, indicating that Cdc7p triggers unwinding (Geraghty et al., 2000). The mcm5/cdc46-bobl mutant bypasses the requirement for the Cdc7p kinase (Hardy et al., 1997) and mutant cells blocked in G1 with α-factor have the same ARS DNA structure as is found in a cdc8 mutant, indicating that Cdc7p dependent unwinding may involve the MCM complex (Geraghty et al., 2000).

1.5 Alternation problem
The ability to alternate S phase with M phase is one of the key problems a cell has to solve. CDKs are required for the onset of both of these events and the regulation of this activity is essential in maintaining the correct genome ploidy. In fact disruption of normal CDK activity is sufficient to alter the dependency of S phase on the completion of mitosis. This was first discovered in a screen for diploidizing mutants, i.e., cells that undergo an additional round of DNA replication in the absence of an intervening mitosis. Two previously identified cdc2 alleles were re-isolated, cdc2-33 and cdc2-M26. Loss of cdc2p from a G2 cell is therefore able to change cdc2p from the mitotic form to the S phase form (Broek et al., 1991). The
significance of the level of kinase activity in determining the cell cycle stage was further demonstrated by two discoveries: first that depletion of the mitotic cyclin, cdc13p, could induce multiple rounds of DNA replication in the absence of mitosis (Hayles et al., 1994) and secondly that the CDK inhibitor, \textit{rum1}, could also induce re-replication when overexpressed (Correa-Bordes and Nurse, 1995; Moreno and Nurse, 1994).

Deletion of the B-type cyclins \textit{cig1} and \textit{cig2} demonstrated that the mitotic cyclin, \textit{cdc13}, alone was able to promote both DNA replication, albeit with a slight delay and mitosis indicating that the level of cdc2p associated kinase activity is the determining factor for whether cells undergo S phase or M phase (Fisher and Nurse, 1996). This work led to the proposal of the quantitative model which suggested that a low level of kinase activity is required to prepare for DNA replication, an increase from a low to moderate level of kinase activity promotes DNA replication, maintenance of this level of activity prevents a subsequent round of replication and the increase to a high level brings about the onset of mitosis (Fig. 1.3) (reviewed in Stern and Nurse, 1996). The mechanisms used to control the cdc2p associated kinase level in a normal cell cycle include availability of a cyclin partner (controlled by transcription and proteolysis), phosphorylation and the presence of CDK inhibitors, all of which have been described previously (Fig. 1.2).

Putative cdc2p substrates involved in both the initiation of replication and mitosis have previously been mentioned, however we have yet to address the substrates phosphorylated by cdc2p after S phase initiation to ensure that DNA replication occurs only once per cell cycle.

It is critical to tightly regulate cdc18 protein levels as overexpression of \textit{cdc18} leads to re-replication. \textit{Cdc18} is controlled both transcriptionally in a cdc10p dependent manner (discussed previously) and post-translationally. Cdc18p contains six cdc2p consensus phosphorylation sites (Kelly et al., 1993) and has been shown to be phosphorylated by cdc2p, probably in association with cig2p (Baum et al., 1998; Jallepalli et al., 1997; Lopez-Girona et al., 1998). Phosphorylation promotes binding of cdc18p to pop1p and pop2p/sud1p, the F-box like components of the SCF complex. These act as recognition factors for phosphorylated cdc18p and, along with the other members of the complex, target cdc18p for degradation via the.
proteasome (Fig. 1.4 E) (Jallepalli et al., 1998; Kominami et al., 1998a; Kominami and Toda, 1997; Wolf et al., 1999b).

Rum1p is also targeted for proteolytic degradation via the SCF after phosphorylation of two threonine residues, T58 and T62, by cdc2p (Benito et al., 1998). When these threonine residues are mutated to alanine, rum1p is stabilized and cells occasionally re-initiate replication to become diploids (Benito et al., 1998). However, this diploidization is more efficient if both rum1p and cdc18p are stabilized, as in a pop1 or pop2/sud1 mutant (Jallepalli et al., 1998; Kominami et al., 1998a; Kominami and Toda, 1997; Wolf et al., 1999b). Proteolysis following cdc2p phosphorylation is therefore a crucial mechanism by which DNA replication is restricted to once per cell cycle.

Another important target of cdc2p phosphorylation is the MCM complex. Cdc21p (mcm4p) contains several cdc2p consensus phosphorylation sites and has been shown to exist in multiple phosphorylation states in fission yeast as well as humans and Xenopus (Coue et al., 1996; Hendrickson et al., 1996; Musahl et al., 1995; Nishitani et al., 2000). Evidence initially suggested that phosphorylation could play an inhibitory role in the chromatin binding of cdc21p as the phosphorylated human Cdc21p appeared less tightly bound to nuclear structures (Musahl et al., 1995), and Xenopus Cdc21p became phosphorylated by Cdc2p during S phase, greatly decreasing its affinity for chromatin (Coue et al., 1996; Hendrickson et al., 1996). More recent data obtained from a mammalian in vitro system suggests that the phosphorylation of Mcm4p by cyclin A/Cdk2 inhibits the helicase activity of the Mcm4p, 6p and 7p complex, thus providing a mechanism for the inhibitory effect of CDKs (Ishimi et al., 2000). The situation in budding yeast is somewhat different as MCM localization is cell cycle regulated (as discussed earlier), being nuclear in G1 and cytoplasmic following S phase. This nuclear exclusion during G2 and mitosis is dependent on Cdc28p associated with either the G1 cyclins (CLNs) or the B-type cyclins (CLBs) (Labib et al., 1999; Nguyen et al., 2000). Thus the precise mechanism used by an organism to prevent re-association of MCMs and hence re-formation of pre-replicative complexes in G2 appears to vary but all involve phosphorylation of Mcm4 by CDKs.
A number of mechanisms have therefore evolved in fission yeast to ensure that M phase always succeeds S phase. These include the periodic transcription and degradation of key proteins such as cdc18p and the inhibition of others by phosphorylation such as cdc21p. Some of these mechanisms are redundant, for example constitutive expression of cdc18 does not affect cell cycle progression (Kelly et al., 1993). A multi-layered regulation system exists to preserve genome integrity by ensuring that DNA replication occurs only once in every cell cycle.

1.6 Completion problem

Checkpoints act to monitor the completion of cell cycle events and prevent cell division until these events have been completed successfully (Hartwell and Weinert, 1989). Checkpoints operate in fission yeast to block the onset of mitosis if DNA replication is not complete or DNA damage is not repaired. The checkpoints are therefore initiated by two different signals but interestingly many of the proteins identified have been found to operate in both checkpoint controls. These are known as the checkpoint rad proteins and include rad1, rad3, rad9, rad17, rad26 and hus1 (Al-Khodairy and Carr, 1992; Enoch et al., 1992; Rowley, 1992).

\( \text{Rad1} \) is predicted to have 3'-5' exonuclease activity and is homologous to RAD17 in \( \text{Saccharomyces cerevisiae} \) (Lydall and Weinert, 1995). \( \text{Rad3} \) is a member of the PI-3 kinase family and is related to \( \text{MEC1} \) in budding yeast and \( \text{ATM} \) in humans (Bentley et al., 1996; Enoch and Norbury, 1995; Savitsky et al., 1995). \( \text{Rad17} \) has some homology to RFC which is required to load PCNA onto DNA. The budding yeast homologue of \( \text{rad17} \) is \( \text{RAD24} \) (Griffiths et al., 1995). A human homologue of \( \text{rad9} \), has been identified (Lieberman et al., 1996), as has \( \text{DDC1} \), a \( \text{Saccharomyces cerevisiae} \) homologue (Longhese et al., 1997). Human and mouse genes have been identified with sequence similarity to \( \text{hus1} \), but \( \text{rad26} \) has no known homologue (Kostrub et al., 1998; Volkmer and Karnitz, 1999).
Table 1.2
Comparison of gene names between *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* of genes involved in the checkpoint pathway

<table>
<thead>
<tr>
<th><em>Schizosaccharomyces pombe</em></th>
<th><em>Saccharomyces cerevisiae</em></th>
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<tbody>
<tr>
<td>rad1</td>
<td>RAD17</td>
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<tr>
<td>rad3</td>
<td>MEC1</td>
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<tr>
<td>rad9</td>
<td>DDC1</td>
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<td>rad17</td>
<td>RAD24</td>
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<td>rad26</td>
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<td>hus1</td>
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<td>cds1</td>
<td>RAD53</td>
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<tr>
<td>chk1</td>
<td>CHK1</td>
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Despite the remarkable similarity of the checkpoint rad mutant phenotypes, the proteins have recently been shown to form sub-complexes and perform distinct functions. Rad17p associates with subunit 3 of RFC which is required for both the replication and damage checkpoints (Shimada et al., 1999). This interaction is conserved as Rad24p also interacts with two RFC subunits, Rfc2p and Rfc5p in *Saccharomyces cerevisiae* (Shimomura et al., 1998). Rad17p remains bound to chromatin throughout the cell cycle (Griffiths et al., 2000) indicating that, along with RFC, it could play a role in sensing damage or stalled replication forks. It has also been hypothesised that it could act as a landing pad for other checkpoint proteins following initiation of the checkpoint signal (Fig. 1.5) (Griffiths et al., 2000).

Hus1p has been shown to interact with rad1p in a *rad9* dependent manner in fission yeast (Kostrub et al., 1998), indicating that the three proteins could form a complex as occurs with the human homologues (Volkmer and Karnitz, 1999). Interestingly, hus1p is phosphorylated in response to DNA damage and this phosphorylation is dependent on the other checkpoint rad genes (Fig. 1.5) (Kostrub et al., 1998). However in humans it is the Rad9p component that is phosphorylated in response to damage (Volkmer and Karnitz, 1999).
The checkpoint signals diverge downstream of the checkpoint rad proteins as one of two protein kinases, chk1p or cds1p, is activated depending on whether the signal is generated by damage or stalled replication (Fig. 1.5). This decision appears to be mediated by rad3p as it is required to initiate but not maintain the chk1p response following DNA damage, but is required for both the initiation and maintenance of the cds1p response (Martinho et al., 1998). Rad3p has also been shown to physically interact with chk1p and cds1p (Martinho et al., 1998) indicating that it acts downstream of the other checkpoint rad proteins (Fig. 1.5). This hypothesis is somewhat controversial as a recent report showed that rad3p stably interacts with and phosphorylates rad26p following DNA damage. This does not require the function of the other checkpoint rad proteins (Edwards et al., 1999). The authors conclude that the rad3p-rad26p complex acts either upstream from, or in a parallel pathway to the other checkpoint proteins and could be involved in detecting the DNA damage, therefore placing rad3p at an earlier stage in the pathway (Fig. 1.5).

The chk1p and cds1p protein kinases transduce the checkpoint signal from the checkpoint rad proteins to the cell cycle machinery, resulting in the inhibition of cdc2p and hence mitosis. Both the damage and DNA replication checkpoints ultimately act to maintain cdc2p in its inactive tyrosine-15 phosphorylated form (Enoch et al., 1991; Rhind et al., 1997; Rhind and Russell, 1998). The phosphorylation state of tyrosine-15 is influenced by the kinases, wee1p and mik1p, and the phosphatase, cdc25p, and their deregulation bypasses the checkpoint controls (Gould et al., 1990; Lundgren et al., 1991; Russell and Nurse, 1986; Russell and Nurse, 1987b). The interaction between the signal transducers and the cell cycle machinery will be explored in greater detail in Chapter 2.
Figure 1.1

The fission yeast life cycle

In the presence of sufficient nutrients haploid fission yeast cells grow mitotically. G2 comprises about 70% of the cell cycle whereas G1, S and M take up 10% each (A). Upon starvation, cells follow an alternative developmental fate and undergo sexual differentiation (B). h+ or h- cells exit the cell cycle in G1 and secrete a mating type specific pheromone. Pheromones initiate conjugation which is immediately followed by meiosis and sporulation, resulting in a zygotic ascus with four haploid spores.
Figure 1.2

Principles of CDK regulation

The CDK is first phosphorylated by CAK (CDK activating kinase) on T167 which enables it to associate with the cyclin subunit (A). CDK activity is also regulated by the inhibitory phosphorylation of the Y15 residue (B) and the reversible binding of the CKI rum1p (C).
Figure 1.3

**B-type cyclins control S phase and mitosis in fission yeast**

The cdc2p kinase activity associated with cig2p peaks early in the cell cycle and brings about the onset of S phase. The kinase activity associated with cdc13p rises from S phase and peaks in mitosis. Cdc13p can fulfil three different functions in association with cdc2p: it is essential for the onset of mitosis, it prevents re-replication, and it compensates for cig2p as the major S phase cyclin in a cig2Δ strain.
Figure 1.4

Model for the initiation of DNA replication in fission yeast

ORC and cdc23p are constitutively bound to DNA throughout the cell cycle (A). Cdc18p, cdt1p and possibly cut5p bind to origins early in G1 (B) and promote the loading of the MCMs and formation of the pre-replicative complex (C). CDK activity is required for the loading of sna41p and the formation of the pre-initiation complex (D). DDK activity is the last requirement before initiation and is thought to promote local unwinding at origins. After initiation, cdc18p is released from origins and phosphorylated by CDK leading to its degradation. MCMs and sna41p are thought to move with the replication forks (E).
Figure 1.5

Model showing how checkpoint signals could be transmitted to the cell cycle machinery

The checkpoint signal is first initiated by either stalled replication forks or DNA damage. The signal is transmitted to the checkpoint rad proteins, possibly via rad17p which is constitutively bound to the DNA and could act as a landing pad for the other checkpoint rad proteins. The pathway diverges again at rad3p which transmits the signal to either cds1p or chk1p depending on whether the signal was initiated by stalled replication or damage. Cds1p and chk1p transduce the signal to the cell cycle machinery which ultimately acts to inhibit cdc2p by Y15 phosphorylation.
Chapter 2

Characterization of cdc18 domains and their ability to perform essential cdc18p functions

Introduction

Cdc18p has a role both in promoting DNA replication and in initiating the checkpoint that acts to prevent mitosis until DNA replication has been successfully completed. In this chapter I used a number of different cdc18p mutants including the N-terminus of the protein, the C-terminus of the protein, and site specific mutants, to discover which domains of the protein are required to carry out these functions. I examined this by assaying the ability of cells to re-replicate and to block mitosis when the mutants were overexpressed. I also investigated the relationship between the mitotic kinase, cdc2p/cdc13p and cdc18p during re-replication. Finally I examined where cdc18p acts in the checkpoint pathway and how the checkpoint initiated by overproduction of cdc18p ultimately acts to inhibit the cdc2p/cdc13p kinase.
Chapter 2. Characterization of cdc18 domains

Results

2.1 The C-terminus of cdc18p is sufficient for cdc18p induced re-replication

It has been shown that cdc18p can induce DNA re-replication and block the onset of mitosis when expressed to high levels (Nishitani and Nurse, 1995). Cdc18p contains several motifs which may be involved in these functions. These include six consensus phosphorylation sites (S/TPxK/R) for the cdc2p kinase, and an NTP binding motif (GxxGxGKT). To investigate which domains of cdc18p are essential for its function, a series of constructs encoding mutated versions of cdc18p were generated (Fig. 2.1). Cdc18-1-141p consists of the first 141 amino acids and includes five out of the six cdc2p consensus phosphorylation sites, while cdc18-150-577p and cdc18-150-577 (T374A)p correspond to the C-terminus of cdc18p, in which five out of the six cdc2p consensus phosphorylation sites are deleted. However they do include the NTP binding motif. The difference between these two constructs is that the sixth cdc2p consensus phosphorylation site is mutated in 150-577 (T374A)p, thus removing the last consensus site for phosphorylation by cdc2p. The final construct, cdc18-NTPp, encodes the complete cdc18p with a mutated NTP binding motif (amino acid residues 204G and 205K are changed to AA). This should inhibit cdc18p from binding to an NTP. In order to investigate whether any of these mutants were functional, wildtype cdc18 and the four mutant constructs were transformed into the temperature sensitive cdc18-K46 mutant. The constructs were then expressed at the restrictive temperature of 36°C to see if they could complement the cdc18-K46 mutant. Only wildtype cdc18 was able to complement. The cells containing the cdc18 mutant constructs were able to undergo at most a few divisions. None of the mutants were therefore able to complement cdc18-K46.

In order to investigate which cdc18p functions are defective in each mutant, their ability to induce re-replication and to block mitosis when expressed to high levels was assayed. Each cdc18 construct was introduced into the pRep3X plasmid, thus placing the genes under the control of the thiamine repressible nmt1 promoter (Maundrell, 1990). This is the strongest nmt promoter and results in substantial
overexpression of the gene. The mutants were transformed into wildtype cells and the phenotypes were examined by microscopy and FACS analysis 20 hours after induction of the promoter at 32°C. Overproduction of the wildtype cdc18p or any of the mutants caused the cells to elongate, unlike cells containing the vector alone (Fig. 2.2 A). These results indicate that all of the cdc18p mutants are capable of blocking mitosis.

Next the ability of the cdc18p mutants to induce DNA re-replication was examined. FACS analysis showed an increase in DNA content of cells over-expressing cdc18-150-577p and cdc18-150-577 (T374A)p. Over 40% of the cells had a DNA content of greater than 4C (Fig. 2.2 B d and e). These mutants, like the wildtype protein, were therefore able to induce re-replication. The C-terminus of cdc18p may even induce re-replication to a greater extent than the wildtype protein. Only a proportion of cells are able to induce re-replication as can be seen from the continued presence of a 2C peak (Fig. 2.2 B b, d and e). This is because cells contain a variable plasmid copy number and a minimum number of plasmids will be required in a cell before re-replication can be induced. Cells overproducing cdc18-1-141p and cdc18-NTPp still elongated to a similar extent as the other mutants, as demonstrated by the forward scatter dot plot. However only a small apparent increase in DNA content was generated (Fig. 2.2 B c and f), and less than 10% of cells had a DNA content of greater than 4C. To verify that cdc18-1-141p and cdc18-NTPp were not causing a low level of re-replication leading to diploidization, cells were plated onto media containing Phloxin B (PB) at the end of the timecourse. Phloxin B is a red dye that allows differentiation between haploid and diploid cells. Only living cells are able to pump out Phloxin B and as there is a greater proportion of dead or dying cells in a diploid colony compared to a haploid colony as diploid cells are more sick, the diploid colonies stain a darker pink. Only pale pink colonies, indicative of haploid cells, grew on the PB plates when cdc18-1-141p and cdc18-NTPp had been expressed indicating that there were no diploid colonies present. The increase in DNA content observed is most likely due to either an increase in background staining or mitochondrial DNA (Sazer and Sherwood, 1990) in the subset of cells expressing the constructs.
Western blots were performed to verify that the cdc18 mutant proteins were expressed in all cases. Antibodies against the C-terminus of cdc18p indicated that cdc18-wtp (66kDa), cdc18-150-577p (48kDa), cdc18-150-577 (T374A)p (48kDa) and cdc18-NTPp (66kDa) were all expressed to equal levels (Fig. 2.2 C, lanes 1-4). Several bands of lower molecular weight are also seen and these are most likely degradation products as cdc18 is a highly unstable protein (Muzi-Falconi et al., 1996). It is surprising that more cdc18p products of lower molecular weight are present when the C-terminus of cdc18p is expressed, as it is more stable than the wildtype protein (Baum et al., 1998).

To detect the N-terminus of cdc18p (cdc18-1-141p), antibodies against the entire protein were used. No band was seen in the vector alone control. However, a cdc18p band of about 16kDa was present in the cdc18-1-141p transformant lane. Endogenous cdc18p was not detected as it is expressed at a much lower level than that induced by the nmt1 promoter 20 hours after derepression (Fig. 2.2 D lanes 1 and 2).

These results indicate that all of the mutants were able to maintain a block over mitosis, while those expressing the C-terminus of the protein, cdc18-150-577p and cdc18-150-577 (T374A)p, could also efficiently promote DNA re-replication.

### 2.2 Cdc18p induced re-replication is not caused by inhibition of the cdc2p/cdc13p mitotic kinase

It has been suggested that DNA re-replication induced by high levels of cdc18p occurs indirectly due to the inhibition of the cdc2p/cdc13p mitotic kinase (Wolf et al., 1996). There are two possibilities for how this inhibition could be achieved. Either cdc18p could directly bind and inhibit cdc2p, or overproduction of cdc18p could saturate the proteolytic machinery that degrades both itself and rum1p (Kominami and Toda, 1997). The latter could subsequently lead to an accumulation of the CDK inhibitor and a decrease in the mitotic kinase activity. These possibilities were investigated in several ways. Firstly, the mitotic kinase activity was assayed in cdc13p immunoprecipitates derived from cells overproducing cdc18-wtp and cdc18-150-577p. The cdc2p kinase activity was followed during a 20
hour time period after induction of the promoter at 32°C (Fig. 2.3 A). High levels of wildtype cdc18p depressed the mitotic kinase activity as previously reported (Nishitani and Nurse, 1995), but cdc18-150-577p did not, despite inducing DNA re-replication. This experiment was repeated but in addition cdc18-1-141p was also overproduced. This enabled us to determine whether the inhibition of the mitotic kinase activity was due solely to the N-terminus of the protein. The cdc2 kinase data was quantified at the 11 and 19 hour timepoints using NIH Image (Fig. 2.3 B). A decrease in the kinase activity was observed between 11 and 19 hours when cdc18-wtp and cdc18-1-141p were overproduced. Cdc18-150-577p overproduction, again, had no effect on the mitotic kinase activity. This led us to the hypothesis that it is the N-terminus of cdc18p that interacts with cdc2p and may be responsible for the inhibition of the kinase activity.

This hypothesis was confirmed when cdc2p was precipitated in order to look for an interaction with either the N-terminus or C-terminus of cdc18p. Cdc2p was precipitated using either suc1p beads or BSA control beads in wildtype cells expressing HA tagged cdc18-1-141p or cdc18-150-577p 16 hours after induction of the medium strength nmt promoter (nmt41) at 32°C. Western blots were performed and probed with anti-HA 12CA5 antibodies (Fig. 2.4 top panel) and anti-cdc2p PSTAIR antibodies (Fig. 2.4 bottom panel). A greater proportion of the C-terminus of cdc18p was found in the total cell extract (lanes 1 and 4) which may reflect a difference in stability of the two mutants. However, only HA cdc18-1-141p was precipitated by the suc1p beads (lanes 2 and 5). Neither cdc18p nor cdc2p were precipitated with the BSA control beads (lanes 3 and 6). This indicates that only the N-terminus of cdc18p is able to interact with cdc2p. The fact that the C-terminus of cdc18p does not interact with cdc2p provides further support that re-replication can occur without cdc18p acting directly on cdc2p to inhibit its activity.

These experiments allow us to conclude that although cdc18p can directly bind cdc2p and depress its activity, it is not through this reduction in cdc2p kinase activity that re-replication is induced. The cdc18-150-577p neither results in a decrease in cdc2p/cdc13p kinase activity nor interacts with cdc2p but is still able to promote re-replication to the same extent as cdc18-wtp.
In order to test the alternative possibility, that overproduction of cdc18p saturates the proteolytic machinery resulting in an increase of rum1p, Western blots were performed with extracts derived from wildtype cells overproducing cdc18-wtp, cdc18-1-141p and cdc18-150-577p 20 hours after induction of the promoter at 32°C. The blots were probed with anti-rum1p (Fig. 2.5 top panel) and anti-α-tubulin antibodies (Fig. 2.5 bottom panel). Levels of rum1p did accumulate when cdc18-wtp and cdc18-1-141p were overproduced (Fig. 2.5 top panel, lanes 1 and 2) but not when cdc18-150-577p was overproduced (Fig. 2.5 top panel, lane 3). Therefore overproduction of cdc18p can result in an increase of rum1p levels but re-replication can still occur in the absence of this, as with cdc18-150-577p. To further confirm this we overexpressed the cdc18 mutants in a rum1Δ background (Fig. 2.6). Re-replication was induced to the same levels in both wildtype cells (Fig. 2.6 A) and rum1Δ cells (Fig. 2.6 B). I therefore conclude that cdc18p induced re-replication does not occur as a result of increased rum1p levels leading to inhibition of the cdc2p/cdc13p mitotic kinase.

As a consequence of these experiments I conclude that cdc18p induced re-replication is not simply due to an inhibition of the cdc2p/cdc13p mitotic kinase.

2.3 Requirement of checkpoint genes for the mitotic block

Cdc18p has previously been shown to have an additional role to that in DNA replication, as deletion of the gene leads to an aberrant mitosis and cytokinesis as cells undergo mitosis in the absence of DNA replication (Kelly et al., 1993). Cdc18p is therefore part of a checkpoint that monitors the state of the cell and restrains mitosis until S phase has been successfully completed. Overproduction of cdc18p results in both re-replication and a block over mitosis, so it is possible that it mimics activation of the DNA replication checkpoint. By expressing cdc18p in checkpoint mutant backgrounds it may be possible to place where cdc18p acts in the checkpoint pathway. Some of the cdc18p mutants are able to inhibit mitosis without inducing re-replication (cdc18-1-141p and cdc18-NTPp). This may allow separation of the replication and checkpoint functions and help determine whether functional domains of cdc18p differ in their ability to activate the checkpoint.
The *cdc18* mutants (Fig. 2.1) were expressed in strains defective in the checkpoint control. Five mutants were used, *rad1-1*, *rad3-136*, *rad9-192*, *rad17-h21* and *hus1-14*, all of which are unable to send the signal preventing mitosis when hydroxyurea (HU), a drug that inhibits ribonucleotide reductase and hence blocks cells in early S phase, is added to the cells (Al-Khodairy and Carr, 1992; Enoch et al., 1992; Rowley, 1992). Cells were scored for elongation, indicating growth without division and therefore a block over mitosis. Cells were considered elongated if they were more than 1.5 times the size of a wildtype cell 20 hours after derepression of the *nmt1* promoter at 32°C. Typically 60% of transformed cells in an exponentially growing population would contain a plasmid and consistently around 60% of wildtype cells transformed with any of the *cdc18* constructs were found to be elongated (Fig. 2.7 A a). In checkpoint mutant backgrounds, cells expressing *cdc18-wtp*, *cdc18-l-141p* and *cdc18-NTPp* were all capable of blocking mitosis. Elongation occurred to the same level as in wildtype cells. However in all the checkpoint mutants, expression of *cdc18-150-577p* and *cdc18-150-577 (T374A)p* did not induce elongation, indicating that these mutants were unable to send the checkpoint signal to prevent mitosis (Fig. 2.7 A b-f). Microscopic examination of cells overproducing *cdc18-150-577p* and *cdc18-150-577 (T374A)p* in a *rad1-1* strain confirmed their inability to send the checkpoint signal as the cells were much smaller and displayed a *cut* (cell untimely torn) phenotype (Fig. 2.7 B d and e). This is indicative of an aberrant mitosis in the absence of completed DNA replication. This results in unequal segregation of the DNA as it may either be torn between the two daughter cells or one cell may be left without any of the genetic material (anucleate). Western blot analysis shows that all of the mutants were expressed (Fig. 2.7 C and D) and therefore that the inability of *cdc18-150-577p* and *cdc18-150-577 (T374A)p* to block mitosis was not due to lack of protein expression. In fact, the protein levels of *cdc18-150-577p* and *cdc18-150-577 (T374A)p* in *rad1-1* cells appear to be about 2 or 3 times higher than those of *cdc18wt p* and *cdc18-NTPp* (Fig. 2.7 C lanes 1-4).

We conclude that high levels of wildtype *cdc18p* and mutants containing the N-terminus can block mitosis even when the checkpoint control is defective. This strongly suggests that *cdc18p* overproduction does not mimic activation of the normal checkpoint control. It seems more likely that as the N-terminus can bind
cdc2p (Fig. 2.4), it inhibits the mitotic kinase and blocks mitosis in a non-specific manner. However, when the N-terminus is deleted as in the cdc18-150-577p and cdc18-150-577 (T374A)p constructs, the block over mitosis is dependent upon an intact checkpoint control. In these cases the high levels of cdc18p do appear to induce activation of the checkpoint. Thus the C-terminus of cdc18p appears to act upstream of the rad and hus genes examined, whilst the N-terminus acts in a rad/hus checkpoint independent manner.

2.4 The NTP binding motif is required both for re-replication and initiation of the checkpoint signal

The NTP mutant cdc18-NTPp is also able to block mitosis when overproduced but does not induce DNA re-replication (Fig. 2.2 A f and B f). There are two possibilities for how cdc18-NTPp could block mitosis. The first is that it is simply due to the presence of the N-terminus of cdc18p, as cdc18-1-141p alone can inhibit mitosis, perhaps by directly binding to cdc2p. The alternative is that cdc18-NTPp could allow a complex to form at the replication origins which is sufficient to trigger a checkpoint signal, but is deficient for initiating replication. To differentiate between these possibilities and test whether the N-terminus region is required for the NTP mutant to block mitosis, a NTP 150-577 mutant was constructed. When cdc18-150-577-NTPp was overproduced in wildtype cells re-replication was not induced (Fig. 2.8 B) whereas overproduction of cdc18-150-577p induced re-replication (Fig. 2.8 A). There was also no block over mitosis as the forward scatter dot plot demonstrates that the cells do not elongate. Therefore, an intact NTP site is required both to induce re-replication and bring about a checkpoint dependent block over mitosis. The cdc18-NTPp mutant must therefore block mitosis via its N-terminal region.

2.5 Requirement for cut5

Another gene, cut5, which is essential for DNA replication has been implicated in the checkpoint (Saka et al., 1994; Saka and Yanagida, 1993). To investigate the effects of the various cdc18p mutants in cut5 mutant cells, they were introduced into the temperature sensitive mutant cut5-580 which blocks DNA replication and
induces mitosis at the restrictive temperature of 36°C. *Cut5-580* cells were transformed with the *cdc18* mutant constructs and grown in the absence of thiamine to allow protein expression for 20 hours at 25°C. The culture was then split and half was shifted to the restrictive temperature of 36°C. The cultures were grown for three further generations and then scored for elongated cells (Fig. 2.9). The cells maintained at the permissive temperature (25°C) elongated to approximately the same extent as wildtype cells expressing the *cdc18* mutants (Fig. 2.7 A a). However, when shifted to 36°C, the C-terminus of *cdc18* was again unable to block cell division. This indicates that the C-terminus of the protein requires *cut5p* to send the checkpoint signal to block mitosis.

### 2.6 Requirement for cell cycle core machinery

The DNA replication checkpoint initiated either by ongoing DNA synthesis, as with re-replication, or a block in DNA synthesis, for example when HU is added to cells, must ultimately act to inhibit the cdc2p/cdc13p kinase and hence prevent mitosis. It does this by utilising the normal cell cycle controls that act in G2 to prevent activation of the kinase until the correct time. This control acts through the inhibitory phosphorylation of the cdc2 tyrosine-15 residue and is regulated by the wee1p/mik1p tyrosine kinases, and the cdc25p phosphatase. As the checkpoint also involves tyrosine-15 phosphorylation (Enoch et al., 1991), it could either activate wee1p or mik1p, or inhibit cdc25p, or both to inhibit cdc2p activity.

To investigate these possibilities I overexpressed the *cdc18* constructs in two strains. The first was a *cdc2* mutant, *cdc2-3w*, which allows cells to enter mitosis without cdc25p (Enoch and Nurse, 1990). If the checkpoint acted to inhibit cdc25p, the cells would be unable to prevent mitosis when the *cdc18* mutants were overproduced as the cells would be able to enter mitosis regardless of cdc25p activity. The cells would therefore continue dividing in a *cdc2-3w* strain and would not be elongated. The cells were grown at 32°C for 20 hours after induction of the promoter and were scored for elongation. All of the mutants produced elongated cells when overexpressed in the *cdc2-3w* strain (Fig. 2.10 A).
Cdc2-3w cdc25Δ cells are longer than cdc2-3w cells due to an increased generation time (Russell and Nurse, 1987b). The fact that cdc2-3w cdc25Δ cells are elongated shows that although cdc2-3w cells do not require cdc25p for mitotic entry, they are still sensitive to changes in cdc25p activity. It was important to confirm that the elongation seen when the cdc18 constructs were overexpressed was a result of a block over mitosis as opposed to an elongated generation time. In order to investigate this I looked at a sample of cdc2-3w cdc25Δ cells and compared them with cdc2-3w cells that had either been transformed with the pRep3X vector or had been overproducing cdcl8-wtp for 20 hours (Fig. 2.10 C). I measured the length of 15 cells in each case and calculated the mean (Fig. 2.10 B). We also calculated the standard deviation to look at the variability and found it to be highest for cdc2-3w cells overproducing cdcl8-wtp. In this case it was 0.912 units, where the average cell length was 4.12 units. We found that cdc2-3w cells overproducing cdcl8-wtp were on average twice the length of cdc2-3w cdc25Δ cells. This indicates that cdc2-3w cells overexpressing the cdc18 mutants are blocking mitosis and do not merely have a longer generation time. We conclude that the DNA replication checkpoint does not act solely through the inhibition of cdc25p.

The second strain used was wee1-50 mik1Δ (Lundgren et al., 1991). At the restrictive temperature for wee1-50 (36°C) the cells do not possess a kinase capable of phosphorylating tyrosine-15 and consequently inhibiting cdc2p. The mutants were transformed into this strain and cultures were grown at the permissive temperature for 20 hours following induction of the promoter. The cultures were then split and half were shifted to 36°C. At 25°C all of the mutants were able to prevent mitosis thus causing cell elongation (Fig. 2.11 A and B a and c). However, at 36°C although those producing cdcl8-wtp (Fig. 2.11 B b), cdcl8-1-141p and cdcl8-NTPp were elongated, nearly all of the cells expressing cdcl8-150-577p (Fig. 2.11 B d) and cdcl8-150-577 (T374A)p were very small and displayed cut nuclei indicating aberrant mitoses. These results indicate that the wee1p/mik1p kinases are required for a response to the checkpoint signal activated by overproducing the C-terminus of cdcl8p which leads to a block over mitosis.
Discussion

Previous work has shown that cdc18p overexpression in fission yeast cells results in re-replication of DNA and induces a checkpoint which blocks mitosis (Nishitani and Nurse, 1995). I show that a series of cdc18p mutants can prevent mitosis, resulting in cell elongation, but only those encoding the C-terminus of cdc18p, cdc18-1-141p and cdc18-1-141 (T374A)p, are able to induce DNA re-replication to a level similar to that found when wildtype cdc18p is overexpressed (Fig. 2.2). These results indicate that the C-terminus of cdc18p with an intact NTP binding motif is able to carry out the re-replication function of cdc18p.

Several lines of evidence have shown that this re-replication can occur in the presence of cdc2p kinase activity. Cdc2p kinase assays using histone H1 as a substrate demonstrate that overproduction of the C-terminus of cdc18p does not cause a decrease in cdc2p kinase activity despite inducing re-replication to a similar extent as cdc18-wtp (Fig. 2.3). However, both cdc18-wtp and cdc18-1-141p do result in a decrease in the kinase activity when overproduced. This result was confirmed when the binding of cdc2p to either the N-terminus or C-terminus of cdc18p was assayed (Fig. 2.4). It was discovered that only the N-terminus was capable of interacting with cdc2p, thus providing a potential mechanism for the decrease in cdc2p kinase activity. An alternative mechanism could be through the accumulation of the CDK inhibitor rumlp. Cdc18p and rumlp are degraded by the same proteolytic pathway (Kominami and Toda, 1997). There was therefore a possibility that overproduction of cdc18p would overwhelm the proteolytic machinery allowing rumlp levels to increase. The re-replication induced by the C-terminus of cdc18p occurred in the absence of an accumulation of rumlp. However an increase in rumlp occurred when constructs containing the N-terminus of cdc18p were overproduced (Fig. 2.5, lanes 1 and 2). This accumulation of rumlp could be sufficient to drive an additional round of replication. This may account for the 4C peak seen on the FACS profile (Fig. 2.2). To address whether this was correct we plated cdc18-1-141p cells onto Phloxin B, 20 hours after induction of the nmt1 promoter. None of the resulting colonies were dark pink, indicative of a diploid population. The continued presence of this 4C peak when cdc18-1-141p
was overproduced in a *rum1Δ* strain (Fig. 2.6) confirms that the increase in DNA is not a small amount of re-replication but is most likely due to either increased background staining caused by cell elongation or mitochondrial DNA as reported previously (Sazer and Sherwood, 1990).

As the C-terminus of *cdc18p* can induce re-replication without a decrease in *cdc2p* kinase levels it indicates that *cdc18p* re-replication does not occur simply by inhibiting the *cdc2p/cdc13p* mitotic kinase, as occurs when the mitotic cyclin, *cdc13p*, is switched off (Hayles et al., 1994) or *rum1p* is overproduced (Moreno and Nurse, 1994). This strengthens the case that *cdc18p* is a key regulator of *S* phase initiation. This result also supports previous work suggesting that *cdc18p* re-replication does not require a decrease in kinase levels since *cdc18* mutated in the first five CDK phosphorylation sites was able to re-replicate even when co-expressed with *cdc13p* to increase *cdc2p/cdc13p* kinase activity (Jallepalli et al., 1997).

I also showed that the C-terminal constructs of *cdc18p*, *cdc18-150-577p* and *cdc18-150-577 (T374A)p*, were unable to maintain the block over mitosis in checkpoint deficient strains (Fig. 2.7). These results indicate that the C-terminus of the protein acts upstream of the checkpoint genes examined and requires the *rad* and *hus* genes to send the checkpoint signal to block mitosis. Mutants containing the N-terminus of *cdc18p* (*cdc18-wtp, cdc18-1-141p* and *cdc18-NTPp*) were able to prevent mitosis in the checkpoint deficient strains. Similarly the mitotic block induced by high levels of the C-terminus of *cdc18p* requires the *cut5* protein while mutants containing the N-terminus of *cdc18p* can block mitosis in the absence of *cut5p* function (Fig. 2.9).

These results suggest that high levels of *cdc18p* can block mitosis by two mechanisms. In the first mechanism the N-terminus region containing five of the *cdc2p* consensus phosphorylation sites binds the *cdc2p* mitotic kinase directly (Fig. 2.4) and blocks mitosis independently of the checkpoint control. I suggest that this is an unphysiological mechanism, resulting from overproduction of the proteins and does not provide any insight into the function of the checkpoint. In the second mechanism the block over mitosis is brought about by the C-terminus which does
operate through the checkpoint control and requires the checkpoint rad/hus genes. The C-terminus region requires an intact NTP site to send the checkpoint signal, and the same site needs to be intact to induce DNA replication. Thus it is likely that the C-terminus block over mitosis is brought about by the induction of DNA replication which then activates the normal checkpoint control. This result suggests that cdcl8p acts at the beginning of the pathway to induce the DNA replication checkpoint and not at the end of the pathway by binding directly to cdcl2p.

It is possible to speculate on the nature of the checkpoint signal; it could be a replication complex on the DNA or the presence of replication intermediates once replication has been initiated. The cdcl8-150-577 (NTP)p mutant expressed in wildtype cells could not induce DNA replication or activate the checkpoint signal as could be seen from the FACS data (Fig. 2.8). As these defects may have been due to the failure to form a complex or fire origins, it was impossible to separate the checkpoint function from that of replication and hence gain further insight into how the signal is initiated.

The C-terminus of cdcl8p alone is therefore able to carry out both of the essential functions of cdcl8p but is still unable to complement the cdcl8-K46 temperature sensitive mutant when expressed at the restrictive temperature. The N-terminus of cdcl8p is thought to play a predominantly regulatory role as it contains 5 out of the 6 cdcl2p consensus phosphorylation sites, the phosphorylation of which targets cdcl8p for degradation (Baum et al., 1998; Jallepalli et al., 1997). However, the N-terminus of cdcl8p is still essential for cell viability. Whether this is due to a requirement for cdcl8p destruction at the end of S phase to allow normal cell cycle progression, or is related to another, as yet unknown function of cdcl8p, will be discussed in more detail in a later chapter.

I have shown that when cdcl8-150-577p or cdcl8-150-577 (T374A)p are overexpressed in the absence of wee1p/mik1p function, cells enter mitosis prematurely resulting in small cells displaying cut phenotypes (Fig. 2.11). This suggests that the checkpoint activated by high cdcl8p levels maintains cdcl2p in its tyrosine-15 phosphorylated state with reduced kinase activity. When the same
mutants are overexpressed in a \textit{cdc2-3w} strain which acts independently of \textit{cdc25p}, the cells are able to maintain a block over mitosis indicating that the checkpoint does not act to inhibit \textit{cdc25p} (Fig. 2.10). This is inconsistent with results obtained when HU is added to a \textit{cdc2-3w} strain as these cells continue to divide when DNA replication is blocked (Enoch and Nurse, 1990). This can be explained if the inhibitory signal sent to indicate ongoing replication is stronger than that found when replication forks are halted by the addition of HU. Alternatively, re-replication induced by high levels of \textit{cdclSp} could be detected by the damage pathway as DNA damage is able to produce a block over mitosis in a \textit{cdc2-3w} strain (Al-Khodairy and Carr, 1992). This point will be addressed in more detail later. The constructs containing the N-terminus of \textit{cdclSp}, \textit{cdclS-wtp}, \textit{cdclS-1-141p} and \textit{cdclS-NTPp}, do not require either \textit{weel/miklp} or \textit{cdc25p} to block mitosis, indicating that the inhibition in this case is not dependent on tyrosine-15 phosphorylation. These results are consistent with our conclusions that the N-terminus of \textit{cdclSp} prevents mitosis by binding directly to \textit{cdc2p}.

Since this work was carried out, further progress has been made into how the checkpoint signal is transduced to ultimately inhibit mitosis. The evidence that the checkpoint is maintained through tyrosine phosphorylation of \textit{cdc2p} has been controversial at times (Barbet and Carr, 1993; Knudsen et al., 1996) but recent work confirms that this phosphorylation is essential to maintain both the DNA damage and DNA replication checkpoints in fission yeast (Rhind et al., 1997; Rhind and Russell, 1998). Two protein kinases provide a link between the \textit{rad} and \textit{hus} proteins and the cell cycle machinery that regulates \textit{cdc2p} activity. These are \textit{cdslp} (Murakami and Okayama, 1995), and \textit{chklp} (Walworth et al., 1993). \textit{Cds1p} is required for the checkpoint arrest induced by stalled replication forks or damage caused during S phase (Lindsay et al., 1998; Murakami and Okayama, 1995). \textit{Chk1p} is primarily involved in the damage checkpoint (Walworth et al., 1993) but is activated by HU if \textit{cds1p} is absent (Lindsay et al., 1998). It has been proposed that \textit{cds1p} prevents the occurrence of DNA damage structures during S phase arrest.

\textit{Cds1p} is thought to bind and phosphorylate \textit{wee1p} in response to HU, however the checkpoint is still maintained in a \textit{weel} mutant. \textit{Mik1p} has also been shown to
accumulate in response to HU but not in a cds1Δ strain, thus both tyrosine kinases contribute to the inhibitory phosphorylation of cdc2p in a cds1p dependent manner (Boddy et al., 1998; Christensen et al., 2000). Both cds1p and chk1p have been shown to phosphorylate cdc25p (Furnari et al., 1999; Zeng et al., 1998) and chk1p is thought to bind cdc25p (Boddy et al., 1998). This phosphorylation occurs at several sites, the major one being Serine 99, and promotes binding to the 14-3-3 protein rad24p (Furnari et al., 1999; Zeng et al., 1998). Binding leads to nuclear exclusion of cdc25p as the complex is actively transported from the nucleus by the exportin crm1p (Lopez-Girona et al., 1999; Zeng and Piwnica-Worms, 1999). This removes cdc25p from the vicinity of its target, cdc2p. Cdc2-3w and cdc2-3w cdc25Δ cells are not hypersensitive to damage suggesting that they are checkpoint proficient and consequently that there is a cdc25p independent signalling pathway. The checkpoint is irradiated if both wee1p and cdc25p are inactivated and thus acts phenotypically as a chk1Δ strain. This suggests that the cdc25p independent pathway acts through wee1p. Wee1p has been shown to be hyperphosphorylated on overexpression of chk1p or UV irradiation and wee1 protein levels have also been shown to increase (O'Connell et al., 1997; Raleigh and O'Connell, 2000).

Given these results, it would be interesting to see whether chk1p or cds1p is activated by cdc18p induced re-replication. As cds1p is not activated during a normal S phase (Lindsay et al., 1998) it is possible that re-replication could be sensed as damage and lead to activation of chk1p. Alternatively, cds1p may not be activated during a normal S phase which is completed within about 10 minutes (Bostock, 1970). Cds1p could therefore be activated in cells held in S phase for a longer period of time, either through addition of HU, inactivation of cdc22p, the small subunit of ribonucleotide reductase, or re-replication. It would also be informative to discover whether re-replication induced by inhibition of the mitotic kinase, through overexpression of rum1p or inactivation of cdc13p, would stimulate the same checkpoint pathway as overexpression of cdc18p. In the former case cells are thought to undergo a normal S phase followed by exit from G2 to G1, thus bypassing mitosis. The latter however, can be thought of as an intra-S re-replication with cells never exiting DNA replication. It is therefore possible that overexpression of cdc18 would generate replication structures recognised as damage rather than ongoing replication. The fact that both the re-replication and
damage checkpoints are maintained in a cdc2-3w strain, as previously mentioned, but the same cells cut on addition of HU may provide a further clue that re-replication could be recognised as damage rather than incomplete replication.

The model in Fig. 2.12 summarises the main conclusions. The N-terminus of cdc18p binds to cdc2p and inhibits mitosis in an unphysiological manner as a result of overexpression. The C-terminus of cdc18p is crucial for both the replication function of cdc18p and for the checkpoint that acts through cut5 as well as the rad and hus genes. Cdc18p also requires an intact NTP binding motif for both of these activities. The checkpoint pathway induced by high levels of the C-terminus of cdc18p exerts its effect through a pathway that requires the activity of the wee1p and mik1p tyrosine kinases, resulting in an inactive tyrosine-15 phosphorylated cdc2p/cdc13p complex.
**Figure 2.1**

*cde18 constructs*

Wildtype *cde18* contains a putative NTP binding motif and six *cdc2p* consensus phosphorylation sites. *cde18-1-141p* possesses the first 141 amino acids. *cde18-150-577p* consists of amino acids 150-577 as does *cde18-150-577 (T374A)p*, however this has an additional mutation from T to A at amino acid 374 in the sixth *cdc2p* phosphorylation site. *cde18-NTPp* consists of the full length protein with the NTP binding motif mutated from GK to AA at amino acids 204-205. The black circles represent *cdc2p* consensus phosphorylation sites, the black rectangle depicts the NTP binding motif and the grey rectangle represents the mutated NTP binding motif.
Figure 2.2

Overproduction of the C-terminus of cdc18p is required and sufficient for DNA replication

Wildtype cells transformed with the cdc18 mutants and a vector control were grown at 32°C for 20 hours after induction of the promoter. (A) Cells were fixed and stained with DAPI. Bar 15 µm. (B) DNA content and cell length was analyzed by FACS.
Figure 2.2 C and D

Protein levels were determined by Western blotting. 25 μg of protein was loaded per lane. Blots were probed with anti-cdc18p C-terminus antibody (C top panel), anti-cdc18p antibody (D top panel) and anti-α-tubulin antibody as a loading control (C and D bottom panels).
Figure 2.3

cdc18p induced re-replication does not require depression of the cdc2p/cdc13p mitotic kinase

(A) Histone kinase assays were performed on cdc13p immunoprecipitates from wildtype cell extracts overproducing cdc18-wtp and cdc18-150-577p. (B) Histone kinase assays were also performed from wildtype extracts overproducing cdc18-wtp, cdc18-1-141p and cdc18-150-577p. The data was quantified at the 11 hour and 19 hour timepoint, using NIH image.
**Figure 2.4**

cdc18-150-577p does not interact with cdc2p directly when overproduced

Western blots were performed on suc1p or BSA control bead precipitates from wild type cells overproducing HA cdc18-1-141p and HA cdc18-150-577p from the nmt41 promoter for 16 hours. 5 μg of total extract and precipitates from 100 μg of extract were run. Blots were probed with anti-HA 12CA5 antibody (top panel) and anti-cdc2p PSTAIR antibody (bottom panel).
Figure 2.5

The C-terminus of cdc18p does not induce increased levels of rum1p when overproduced

Protein levels were determined by Western blots performed from extracts of wild type cells overproducing cdc18-wtp, cdc18-1-141p and cdc18-150-577p, 20 hours after induction of the promoter. Blots were probed with anti-rum1p antibody (top panel) and anti-α-tubulin antibody as a loading control (bottom panel).
Figure 2.6

cdc18p induced re-replication can occur in the absence of rum1p

Wildtype cells (A) and rum1Δ cells (B) were transformed with the cdc18 mutants and a vector control and were grown at 32°C for 20 hours after the induction of the promoter. DNA content and cell length were analysed by FACS.
Figure 2.7
The cdc18p C-terminus is unable to block mitosis when overproduced in a checkpoint deficient background

Wildtype cells and checkpoint deficient strains transformed with a vector control and the cdc18 constructs were grown at 32°C for 20 hours after induction of the promoter. (A) 300 cells were scored for elongation. (B) radl-1 cells were fixed and stained with DAPI. Bar 15 μm.
Figure 2.7 C and D

Protein levels were determined by Western blotting in radl-1 cell extracts. 25 μg of protein was loaded per lane. Blots were probed with anti-cdc18p C-terminus antibody (C top panel), anti-cdc18p antibody (D top panel) and anti-α-tubulin antibody as a loading control (C and D bottom panel).
An intact NTP site is required both to induce re-replication and to initiate the checkpoint

Wildtype cells overproducing cdc18-150-577p (A) and cdc18-150-577-NTPp (B) were grown for 20 hours after the induction of the promoter. DNA content and cell length were analysed by FACS.

Figure 2.8
The C-terminus of cdc18p also requires cut5p to prevent mitosis

cut5-580 mutant cells were transformed with the cdc18 constructs and a vector control and were grown at 25°C for 20 hours after induction of the promoter. The cultures were then split and half were shifted to the restrictive temperature of 36°C. They were allowed to grow for a further 3 generations (12 hours at 25°C and 7 hours plus 1 hour for temperature shift recovery at 36°C) before 300 cells were scored for elongation.
Figure 2.10

The checkpoint induced by cdc18p overproduction does not act solely through the cdc25p phosphatase

cdc2-3w cells were transformed with the cdc18 constructs and a vector control. (A) cdc2-3w cells were grown at 32°C for 20 hours after the induction of the promoter and 300 cells were scored for elongation. (B) cdc2-3w cells expressing a vector control, and overproducing cdc18-wtp, 20 hours after induction of the promoter at 32°C and cdc2-3w cdc25Δ cells were measured for cell length and the mean was calculated. (C) Cells were fixed and stained with DAPI. Bar 15μm.
The wee1p/mik1p tyrosine kinases are required for the DNA replication checkpoint that acts through cdc18p

wee1-50 mik1Δ cells were transformed with the cdc18 mutants and a vector control. (A) wee1-50 mik1Δ cells were grown at 25°C for 20 hours following induction of the promoter. The cultures were then split and half were shifted to the restrictive temperature of 36°C. Cells were grown for 3 generations then 300 were scored for elongation for each point. (B) wee1-50 mik1Δ cells overproducing cdc18-wtp at 25°C (a) and 36°C (b), and cdc18-150-577p at 25°C (c) and 36°C (d) were also fixed and stained with DAPI. Bar 15 μm.
Figure 2.12

Model for the role of cdc18p in the DNA replication checkpoint

The C-terminus of cdc18p acts upstream of the rad and hus genes in the DNA replication checkpoint. The pathway requires the function of the wee1p and mik1p tyrosine kinases to inhibit the cdc2p/cdc13p kinase and hence mitosis. The N-terminus of cdc18p can prevent mitosis independently of the rad and hus checkpoint genes, probably via a direct interaction with cdc2p.
Chapter 3

A functional analysis of cdc18 homologues in Schizosaccharomyces pombe

Introduction

Cdc18 has homologues in Saccharomyces cerevisiae, Xenopus laevis and humans. In this chapter I investigated their ability to complement the cdc18-K46 temperature sensitive mutant. I also determined whether they, like cdc18, are able to induce re-replication when overexpressed, either in the presence or absence of wildtype cdc18p function. The effect of co-expression of cdt1 with cdc18 and its homologues was also examined in both the complementation and re-replication assay as it is known to act co-operatively with cdc18p to promote replication.
Results

3.1 Cdc18 has homologues in *Saccharomyces cerevisiae*, *Xenopus laevis* and *Homo sapiens*

*Cdc18* has been found to have homologues in several other organisms that are both structurally and functionally similar. The gene products are called Cdc6p in other organisms and to avoid confusion they will be designated ScCdc6p (*Saccharomyces cerevisiae*), XICdc6p (*Xenopus laevis*) and HsCdc6p (*Homo sapiens*). The *Saccharomyces cerevisiae* gene was isolated by complementation of a temperature sensitive *cdc6* mutant from a genomic library (Lisziewicz et al., 1988; Zhou et al., 1989). The *Xenopus laevis* homologue was cloned using degenerate PCR primers, based on sequence conserved between the budding and fission yeast genes, to amplify a segment of its cDNA. This PCR product was then used to isolate a full length cDNA (Coleman et al., 1996). A similar method was used to clone the human gene (Sanders Williams et al., 1997). This was also identified independently in a two-hybrid screen as a PCNA interacting protein (Saha et al., 1998). Recently a mouse homologue has also been identified (Berger et al., 1999).

The proteins share approximately 30% identity (Fig. 3.1) and have several motifs in common. They all possess a putative nuclear localization signal and several consensus sites for CDK phosphorylation which indicates that their localization and activity could be regulated in a similar manner. The presence of both a NTP binding and hydrolysis motif in all homologues could indicate a shared function (Coleman et al., 1996; Kelly et al., 1993; Lisziewicz et al., 1988; Saha et al., 1998; Sanders Williams et al., 1997; Zhou et al., 1989). In fact Cdc18/6p appears to have a role in promoting DNA replication in all organisms. A more precise function has been found in yeast and *Xenopus* as Cdc18/6p can specifically load the MCM family of proteins onto chromatin thus establishing the pre-replicative complex (Aparicio et al., 1997; Coleman et al., 1996; Donovan et al., 1997; Liang and Stillman, 1997; Nishitani et al., 2000; Tanaka et al., 1997). Work with a mammalian cell-free system demonstrated a similar dependence on Cdc6p for MCM loading in humans (Stoeber et al., 1998).
As the *cdc18* homologues appear to share sequence identity, functional motifs and perform a similar cell cycle role I decided to examine whether the homologues could functionally substitute for *cdc18* in fission yeast. As I was aware that the *cdtl* protein acted cooperatively with *cdc18p* to promote DNA replication in fission yeast (Nishitani et al., 2000), I decided to co-express *cdtl* with the *cdc18* homologues in the assay for complementation to promote the chance of a rescued phenotype.

### 3.2 ScCDC6 and XlCdc6 initially appeared to complement *cdc18-K46*

As I was unaware of the effect the *cdc18* homologues may have when expressed in fission yeast I wanted to be able to control their expression. I therefore used the *nmt* thiamine regulatable promoter (Maundrell, 1990). Mutations in the TATA box of the *nmt* promoter have been made which alter the levels of transcript produced but not the thiamine repressibility. This allows a range of promoter activity with *nmt81* producing expression levels approximately 12 times lower than *nmt41* which in turn expresses at about 6 times less than *nmt1* (Basi et al., 1993). I knew that *nmt81-cdc18* rescued the *cdc18-K46* mutant when expressed from the multicopy plasmid Rep81 and that high levels of expression under the control of the *nmt1* promoter resulted in lethality caused by re-replication of the DNA (Nishitani and Nurse, 1995). I therefore decided to clone the *cdc18* homologues into the vectors containing the two lower strength promoters, Rep81 and Rep41. *Cdt1* was cloned into both Rep42 and Rep82, vectors with the same promoter as Rep41 and Rep81 but having the fission yeast *ura4* gene as a marker rather than the budding yeast *LEU2* gene.

The strategy used to investigate whether the *cdc18* homologues can complement the *cdc18-K46* mutant is outlined in Figure 3.2. The *cdc18* gene and its homologues were transformed into the *cdc18-K46* mutant, either with or without the *cdtl* gene. *Cdt1* was also transformed alone, as were the vectors on their own as a negative control. The *cdc18-K46* strain also contained the *leu1-32* and *ura4-D18* mutations so cells containing specific plasmid combinations were selected by their ability to grow on different media. Cells transformed with both a Rep41/81 and Rep42/82
vector were grown on minimal media containing thiamine, so both plasmids must be present to complement the auxotrophy but gene expression is repressed. Minimal media supplemented with uracil and thiamine selected for a Rep41/81 vector and minimal media supplemented with leucine and thiamine selected for a Rep42/82 vector. Transformants were grown at 25°C, the permissive temperature for \textit{cdc18-K46}. Colonies formed after approximately four days at which stage cells were replica plated to minimal media containing the same supplements as before but either in the absence or presence of thiamine. This allowed the promoter to be expressed or repressed to provide a greater range of expression levels. The \textit{nmt41} promoter is expressed approximately 200 fold less in the presence of thiamine than the absence, thus producing a lower expression level than from \textit{nmt81} in the absence of thiamine (Basi et al., 1993).

The cells were incubated at 25°C for a further 24 hours before they were replica plated again, this time to media containing both leucine and uracil, thiamine if present before and Phloxin B. Cells were incubated at 36°C overnight before the plates were visually screened for colonies that were able to complement the \textit{cdc18-K46} mutant at its restrictive temperature of 36°C. Phloxin B helped to discriminate between dividing cells and those that were dying due to an inability to complement the \textit{cdc18-K46} defect as dying cells stain a darker pink. To make the screening easier the media was fully supplemented so that both the cells that do not complement and cells that have lost one of the plasmids will have a \textit{cdc} phenotype.

The visual screen initially produced several complementing combinations when thiamine was absent from the plates. Both Rep41-cdc18 and Rep81-cdc18 could complement the \textit{cdc18-K46} mutation. They therefore acted as a positive control for the experiment. Rep41-XICDC6 and Rep42-cdt1 together appeared to complement the defect well and Rep81-ScCDC6 alone complemented less well. The latter cells were longer and the population was more heterogeneous but they were still dividing as judged by the presence of septa. Cells from both these transformants were patched from the original transformation plates to allow further analysis.
3.3 Ability of Rep81-ScCDC6 and Rep41-XlCDC6+Rep42-cdt1 to form colonies at 36°C

In order to assess further the ability of the homologues to complement the cdc18-K46 mutant, their ability to allow formation of colonies at 36°C was assayed (Fig. 3.2). Rep81-ScCDC6 cells were grown at 25°C in selective media as were Rep81 cells as a negative control and Rep81-cdc18 cells as a positive control. Rep41-XlCDC6+Rep42-cdt1 cells were also grown in selective media at 25°C alongside Rep41+Rep42, and Rep41-cdc18+Rep42 as controls. Cells were counted using a haemocytometer and 1000 cells were plated from each culture onto two plates. One was placed at 25°C and the other at 36°C. The number of colonies formed at both temperatures was monitored over a period of several days and the number after 7 days is presented in Table 3.1.

Neither the Rep81 or Rep41+Rep42 containing cells were able to promote colony formation at 36°C. Only 2% and 5%, respectively, of the colonies formed at 25°C also formed at 36°C. Expression of both Rep81-cdc18 and Rep41-cdc18+Rep42 permitted colony formation at 36°C, 70% and 77% respectively of the number of colonies growing at 25°C. As these plasmid combinations acted as negative and positive controls it enabled us to determine the significance of the number of colonies formed at 36°C when Rep81-ScCDC6 and Rep41XlCDC6 and Rep42-cdt1 were expressed. Rep81-ScCDC6 and Rep41-XlCDC6+Rep42-cdt1 transformants also formed colonies at 36°C, 54% and 62% respectively of the number of colonies growing at 25°C. This analysis indicated that both Rep81-ScCDC6 and Rep41-XlCDC6 when co-expressed with Rep42-cdt1 were able to substitute for cdc18 function.

The total number of colonies formed when both Rep41 and Rep42, and Rep41-cdc18 and Rep42 were expressed was 5-8 fold lower than expected. However this did not affect the comparison between the number of colonies formed at the permissive and restrictive temperature as the percentage was similar between the two experiments using the negative controls (Rep81 and Rep41+Rep42) and the positive controls (Rep81-cdc18 and Rep41-cdc18+Rep42).
cdcl8-K46 cells transformed with cdcl8 and homologues | Number of colonies formed at specified temperature | % colonies formed 36°C colonies formed 25°C |
--- | --- | --- |
Rep81 | 1028 | 23 | 2 |
Rep81-cdc18 | 992 | 697 | 70 |
Rep81-ScCDC6 | 968 | 523 | 54 |
Rep41 and Rep42 | 218 | 11 | 5 |
Rep41-cdc18 and Rep42 | 123 | 95 | 77 |
Rep41-X1CDC6 and Rep42-cdtl | 1132 | 707 | 62 |

Table 3.1

Rep81-ScCDC6 and Rep41-X1CDC6+Rep42-cdtl appeared to complement cdcl8-K46 when the ability to form colonies was assayed

cdcl8-K46 cells transformed with cdcl8 and homologues that initially appeared to complement at 36°C were assayed by their ability to form colonies at 25°C and 36°C. 1000 cells were plated and the number of colonies formed after 7 days was counted.
In order to assess whether the homologues could complement the *cdc18-K46* defect as well as *cdc18*, cells were picked from the colonies and DAPI staining was performed (Fig. 3.3). Rep81-cdc18 appeared to grow equally well at 25°C and 36°C as judged by cell and nuclear morphology (Fig. 3.3 A, a and c). However, Rep81-ScCDC6 looked less healthy at 36°C (Fig. 3.3 A, d). There were still growing and dividing cells when Rep81-ScCDC6 was expressed but there were also some elongated cells containing an abnormal nucleus. This is consistent with the initial result that Rep81-ScCDC6 complemented less well than Rep81-cdc18. Rep41-XICDC6 and Rep42-cdt1 however, appeared to grow as well at 36°C as Rep41-cdc18 and Rep42 did at both 25°C and 36°C (Fig. 3.3 B), indicating that it complements effectively.

### 3.4 Ability of Rep81-ScCDC6 and Rep41-XICDC6+Rep42-cdt1 to grow in liquid media at 36°C

A more stringent test for complementation was also used. In this, cells were grown to exponential phase in liquid minimal media at 25°C. Cultures were then split. Half continued to grow at 25°C whereas the other half were shifted to 36°C, the restrictive temperature for the *cdc18-K46* mutant (Fig. 3.2). Their ability to complement was assayed by cell number increase over a 10 hour period (Fig. 3.4 A and 3.5 A) and by their cellular morphology (Fig. 3.4 B and 3.5 B). Rep81, Rep81-cdc18 and Rep81-ScCDC6 were all able to grow with similar kinetics at 25°C (Fig. 3.4 A, a). At 36°C Rep81-cdc18 continued to grow exponentially but cell number stopped increasing after 5 hours when both Rep81 and Rep81-ScCDC6 were expressed (Fig. 3.4 A, b). Cells expressing Rep81 and Rep81-ScCDC6 at 36°C have a distinct cellular morphology when stained with DAPI (Fig. 3.4 B, d and f), cells are elongated, often have several septa and have abnormal nuclei. This indicates that Rep81-ScCDC6 is unable to complement the *cdc18-K46* defect when assayed in this way. Rep41-XICDC6 and Rep42-cdt1 behaved similarly to Rep41 and Rep42, and consequently to Rep81-ScCDC6, when the ability to complement in liquid media at 36°C was tested. Cells stopped growing after approximately 5 hours (Fig. 3.5 A b), and DAPI stained cells had the same abnormal morphology as described above (Fig. 3.5 B d and f). I diluted the cultures in pre-warmed media and grew them overnight at the same temperature to determine whether the homologues could
complement if incubated for a longer period of time. There was no further growth when Rep81, Rep41, Rep81-ScCDC6 or Rep41-CdCDC6 and Rep42-cdt1 were expressed. I therefore conclude that Rep81-ScCDC6 and Rep41-XICDC6 expressed with Rep42-cdt1 are unable to complement \( cdc18-K46 \) at the restrictive temperature when the ability to grow in liquid media is assayed.

In order to verify this result I attempted to duplicate it. However, despite repeating the transformation followed by the visual screen at the restrictive temperature several times, I was unable to identify Rep81-ScCDC6 and Rep41-XICDC6+Rep42-cdt1 as complementing the \( cdc18-K46 \) mutant. Only Rep81-cdc18 and Rep41-cdc18+Rep42 were isolated as having the ability to complement. I varied several factors when repeating the experiment in order to duplicate my first result. Several transformation protocols were used, including the lithium acetate procedure, electroporation and protoplasting. I also used different plasmid concentrations as it was possible that the ability to complement was very sensitive to precise protein levels and perhaps the balance between the \( cdc18 \) homologue and \( cdtl \). I therefore kept the amount of \( cdc18 \) or its homologue constant at 1\( \mu \)g and varied the \( cdtl \) level from 0.3\( \mu \)g, to 1\( \mu \)g, to 3\( \mu \)g per transformation. However none of these changes affected the outcome which was that I was unable to repeat the initial results. The possible reasons for this will be discussed later.

3.5 Overexpression of \( ScCDC6 \) induces re-replication to a similar level as \( cdc18 \)

I decided to assess the functional similarity of \( cdc18 \) and its homologues by an additional method; the ability to induce re-replication when overexpressed. \( Cdc18 \) and the three homologues were expressed from the \( nmt41 \) promoter, with either Rep42-cdt1 (Fig. 3.6 A) or Rep82-cdt1 (Fig. 3.6 B) as \( cdtlp \) is known to act cooperatively with \( cdc18p \) in a re-replication assay (Nishitani et al., 2000). Cells were grown to exponential phase in thiamine containing media. The thiamine was then washed out to induce expression from the \( nmt \) promoters. Cells were grown for 20 hours before being fixed and analysed by FACS. I discovered that \( cdtl \) alone did not promote re-replication. The human and \( Xenopus \) homologues were also unable to promote re-replication when co-overexpressed with \( cdtl \). These
constructs were also unable to invoke a checkpoint as the forward scatter demonstrates that cell length did not increase. However, both Rep41-cdc18 and Rep41-ScCDC6 induced re-replication to similar extents when co-overexpressed with cdt1, and could maintain a block over mitosis as judged by the elongated cells in the forward scatter.

There were two possible explanations for ScCdc6p being able to induce re-replication. The first was that ScCdc6p had an intrinsic ability to promote re-replication when co-expressed with cdt1p. The alternate explanation was that overproduction of ScCdc6p allowed interaction with and saturation of the proteolytic system, thus allowing accumulation of the endogenous cdc18p. The re-replication would therefore be induced by the endogenous cdc18p.

3.6 ScCdc6p induced re-replication requires cdc18p function but does not increase its endogenous protein levels

In order to differentiate between these alternatives I expressed Rep41-cdc18 and Rep41-ScCDC6 with either Rep42, Rep42-cdt1 or Rep82-cdt1 in a cdc18-K46 mutant background. Cells were grown at the permissive temperature of 25°C for 18 hours following derepression of the promoter by thiamine wash-out. Cell cultures were split and half was shifted to the restrictive temperature of 36°C. Cells were grown for a further 2.5 generations which amounts to 10 hours at 25°C and 5 hours 50 minutes plus 1 hour for temperature shift recovery at 36°C. Cells were then analysed by FACS (Fig. 3.7 A), DAPI staining (Fig. 3.7 B) and Western blotting (Fig. 3.7 C and D). I showed that Rep41-cdc18 is able to promote re-replication to a similar extent at both 25°C and 36°C. However, although Rep41-ScCDC6 can promote re-replication at 25°C, the re-replication is abolished at 36°C. This suggests that the endogenous cdc18p provides an essential function for this ScCdc6p induced re-replication. However, cdc18p levels do not accumulate when Rep41-ScCDC6 is overexpressed (Fig. 3.7 D upper panel) and in fact the protein level remains similar to wildtype (Fig. 3.7 C lanes 1 and 2). Wildtype cdc18p function is therefore required for the re-replication induced when ScCdc6p is co-overproduced with cdt1p but this re-replication is not caused by an increase in the endogenous protein levels.
Discussion

Proteins that are both structurally and functionally homologous to cdc18 have been identified in several organisms. In experiments to examine the ability of homologues to complement the cdc18-K46 temperature sensitive mutant I identified two situations in which a cdc18 homologue was able to complement the cell cycle defect. The first was when the Saccharomyces cerevisiae homologue, ScCDC6, was expressed at low levels using the nmt81 promoter. The second was when the Xenopus laevis homologue, XICDC6, was co-expressed with cdt1 using the medium strength promoters in the Rep41 and Rep42 vectors respectively (Table 3.1 and Fig. 3.3). However, I have been unable to repeat this experiment which suggests that complementation is very sensitive to specific conditions.

Work from several different labs has given conflicting results about the ability of cdc18 homologues to functionally rescue cdc18 mutants. It has been reported that ScCDC6 is unable to functionally substitute for cdc18 (Dutta and Bell, 1997; Wolf et al., 1999a). However another recent study states that ScCDC6 is able to complement both a cdc18-K46 mutant and a cdc18A strain (Sanchez et al., 1999).

One possibility for the discrepancies could be the protein level of the cdc18 homologues. It is known that overexpression of cdc18 results in re-replication of the DNA and is lethal to the cells. For this reason only the medium and low level promoters were used in our experiments. However, the work by Sanchez et al. states that the nmt1 promoter was used and that high levels of ScCdc6p are required to achieve complementation. The problem with trying to compare these results is that different ScCDC6 cDNAs may have been used which may affect expression level even from the same promoter. An example of how small changes in the cDNA sequence can have dramatic consequences for gene expression is that of the original cdc18 cDNA isolated (Kelly et al., 1993) compared to one isolated later that contained an extra 89 nucleotides upstream from the start site (Nishitani and Nurse, 1995). This additional sequence was sufficient to allow re-replication when expressed using the nmt1 promoter. An alternative explanation for why the full strength nmt1 promoter was required for complementation of cdc18 could be
Sanchez also doesn't look at endogenous cdc18 protein level and cdc15-146 is thought to rescue defect when ole-1 doesn't help rescue it though.
because an integrated copy of \textit{nmt1:ScCDC6} was used, whereas I used a multicopy plasmid. Several lower level expressing plasmids could allow the same level of protein production as can be achieved from one integrated higher expression level plasmid. The only way to come to a firm conclusion about this would be to tag both constructs and directly compare the protein expression levels within a cell population that is either able or unable to complement the \textit{cdc18} gene. The cDNAs could also be exchanged and the experiments repeated to see if the same results are obtained. At present it is impossible to conclude whether the \textit{ScCDC6} and \textit{XICDC6} gene products are able to fully complement the \textit{cdc18-K46} mutant.

Interestingly, the presence of \textit{cdt1p} was of no benefit when looking for complementation with \textit{ScCDC6}, an organism that does not appear to possess a \textit{cdt1} homologue. However, even though \textit{XICDC6} was only found to complement once, it only did so when co-expressed with \textit{cdt1}. As a homologue of the fission yeast \textit{cdt1} gene has recently been identified in \textit{Xenopus laevis} (Maiorano et al., 2000) it would be interesting to see whether co-expression of the \textit{Xenopus laevis cdc18} and \textit{cdt1} homologues would result in complementation of the \textit{cdc18-K46} mutation in a more consistent manner. Furthermore, as the human \textit{cdc18} homologue was also unable to complement, it may be interesting to repeat the experiment in the future if a human \textit{cdt1} homologue is cloned. The sequence of a 5' truncated human cDNA encoding a putative \textit{cdt1} homologue can be found in the databases.

The mechanism by which \textit{ScCdc6p} is able to promote re-replication has also been somewhat controversial (Sanchez et al., 1999; Wolf et al., 1999a). Wolf et al. state that \textit{ScCdc6p} induced re-replication requires only amino acids 2-73. This part of the protein is sufficient to allow an interaction with both \textit{cdc2p} and \textit{pop2p}, thus overexpression results in titration of the proteolytic machinery that degrades \textit{rum1p} and \textit{cdc18p}, allowing both proteins to accumulate. However, the re-replication still occurs in a \textit{rum1A} strain and the \textit{cdc18p} levels appear to increase only by about 2 fold when the N-terminus of \textit{ScCDC6} is overexpressed. This level of increase would not be inconsistent with our results (Fig. 3.7 C lane 1 and D lane 1) when \textit{ScCDC6} is overexpressed. It seems unlikely that a doubling of \textit{cdc18p} caused by \textit{ScCDC6} overexpression could produce the same level of re-replication as that found when \textit{cdc18} is overexpressed (Fig. 3.7 C lane 3). Wolf et al. discuss
this point, stating that both pop1 rum1 and pop2 rum1 double mutants are able to maintain a normal genome ploidy despite levels of cdc18p as high as those in cells overexpressing ScCdc6p. They therefore conclude that accumulation of rum1p appears to be the key event to permit re-replication. As re-replication can still occur in a rum1Δ strain, they suggest that in this case, re-replication could be due to sequestration of cdc2p by cdc18p or ScCdc6p, preventing the kinase from phosphorylating substrates required for inhibiting re-replication. Although I can not rule out this possibility, I would propose a more direct role for ScCdc6p in promoting re-replication as I have shown in my work that overexpressing the N-terminus of cdc18p does not result in re-replication, despite being able to inhibit the cdc2p/cdc13p kinase (Fig. 2.3), bind to cdc2p (Fig. 2.4) and allow accumulation of rum1p (Fig. 2.5).

In comparing the work of Sanchez et al. with that of my own the question of expression levels is, once again, paramount. In order to achieve significant re-replication, an integrated nmt1:5cC0Cb strain had to also possess a multicopy vector carrying the transcription factor ntf1, known to regulate expression from the nmt1 promoter (Tang et al., 1994). This increased ScCdc6p levels sufficiently to allow re-replication. The fact that the nmt1ScCOCb strain is unable to re-replicate alone is presumably related to the fact that ScCOib is integrated and also perhaps that they could be using a different cDNA, as discussed previously.

The main difference between my results and those of Sanchez et al. arises when the ability of ScCdc6p to induce re-replication when the endogenous cdc18 function is compromised is examined. Sanchez et al. examined this in both a cdc18-K46 and cdc18Δ background and found in both cases that re-replication occurred to the same extent as when cdc18p was present. I found that re-replication induced by overexpression of ScCDC6 was compromised in a cdc18-K46 mutant at 36°C (Fig. 3.7). However, the experimental systems used were different. I used lower expression levels of ScCDC6 but maximised the ability to induce re-replication by co-expressing cd18. It is possible that ScCdc6p is less effective at interacting with a fission yeast protein or performing an essential function and therefore when it is expressed at relatively low levels, as in my experiments, cdc18p is required to carry out that function. However, increased expression of ScCdc6p could
compensate for the fact that it functioned less effectively and cdc18p would not be required. A possible explanation for this could be related to the fact that cdc18p interacts with cdt1p (Nishitani et al., 2000). There is no cdt1 gene in budding yeast so ScCdc6p may interact more weakly with cdt1p. Cdc18p could therefore promote the interaction to allow re-replication when ScCdc6p is expressed at a lower level. As with the ability to complement, it may be interesting to overexpress the cdc18 homologue with the cdt1 homologue from a particular organism and see whether the proteins are then able to promote re-replication.

It would appear that none of the cdc18 homologues examined here can functionally substitute for cdc18. Despite the fact that the cdc18 homologues behave similarly in different organisms, there could be changes in both function and regulation. Some of these differences will be discussed later (Chapter 5).

One of the major differences between the organisms which could be directly relevant to the function of cdc18p is origin specification. It is known that the definition of an origin varies greatly between different organisms. This suggests that cdc18 homologues could be unable to recognise Schizosaccharomyces pombe origins. Alternatively, higher eukaryotes may have a more complex pre-replicative complex, possibly involving more proteins. These could be required for the cdc18 homologue to interact at origins. When these are absent, as in fission yeast, the homologue may be unable to bind. As progression is made in this direction, we may be able to discover if either of these hypotheses are correct.
Figure 3.1

Sequence alignment of cdc18p and homologues

The cdc18p sequence is aligned with HsCdc6, XICdc6 and ScCdc6. $\square$ denotes identity between all four sequences, $\blacksquare$ denotes identity between three sequences and $\blacklozenge$ denotes similarity between at least three sequences.
Transform *cdc18-K46* cells with *cdc18* and homologues

Plate on min+T (leu/ura) grow at 25°C for ~ 4 days (until colonies formed)

Replica plate to min (leu/ura) to induce promoter and min+T (leu/ura) grow at 25°C overnight

Replica plate from min to min+leu+ura+PB and from min+T to min+leu+ura+T+PB grow at 36°C overnight

Screen visually for colonies that complement *cdc18-K46* at 36°C, patch colonies to min+T

Test ability to complement further by:

- grow cells in min at 25°C
- grow cells in min at 25°C

Plate 1000 cells on min at 25°C Plate 1000 cells on min at 36°C

Split culture and grow half at 25°C and half at 36°C

Count number of colonies formed and look at cells with DAPI staining

(Table 3.1 and Figure 3.3)

Follow cultures by cell number and DAPI staining for 10 hrs

(Figures 3.4 and 3.5)

*Figure 3.2*

*Strategy used to investigate whether the cdc18 homologues complemented the cdc18-K46 temperature sensitive mutant*
Figure 3.3

Rep81-ScCDC6 and Rep41-XICDC6+Rep42-cdt1 appeared to complement cdc18-K46 when the ability to form colonies at 36°C was assayed

cdc18-K46 cells transformed with cdc18 and homologues that initially appeared to complement at 36°C were further assayed by their ability to form colonies at 25°C and 36°C. Colonies were picked and DAPI staining was performed. (A) Rep81-cdc18 at 25°C (a) and 36°C (c), Rep81 (b) and Rep81-ScCDC6 (d) at 36°C. (B) Rep41-cdc18+Rep42 at 25°C (a) and 36°C (c), Rep41+Rep42 (b) and Rep41-XICDC6+Rep42-cdt1 (d) at 36°C. Bar 15μm.
Figure 3.4

Rep81-ScCDC6 did not appear to complement cdc18-K46 when the ability to grow in liquid media at 36°C was assayed.

cdc18-K46 cells transformed with cdc18 and homologues that initially appeared to complement at 36°C were grown in liquid media at 25°C. The cultures were then split and half were shifted to 36°C. (A) Cell number was followed for 10 hours after the cultures were split at 25°C (a) and 36°C (b). (B) Cells were fixed and stained with DAPI 10 hours after the cultures were split. Bar 15µm.
Figure 3.5

Rep41-XICDC6 and Rep42-cdt1 did not appear to complement cdc18-K46 when the ability to grow in liquid media at 36°C was assayed

cdc18-K46 cells transformed with cdc18 and homologues that initially appeared to complement at 36°C were grown in liquid media at 25°C. The cultures were then split and half were shifted to 36°C. (A) Cell number was followed for 10 hours after the cultures were split at 25°C (a) and 36°C (b). (B) Cells were fixed and stained with DAPI 10 hours after the cultures were split. Bar 15μm.
**Figure 3.6**

ScCdc6p can induce re-replication to similar levels as cdc18p when co-overproduced with cdt1p

Wildtype cells were transformed with *cdc18* or homologues behind the *nmt4I* promoter (Rep41) and cdt1 behind either *nmt4I* (Rep42) (A) or *nrntS*! (Rep82) (B). Cells were grown for 20 hours after induction of the promoter. DNA content and cell number was analysed by FACS.
**Figure 3.7**

ScCdc6p can promote re-replication without the increase of endogenous cdc18p levels

cdc18-K46 cells were transformed with cdc18 or ScCDC6 and cdt1 and were grown for 18 hours at 25°C before being split and half shifted to 36°C. Cells were grown for a further 2.5 generations (10 hours at 25°C and 5 hours 50 minutes plus 1 hour for temperature shift recovery at 36°C). (A) DNA content and cell length were analysed by FACS.
Figure 3.7 B

(B) Cells were fixed and stained with DAPI; Rep41 and Rep42 at 25°C (a) and 36°C (b), Rep41-cdc18 and Rep42 at 25°C (c) and 36°C (d), Rep41-cdc18 and Rep42-cdt1 at 25°C (e) and 36°C (f), Rep41-cdc18 and Rep82-cdt1 at 25°C (g) and 36°C (h), Rep41-ScCDC6 and Rep42 at 25°C (i) and 36°C (j), Rep2-ScCDC6 and Rep42-cdt1 at 25°C (k) and 36°C (l), Rep41-ScCDC6 and Rep82-cdt1 at 25°C (m) and 36°C (n). Bar 15 μm.
Figure 3.7 C and D

Protein levels were determined by Western blotting. 50μg of protein was loaded per lane. Blots were probed with anti-cdc18p antibody (C and D top panel) and anti-α-tubulin antibody as a loading control (C and D bottom panel).
Chapter 4

A mutational analysis of \textit{cdc18}

\textbf{Introduction}

Cdc18p has a number of structural motifs potentially important for its function and regulation. In order to investigate the role of these motifs I constructed a strain in which \textit{cdc18} is under the control of a repressible promoter and made site specific mutants of \textit{cdc18} integrated at a different locus where they were expressed from the endogenous \textit{cdc18} promoter. I examined whether the mutants were viable when cells were depleted for wildtype \textit{cdc18}. Mutants that were unable to grow in the absence of cdc18p were physiologically and biochemically characterized to describe their defects.
Results

4.1 Characterization of the 81-cdc18 S/O strain

Cdc18p contains several structural motifs that are conserved between cdc18 homologues in different organisms, indicating that they could be important for cdc18p function. Mutagenesis of these motifs should illuminate both how cdc18p is regulated and what its precise role in the cell cycle could be.

In order to carry out a physiological analysis it was important that:
1) wildtype cdc18p could be repressed in order to investigate any potential defects of the mutants
2) mutants were expressed at wildtype levels
3) wildtype and mutant copies of cdc18p could be distinguished.

To create a strain in which the wildtype copy of cdc18p could be repressed I first chose which promoter would be suitable. The nmt promoters are repressed in the presence of thiamine and have been mutated to express at three levels (Basi et al., 1993; Maundrell, 1990). The strongest promoter nmt1, causes re-replication in fission yeast when used to overexpress cdc18 (Nishitani and Nurse, 1995, Chapter 2). Both nmt41-cdc18 and nmt81-cdc18 are capable of complementing the cdc18-K46 temperature sensitive mutation when expressed from a multicopy plasmid but nmt41-cdc18 also results in a small amount of re-replication (Chapter 3). I therefore used the nmt81 promoter. The strain was made using a homologous integration technique (Bahler et al., 1998) to replace the endogenous promoter with that of nmt81. The strain is designated 81-cdc18 S/O as the nmt81-cdc18 gene can be Switched Off on the addition of thiamine.

To characterize the strain, 81-cdc18 S/O cells were grown to exponential phase at 32°C in the absence of thiamine. Thiamine was then added to the culture and the phenotype was analysed by FACS, DAPI staining and Western blotting (Fig. 4.1). Cdc18p levels were undetectable within an hour of thiamine addition (Fig. 4.1 C lanes 2 and 3). This corresponded with an accumulation of cells with a 1C DNA
Chapter 4 Mutational analysis of cdc18

content (Fig. 4.1 A b). By 3 hours almost all of the cells had a 1C DNA content and by 4 hours a proportion of cells had a DNA content of less than 1C (Fig. 4.1 A, d and e). Cells initially accumulate in G1 as they are unable to initiate DNA replication in the absence of cdc18p. However, these cells are also unable to send the checkpoint signal to inhibit mitosis so they undergo a nuclear division followed by septation in the absence of DNA replication giving rise to some cells with a DNA content of <1C (Kelly et al., 1993). This can be visualised by DAPI staining cells from the 3 and 4 hour timepoints when cells possessing a cut phenotype are first seen (Fig. 4.1 B, d and e). The protein disappears rapidly due to its short half life (Muzi-Falconi et al., 1996) and the cell phenotype after addition of thiamine is equivalent to a cdc18Δ strain (Kelly et al., 1993). One thing to note is that the expression level produced from the nmt81 promoter is several fold lower than the wildtype protein level. An 81-cdc18 S/O cell population is more heterogeneous in cell length as the cells are slightly sick. However, cells are viable and the lower expression level aids a complete depletion of the protein when the promoter is switched off.

4.2 A 5.2kb genomic fragment of cdc18 complements the 81-cdc18 S/O strain

In order to tackle the problem of expressing the mutants at wildtype levels, I used a 5.2kb genomic fragment of cdc18 (a gift from José Ayté) cloned from the c14C8 cosmid. A Sac1/ApaL1 digest was performed on the cosmid and the fragment was cloned into the multicopy plasmid pIRT2 on a Sac1/Sma1 digest. This fragment contained 2.5 kb of upstream promoter sequence, the 1.8kb Open Reading Frame and approximately 0.9kb of downstream sequence. It was possible that this genomic DNA fragment (gcdc18) would be able to rescue the 81-cdc18 S/O phenotype when thiamine was present as wildtype expression of the gene could be produced from the endogenous promoter. I also cloned the genomic fragment of cdc18 into the pJK148 vector which does not carry an ARS and so can not be maintained in a cell population unless integrated. pJK148 also contains the leu1 marker and integrates preferentially at the leu1 locus on transformation. I transformed both pIRT2-gcdc18 and pJK148-gcdc18 into 81-cdc18 S/O cells alongside pJK148 as a negative control. Presence of the plasmids was selected by
the ability to grow on media lacking leucine as the strain is auxotrophic for leucine. Transformants were grown to exponential phase at 32°C in media lacking thiamine. Thiamine was then added and cells were followed for 10 hours by FACS, cell number and DAPI staining (Fig. 4.2). Cells transformed with pJK148 are unable to complement the 81-cdc18 S/O strain. The FACS profile demonstrates that cells accumulate with a 1C DNA content with the same kinetics as the 81-cdc18 S/O strain (Fig. 4.2 A, a to f and Fig. 4.1 A). Cell number data suggests that cells stop dividing 5 hours after thiamine addition and this timing corresponds to an accumulation of cells with a cut phenotype (Fig. 4.2 B and C, a to c). Both pIRT2-gcdc18 and pJK148-gcdc18 are able to complement the 81-cdc18 S/O strain in the presence of thiamine. The FACS data shows that when either pJK148-gcdc18 (Fig. 4.2 A, g to i) or pIRT2-gcdc18 (Fig. 4.2 A, j to l) are expressed the cells can maintain a 2C DNA content. The cell number data demonstrates that cells continue to divide (Fig. 4.2 B) and the DAPI staining that they are of a wildtype size and morphology (Fig. 4.2 C, d to i). pIRT2 is a multicopy plasmid so cells would contain a variable number of plasmids and some may have lost the plasmid. Plasmid loss results in small starved cells, which can be seen on the FACS profile as a 1C peak (Fig. 4.2 A, k and l). This could also explain the slower growth rate of pIRT2-gcdc18 compared to pJK148-gcdc18 as pJK148-gcdc18 is probably integrated so would have a constant copy number.

Having confirmed that a genomic fragment of cdc18, one expressed under its own promoter, could complement the 81-cdc18 S/O strain, the next problem of distinguishing between the wildtype and mutant copies of cdc18p could be addressed. The genomic cdc18 fragment was tagged at the C-terminus so expression from the endogenous promoter would not be affected. A triple HA tag was used as it was known that introducing this 3' to the cdc18 gene using the homologous recombination method (Bahler et al., 1998) resulted in a fully functional gene product (B. Baum, unpublished results). The cdc18 stop codon was replaced by a NotI restriction site using site-directed mutagenesis. The triple HA tag was then cloned with a NotI digest and orientation was confirmed by sequencing. The mutants were constructed by site-directed mutagenesis of pJK148-gcdc18-3HA.
4.3 Mutant construction

Cdc18p possesses a number of structural motifs (Fig. 4.3). These include the highly conserved Walker A and Walker B box motifs which are essential for stable binding and hydrolysis of nucleotides in a number of proteins (Walker et al., 1982). The Walker A motif contains the consensus sequence GxxGxGKT (as described in Chapter 2). Structural analysis has shown that this motif forms a loop that binds nucleotides. The Walker B motif contains the consensus sequence DExD. The conserved residues play a role in coordinating the magnesium ion (Guenther et al., 1997; Story and Steitz, 1992). Many of the proteins that contain the Walker A motif are proteins in which hydrolysis is coupled to the binding of another molecule, (DNA, RNA, protein). In the RecA protein, ATP and ADP have been found to stabilize different conformations (Fujita et al., 1997; Story and Steitz, 1992). The conformation stabilized by ATP has a much higher affinity for DNA. This provides an indication as to how NTP binding and hydrolysis could relate to cdc18p function. ATP binding could be required for cdc18p binding to DNA and hydrolysis could then result in the release of cdc18p from chromatin, although whether this occurs before initiation of replication, acts as the trigger for initiation or occurs later is currently unknown.

The Walker A motif was mutated so the conserved lysine residue was changed to the negatively charged glutamate residue. This amino acid substitution had previously been shown to have the most dramatic phenotype when made in the *Saccharomyces cerevisiae* homologue Cdc6p (Perkins and Diffley, 1998; Wang et al., 1999; Weinreich et al., 1999). The conserved glutamate of the Walker B motif was mutated to a glycine. This mutant had originally been identified as a dominant negative when overexpressed in budding yeast (Perkins and Diffley, 1998).

The major role of cdc18p is thought to be to load the MCM family of proteins onto chromatin (Nishitani et al., 2000). For this to occur cdc18p must be localized in the nucleus. Immunofluorescence data shows that the overexpressed cdc18 protein localizes to the nucleus (Nishitani and Nurse, 1995) and a more recent study reports that a myc-tagged cdc18p expressed at wildtype levels is located in the nucleus (Nishitani et al., 2000). Cdc18p contains two putative bipartite nuclear
localization signals (Fig. 4.3) which could be responsible for the correct localization of the protein and hence have a direct effect on the ability of cdc18p to perform its designated function. Bipartite nuclear localization signals (NLS) contain two basic residues (lysine or arginine) followed by a spacer of 10 or 11 amino acids followed by at least 3 basic residues out of the next 5 (Robbins et al., 1991). Cdc2p consensus phosphorylation sites are often found close to bipartite nuclear localization sequences, indicating that localization may be influenced by cdc2p phosphorylation (Robbins et al., 1991; Takei et al., 1999). Mutating the basic residues of the proximal arm of the bipartite NLS to neutral residues has been found to exclude the cytokine interleukin-5 from the nucleus (Jans et al., 1997). I therefore mutated the two putative nuclear localization signals as indicated:

NLS1 \[ \text{RKRxxxxxxxxxxxKRxK} \rightarrow \text{AAAxxxxxxxxxxxKRxK} \]

NLS2 \[ \text{KKxxxxxxxxxKxxKR} \rightarrow \text{AAxxxxxxxxxxKxxKR} \]

I also combined the two mutants in case the sequences acted redundantly.

Finally a mutant was constructed in which all 6 of the cdc2p consensus phosphorylation sites, S/TPxK/R, were mutated so the S/T residue was changed to alanine. Stepwise mutagenesis was performed to construct both the NLS1+NLS2 and P1-6 mutants. The plasmids were sequenced after each round of mutagenesis to confirm that the expected mutation had been successfully introduced without the acquisition of new mutations. Phosphorylation is known to be required to target cdc18p for degradation via the SCF pathway (Baum et al., 1998; Jallepalli et al., 1997; Kominami and Toda, 1997). I was therefore interested to see whether a stable cdc18p would interfere with the normal alternation of S and M phase.
Table 4.1

Construction of mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Motif</th>
<th>Amino acid substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>WA</td>
<td>GxxGxGKT</td>
<td>K→E</td>
</tr>
<tr>
<td>WB</td>
<td>DExD</td>
<td>E→G</td>
</tr>
<tr>
<td>NLS1</td>
<td>RKRxxxxxxxxxxKRxK</td>
<td>RKR→AAA</td>
</tr>
<tr>
<td>NLS2</td>
<td>KKxxxxxxxxxxKxxKR</td>
<td>KK→AA</td>
</tr>
<tr>
<td>P1-6</td>
<td>S/TPxK/R</td>
<td>S/T→A</td>
</tr>
</tbody>
</table>

The mutants were constructed by site-directed mutagenesis of the pJK148-gcdc18-3HA plasmid as previously mentioned, and were then transformed into the 81-cdc18 S/O strain. Transformants were selected by conversion of the 81-cdc18 S/O strain to leucine prototrophy. To increase the probability of integration at the leu1 locus, the plasmid can be linearised by digesting with a restriction enzyme that cuts within the leu1 gene. However, due to the presence of the genomic cdc18 fragment in the plasmid there was no restriction site available that was present only once in the leu1 gene and not elsewhere in the plasmid. I was therefore unable to improve the chance of integration at the leu1 locus in this way. The presence of the 5.2kb of genomic DNA in the plasmid also provides a large region of homology with the cells genomic DNA and produces a second site where homologous recombination would result in integration of the plasmid. These factors may have been responsible for the low frequency of integration at the leu1 locus (approximately 1:50 integrants were integrated at the leu1 locus). Many transformants were screened by Southern blotting to ensure that the plasmids were integrated at the leu1 locus.

DNA was prepared from transformants and a BamH1 digest was performed. This was followed by Southern blotting. Blots were probed with cdc18 and leu1 (Fig. 4.4). A BamH1 digest produces a band of 9.1kb at the cdc18 locus which should remain unchanged. The leu1 locus corresponds to a 14.8 kb band. On integration of the pJK148-gcdc18-3HA plasmid at the leu1 locus, this 14.8kb band is converted to four bands following BamH1 digestion (Fig. 4.4 A). A leu1 probe made with only
the open reading frame recognises a 4.2kb and 17.7kb band (Fig. 4.4 B top panel). When the vector pJK148 is integrated as a control, the *leu1* probe still recognises bands of the same size. This is because the cloning of gcdc18 into pJK148 deleted a BamH1 site 1.1kb downstream of the *leu1* gene. Without the presence of the gcdc18 fragment, this BamH1 site still exists, thus bands of 4.2kb and 17.7kb are recognised, as seen on the *leu1* Southern (Fig. 4.4 B, top panel, lane 2) but the 1.4kb and 2.9 kb bands are not produced as they are specific to the genomic *cdc18* fragment. A *cdc18* probe therefore recognises the 9.1kb band at the endogenous locus and the 2.9kb band when the mutant is integrated at *leu1* (Fig. 4.4 B, bottom panel, lanes 3, 4 and 6 to 9).

The exception to this band pattern occurred when the Walker B (WB) mutant was integrated (Fig. 4.4 B, lane 5). The *leu1* probe recognised bands of the correct size, as did the *cdc18* probe for the 2.9kb band. This indicated that the pJK148-gcdc18-3HA-WB plasmid was integrated correctly at the *leu1* locus. However, there was a change in band size at the endogenous *cdc18* locus, from 9.1kb to approximately 7kb. This could mean one of two things, either a new BamH1 site had been introduced or 2kb of DNA had been deleted within the original BamH1 fragment. A 2kb deletion could affect whether the wildtype gene was present and whether the promoter was present that allows the gene to be switched off by thiamine. I have therefore tried to remake the strain although so far to no avail. This has been attributed to the low frequency of integration at the *leu1* locus as previously described. In the meantime I have performed a characterization of the *81-cdc18 S/O-WB* strain and shown that the full length, wildtype cdc18 protein is both produced and switches off in response to thiamine, as expected.

4.4 Complementation of mutants

In order to characterize the phenotype of the *cdc18* mutants the first thing to establish was whether they were able to rescue the lack of wildtype cdc18 protein when it was switched off. The *81-cdc18 S/O* strains containing the vector control, the wildtype cdc18p or one of the 7 mutants were grown to exponential phase at 32°C in media lacking thiamine. The cell cultures were then split and thiamine was added to half. This switches off the endogenous *cdc18* gene resulting in the rapid
depletion of wildtype cdc18 protein. Samples were taken every hour for 10 hours for cell number, FACS analysis, DAPI staining and to make protein extracts. The phenotype of each strain will be described in turn.

81-cdc18 S/O-JK cells contain the vector pJK148. This strain differs from the strain described previously (Fig. 4.2) and we have confirmed integration at the leu1 locus in this case. The phenotype is, however, equivalent. The 81-cdc18 S/O-JK cells are unable to continue growing in the presence of thiamine as the only copy of cdc18 in the cell has been switched off. Cell number stops increasing exponentially between 4 and 5 hours after the addition of thiamine (Fig. 4.5 A). The FACS profile shows that a 1C peak begins to accumulate by 1 hour after the addition of thiamine and by 5 hours all of the cells have a DNA content of 1C or less than 1C (Fig. 4.5 B). Likewise the DAPI staining demonstrates that many cells with a cut phenotype are present 5 hours post thiamine addition (Fig. 4.5 C). This data demonstrates that the 81-cdc18 S/O-JK strain acts as both the 81-cdc18 S/O strain and the 81-cdc18 S/O- pJK148 strains (Fig. 4.1 and Fig. 4.2).

Western blots were performed to look for depletion of the wildtype protein after the addition of thiamine. As cdc18p is an unstable protein, wildtype expression levels can be difficult to detect. I discovered that detection was improved by using protein extracts made from fresh cells rather than frozen pellets. However, a cross-reacting band of almost exactly the same size as cdc18p is also present. As I was looking for the loss of wildtype protein after thiamine addition it was extremely important to separate these two bands. A longer gel system was used to allow greater separation of cdc18p and the cross-reacting band and control extracts of cdc18-HA cells and wildtype cells were also run to demonstrate that the lower band was cross-reacting and not a different cdc18p isoform. The cdc18-HA strain possesses one copy of cdc18 tagged at the C-terminus with 3HA at the endogenous locus. The anti-cdc18p antibody recognises two bands with both the cdc18-HA and wildtype strains (Fig. 4.5 D). Both possess a lower molecular weight band which I believe is a protein that cross-reacts with the cdc18p antibody. Cdc18-HA cells possess a band of a higher molecular weight than the wildtype protein as the tag increases the molecular weight of the protein by approximately 4.5kDa (Fig. 4.5 D, lanes 1 and 2, upper panel). The cdc18-HA band is also recognised by an anti-HA
antibody (Fig. 4.5 D, lane 1, middle panel). The tubulin Western confirms that the proteins were equally loaded (Fig. 4.5 D, lanes 1 and 2, bottom panel). Having confirmed that the lower band is a cross-reacting protein I examined the 81-cdc18 S/O-JK extracts made either in the absence of thiamine or 1 hour after thiamine addition (Fig. 4.5 D, lanes 3 and 4). The cross-reacting band recognised by the cdc18p antibody is present in both lanes (upper panel) but a slightly higher molecular weight band of the same size as the wildtype protein (lane 2) is present in the absence of thiamine (lane 3) and disappears when thiamine is added (lane 4). No protein is seen using the anti-HA antibody as there is no tagged protein present in the cells (middle panel). The anti-tubulin control confirms equal protein loading (Fig. 4.5 D bottom panel).

The 81-cdc18 S/O-wt strain continues to grow in the presence of thiamine as the cdc18-3HA expressed from the endogenous promoter at the leu1 locus is able to rescue the depletion of cdc18p. Cell number increase is the same in both the presence and absence of thiamine (Fig. 4.6 A). FACS analysis shows that cells maintain a 2C DNA content indicating no G1 arrest (Fig. 4.6 B) and DAPI staining shows that the cells have normal nuclear morphology 10 hours after the addition of thiamine (Fig. 4.6 C). Western blots demonstrate that the cdc18-3HA protein is produced as there is a protein of a higher molecular weight recognised by both the cdc18p and the anti-HA antibodies (Fig. 4.6 D). The wildtype protein also disappears on the addition of thiamine but the cross-reacting band remains (Fig. 4.6 D lanes 1 and 2). The anti-tubulin Western demonstrates that equal amounts of protein were loaded (Fig. 4.6 D bottom panel).

The 81-cdc18 S/O-WA strain appears to act identically to the 81-cdc18 S/O-JK strain (Fig. 4.7). Cells arrest with exactly the same kinetics after thiamine addition as can be seen from the cell number graph (Fig. 4.7 A). The FACS profiles and DAPI staining confirm that the two strains act similarly as cells accumulate initially in G1 before undergoing another division in the absence of DNA replication, thus producing cells with a cut phenotype (Fig. 4.7 B and C). Western blotting shows that the cdc18-WA-3HA protein is produced (Fig. 4.7 D, upper and middle panels). The wildtype protein is also depleted 1 hour after the addition of thiamine (Fig. 4.7 D, upper panel). An anti-tubulin Western demonstrates that the proteins are
equally loaded (Fig. 4.7 D, bottom panel). It therefore appears that the cdc18-WA-3HAp is produced but is non-functional, unable to either initiate DNA replication or the checkpoint that inhibits mitosis until DNA replication is completed. The cells act identically to the 81-cdc18 S/O-JK strain after thiamine addition.

81-cdc18 S/O-WB is also unable to rescue the depletion of wildtype cdc18p when thiamine is added to an exponentially growing population of cells. However, the terminal phenotype is different to the Walker A mutant. 81-cdc18 S/O-WB cells arrest earlier than 81-cdc18 S/O-JK and 81-cdc18 S/O-WA cells. Cell division slows by 3-4 hours rather than 4-5 hours (Fig. 4.8 A). The reason that the 81-cdc18 S/O-WB cells stop dividing earlier is due to their cell cycle arrest point. The 81-cdc18 S/O-WB cells arrest within S phase, unlike both 81-cdc18 S/O-JK and 81-cdc18 S/O-WA cells which are unable to initiate either DNA replication or the checkpoint so continue into the next mitosis and arrest after that. The cdc18-WB-3HA protein appears to be able to perform bulk DNA synthesis as the FACS profile demonstrates that cells arrest with a 2C DNA content (Fig. 4.8 B). The fact that cells arrest indicates that S phase is not completed and a checkpoint may have been initiated. The DAPI staining strengthens this idea as cells are elongated (Fig. 4.8 C). However by later timepoints cells appear to have entered mitosis as septa are present and the nuclei look fragmented and abnormal (Fig. 4.8 C, d). The FACS data also indicates this as the 10 hour timepoint (Fig. 4.8 B, d) has a very broad peak indicating that the cells possess a variable DNA content. This could be because the Walker B mutant has a partially deficient checkpoint and the cells eventually leak through and enter mitosis.

Western blot analysis demonstrates that the full length wildtype protein is produced and that it is depleted within 1 hour of the promoter being switched off (Fig. 4.8 D, upper panel). The Western data also demonstrates that the cdc18-WB-3HA protein is produced (Fig. 4.8 D, upper and middle panel) and the tubulin Western shows that the equal amounts of protein were loaded (Fig. 4.8 D bottom panel).

The 81-cdc18 S/O-N1 strain is able to continue growing after the wildtype protein is depleted, indicating that this putative nuclear localization signal is not required for
cdc18p function. Cell number continues to increase with the same kinetics after thiamine addition (Fig. 4.9 A). FACS analysis shows that the cells maintain a 2C DNA content and the forward scatter indicates that there is no change in cell length (Fig. 4.9 B). This was confirmed by DAPI staining as cells have normal nuclear morphology 10 hours after thiamine addition (Fig. 4.9 C). The Western blot analysis shows that the cdcl8 wildtype protein is absent 1 hour after addition of thiamine and that the cdcl8-N1-3HA protein is produced (Fig. 4.9 D, upper and middle panels), thus the ability to grow in the presence of thiamine is not due to the persistence of wildtype cdcl8 protein but due to the presence of cdcl8-N1-3HAp. These results suggest that NLS1 is not essential and that NLS2 may be the functional nuclear localization signal.

The 81-cdcl8 S/O-N2 strain was examined and cells were shown to grow equally well in the presence and absence of thiamine (Fig. 4.10). Cell number increases at the same rate (Fig. 4.10 A), cells have a 2C DNA content (Fig. 4.10 B), DAPI staining demonstrates that nuclear morphology does not alter after the addition of thiamine (Fig. 4.10 C) and Western blot analysis shows that the wildtype cdcl8 protein is depleted, the cdcl8-N2-3HA protein is produced and equal amounts of protein are loaded (Fig. 4.10 D). As NLS2 was also not essential another possibility was that the two putative nuclear localization signals acted redundantly and both had to be mutated to inhibit cdcl8p nuclear localization and hence function.

The 81-cdcl8 S/O-N1+N2 strain combined both of the mutated putative nuclear localization signals but the protein was still fully functional as judged by the ability to maintain growth after thiamine addition. Cell number increased with the same kinetics in the presence and absence of thiamine (Fig. 4.11 A), cells maintain a 2C DNA content (Fig. 4.11 B) and DAPI staining shows that cells had normal nuclear morphology (Fig. 4.11 C). The wildtype protein is switched off in response to thiamine and the triple HA tagged mutant protein is produced (Fig. 4.11 D, upper and middle panels). These data indicate that neither of the putative bipartite nuclear localization signals are required for cdcl8p function.

The final mutant strain examined was 81-cdcl8 S/O-P1-6, in which all 6 of the putative cdcl8p consensus sites were mutated. As phosphorylation of cdcl8p by
cdc2p is known to target the protein for degradation by the SCF pathway (Baum et al., 1998; Jallepalli et al., 1997; Kominami and Toda, 1997), it was possible that the cdc18-P1-6-3HA protein would be more stable and the prolonged presence of the protein could disturb normal cell cycle progress from S phase to G2 to mitosis. However, this was not the case. Cell number increased with normal kinetics in the presence or absence of thiamine (Fig. 4.12 A). Cells maintained a 2C DNA content (Fig. 4.12 B) and DAPI staining demonstrated that the nuclei looked wildtype (Fig. 4.12 C). The wildtype protein is depleted shortly after thiamine addition (Fig. 4.12 D, top panel). The cdc18-P1-6-3HA protein does not appear to be noticeably more abundant than the wildtype cdc18 protein or other mutants expressed from the same promoter, when a comparison of relative abundance is made between the mutant protein, and the cross-reacting band and the wildtype protein (for example Fig. 4.9 D top panel, compared with Fig. 4.12 D top panel; all samples were run on the same gel). It would therefore appear that the degradation of cdc18p at the end of S phase is one control that acts redundantly with others to prevent re-initiation of DNA replication.

As most of the mutants constructed were able to sustain normal growth when the wildtype cdc18 was switched off, I decided to focus my investigation on the 81-cdc18 S/O-WA and 81-cdc18 S/O-WB strains which were unable to rescue the 81-cdc18 S/O strain in the presence of thiamine.

4.5 Quantitative analysis of the NTP binding (WA) and hydrolysis (WB) mutants

As previously described the phenotype of the 81-cdc18 S/O-WA strain is indistinguishable from that of the 81-cdc18 S/O-JK strain after thiamine addition suggesting that the cdc18-WA-3HA protein is non-functional. The 81-cdc18 S/O-WB strain is capable of initiating DNA replication when the wildtype protein is depleted by thiamine addition as the cells arrest with a 2C DNA content and become elongated indicative of cell cycle arrest. However, by 10 hours after thiamine addition cells contain septa and fragmented nuclei indicating that the cell cycle arrest has not been maintained and cells have attempted mitosis. I quantified these observations in order to better understand the nature of the mutant defect.
Chapter 4 Mutational analysis of cdc18

Cells fixed in 70% ethanol during the complementation timecourse described previously were rehydrated in water. They were then stained with either calcofluor to visualise septa, or DAPI to stain the DNA. The septation index and percentage of abnormal nuclei were then determined at each timepoint (Fig. 4.13). Septation index remained relatively constant after addition of thiamine in the 81-cdc18 S/O-JK and 81-cdc18 S/O-WA strains. This can be explained as both strains are unable to initiate either DNA replication or the checkpoint to inhibit mitosis, so cells continue into mitosis and septation. Cytokinesis will proceed at a slow rate so cell number increases slightly and a small proportion of the cell population contain a septum. The 81-cdc18 S/O-WB strain undergoes a transient decrease in the percent of septated cells corresponding to the early arrest. This was confirmed by the cell number data which showed a delay in cell number increase (Fig. 4.8 A). By 5 hours after the addition of thiamine, the septation index began to increase so that by 10 hours it was at a similar level to that found in the other strains (Fig. 4.13 A).

The criteria used to determine nuclear abnormality was:
1) cells with a cut phenotype where the septum bisects a nucleus that has not been segregated to the two cell poles
2) cells where one daughter has not received any genetic material and therefore does not contain a nucleus
3) fragmented nuclei with several DAPI stained areas, some of which have a normal nuclear morphology but are extremely small, other areas are diffuse, spreading through the cell. Background staining always appears much higher in these cells.

The 81-cdc18 S/O-JK and 81-cdc18 S/O-WA strains have a similar percentage of abnormal nuclei. After thiamine addition, abnormal nuclei start to appear by 4 hours in both strains and the curve rises steeply so by 7 hours 80% of cells have a nucleus with an abnormal morphology and by 9 hours almost 100% of cells possess abnormal nuclei (Fig. 4.13 B). The 81-cdc18 S/O-WB strain also accumulates cells with abnormal nuclei but with a delay of approximately 2 hours compared to the Walker A mutant. The curve is also less steep. Cells with abnormal nuclei start to increase after 4 hours in the presence of thiamine. This corresponds to the point
at which the septation index starts to increase. By the 9 and 10 hour timepoints the percentage of cells with abnormal nuclear material is about 80%. These data show that the cdc18-WB-3HA protein is able to initially block mitosis but not maintain the block so the cells eventually enter mitosis resulting in abnormal nuclear morphology (Fig. 4.13 B and 4.8 C).

4.6 The 81-cdc18 S/O-WB and cdc18-K46 mutants arrest with the same kinetics but different terminal phenotypes

81-cdc18 S/O-WB cells have a similar phenotype after the addition of thiamine as the cdc18-K46 temperature sensitive mutant at the restrictive temperature. Both mutants elongate and arrest with a 2C DNA content. The two strains were therefore compared to investigate whether the inability to maintain the block over mitosis for a prolonged period, as seen in the Walker B mutant, was common to both strains. The strains were grown to exponential phase at 25°C, the permissive temperature for the cdc18-K46 mutant, then both were shifted to 36°C and thiamine was simultaneously added to the 81-cdc18 S/O-WB cells. Cells were incubated for 8 hours at 36°C and samples were taken at hourly intervals for FACS analysis, cell number, septation index and the percentage of abnormal nuclei (Fig. 4.14).

The FACS analysis demonstrates that both strains maintain a 2C DNA content (Fig. 4.14 A). The FACS peak does drift somewhat throughout the timecourse, presumably due to cell elongation and increased background staining (Sazer and Sherwood, 1990). In both mutants the cell number increase stops by 4 hours after the shift to 36°C and addition of thiamine. However, the cdc18-K46 cells begin to increase in cell number again from 6 hours onwards (Fig. 4.14 B). This is presumably due to the leakiness of the temperature sensitive mutant. Septation index and the percentage of cells with abnormal nuclei were scored after rehydrating fixed cells in water and staining with calcofluor and DAPI respectively. The septation index demonstrates that both strains undergo an initial decrease in the percentage of septated cells as they arrest. However, the septation index begins to increase for cdc18-K46 cells as they leak through the block at 6 hours (Fig. 4.14 C). The percentage of abnormal nuclei also begins to increase at this point for the cdc18-K46 strain (Fig. 4.14 D). This would be expected as the cells
have been unable to complete DNA replication successfully but have entered mitosis. The 81-cdc18 S/O-WB mutant strain accumulates cells with abnormal nuclei at a much earlier stage, despite the septation index not increasing until 7-8 hours. By 5 hours after the addition of thiamine, 1 hour after cells have arrested, the percentage of abnormal nuclei has increased to 25%. It then continues to increase dramatically and by 8 hours after the addition of thiamine, almost 95% of cells have an abnormal nuclear morphology. This indicates that mitosis is not restrained in these cells as it is in cdc18-K46 cells so the checkpoint signal may not be initiated. This result is somewhat different to the expected result, as the initial data suggested that the cdc18-WB-3HA protein was able to initiate but not complete DNA replication and initiated a checkpoint signal that led to a block over mitosis.

To investigate this further I examined the requirement for the checkpoint proteins in the initial block over mitosis in the Walker B mutant. The 81-cdc18 S/O-WB strain was crossed with a rad3-136 mutant strain in order to isolate a checkpoint defective 81-cdc18 S/O-WB rad3-136 strain. If the Walker B mutant cells do require an intact checkpoint to establish the initial block that allows cell elongation, less elongation would be expected after addition of thiamine in the checkpoint defective strain as the cells would not arrest. The septation index would not decrease and cells would accumulate abnormal nuclei at an earlier timepoint as mitosis would not be inhibited. Unfortunately, I was unable to make this strain as 81-cdc18 S/O cells did not form colonies after germination. The spores are able to germinate but cells can undergo at most two or three divisions. This might be due to the low level of cdc18p in the cells. I have already stated that the cdc18 protein levels produced from the nmt81 promoter are lower than wildtype levels but that this level is sufficient for a mitotic cell cycle. However, it does not appear to be sufficient for cells to re-enter the mitotic cell cycle effectively after mating. The inability to cross the strain has meant that I have been unable to positively conclude that a checkpoint signal is not sent to allow the initial cell cycle block. My results do suggest that the mitotic checkpoint may not be initiated in the Walker B mutant, however this needs further investigation.
4.7 The 81-cdc18 S/O-WB strain exhibits a delay over S phase initiation after release from a HU block

To gain further insight into the defect of the 81-cdc18 S/O-WB strain, a Hydroxyurea (HU) block and release experiment was performed to compare the kinetics of S phase entry in a synchronous culture in the following strains: 81-cdc18 S/O-JK that is unable to complement the depletion of wildtype cdc18p after addition of thiamine, the 81-cdc18 S/O-wt strain that does complement as wildtype protein is still produced and the 81-cdc18 S/O-WB strain that I wish to investigate. All three strains were grown to exponential phase at 32°C, then HU was added to block cells at the beginning of S phase and thiamine was added 3 hours after the addition of HU to switch off the wildtype cdc18 gene. Cells were released from the HU block by filtering and washing three times in minimal media containing thiamine then resuspending them in thiamine containing media to maintain depletion of wildtype cdc18p. Cells were incubated at 32°C to allow growth and timepoints were taken for FACS analysis every 20 minutes for 3 hours (Fig. 4.15 A). These cells were also rehydrated and stained with calcofluor and DAPI so they could be scored for septation (Fig. 4.15 B) and percentage of binucleate cells (Fig. 4.15 C) to assess the synchrony of release into S phase.

All three strains complete the first S phase 40 to 60 minutes after release from the HU block. The 81-cdc18 S/O-JK cells start to accumulate with a 1C DNA content at 100 minutes. This increases to approximately 60% of the cells by 120 minutes and by 140 minutes 90% of the cells are in G1 (Fig. 4.15 A, a). Cells are able to complete the first S phase as wildtype cdc18p was present in the previous G1 to allow formation of the pre-replicative complex at origins. Cells undergo mitosis between 80 and 100 minutes after release from the HU block as can be seen by the increase in the percentage of binucleates (Fig. 4.15 C). Mitosis occurs soon after completion of S phase as HU blocked cells elongate allowing them to meet the size requirement for mitosis, cells therefore spend the minimum time required in G2 (Fantes and Nurse, 1978). The lack of wildtype cdc18p after mitosis prevents formation of pre-replicative complexes and hence initiation of DNA replication. Therefore when cells begin to septate, between 100 and 120 minutes after release from the HU block (Fig. 4.15 B), the G1 population of cells is first visualised. The
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81-cdc18 S/O-wt strain produces wildtype cdc18-3HA protein as the cells exit mitosis approximately 80 minutes after HU release as judged by the peak of binucleate cells (Fig. 4.15 C). The septation index peaks at 100 minutes (Fig. 4.15 B) and a 4C DNA content shoulder is seen at this stage on the FACS profile as cells initiate DNA replication simultaneously with septation (Fig. 4.15 A, b). The cells remain with a 2C DNA content for the rest of the timecourse. Both the 81-cdc18 S/O-JK, which is unable to undergo S phase but can still enter mitosis, and 81-cdc18 S/O-wt strains show an increase in both the septation index and percent of binucleate cells from 140 minutes as cells are still long enough to meet the size requirement and the second mitosis is initiated relatively quickly.

The 81-cdc18 S/O-WB strain also shows a peak of binucleate cells 100 minutes after release from the HU block and the septation index peaks between 100 and 120 minutes after release (Fig. 4.15 B and C). Therefore the kinetics of the first mitosis are equivalent in all three strains. However, the FACS profile is somewhat different to both of the other strains (Fig. 4.15 A, c). Cells predominantly have a 2C DNA content at 100 minutes, by 120 minutes approximately 50% of the cells have a DNA content of less than 2C and by 140 minutes most cells have a DNA content of less than 2C. The DNA content then gradually increases so by 180 minutes is between 1C and 2C. The cdc18-WB-3HA protein is therefore able to initiate DNA replication but with a delay of approximately 60 minutes compared to wildtype cells. The second mitosis is also delayed but only by approximately 40 minutes as both the septation index and percent of binucleate cells starts to increase at the 180 minute timepoint.

My previous data would suggest that although these cells have a 2C DNA content, DNA synthesis has not been successfully completed as we know that these cells are unable to properly segregate the DNA when mitosis is attempted. Cells therefore appear with abnormal nuclei. A checkpoint signal may not be sent in these cells as the delay over initiation of the second mitosis compared to the other strains is only 40 minutes. The DNA does not appear to be recognised as incompletely replicated. This could suggest a more direct link between the wildtype cdc18p and initiation of the checkpoint signal. This will be discussed in more detail later.
4.8 Does cdc18p interact directly with the MCM proteins

As the main function of cdc18p is thought to be to load the MCM proteins onto chromatin it is possible that they interact at some stage of the cell cycle. I immunoprecipitated cdc18p to see if it was able to interact with the Mcm4p homologue, cdc21p, and vice versa. The intention was to look for an interaction between cdc21p and the wildtype cdc18p and then investigate whether this interaction was either weaker or lacking when the NTP binding (WA) and hydrolysis (WB) mutants were expressed.

I initially tried to immunoprecipitate cdc18-3HAp and cdc21p from exponentially growing cells using antibodies to the HA tag and a cdc21 polyclonal antibody (Nishitani et al., 2000). Three strains were used, 81-cdc18 S/O-JK, 81-cdc18 S/O-wt and orp1-HAp as a positive control for the anti-HA immunoprecipitation. The orp1-HAp has previously been shown to immunoprecipitate with anti-HA antibodies (Grallert, 1996). Cells were grown to exponential phase at 32°C, harvested and protein extracts were made. Immunoprecipitations (IPs) were performed using 5mg of soluble protein. 5μl of either the 16B12 monoclonal anti-HA antibody or the cdc21p polyclonal antibody was used for each IP. Either Protein G or Protein A beads respectively, were then added to the extract/antibody mix. Protein A beads alone were included with the cdc21p IP as a negative control as a cdc21Δ strain is not viable and the MCM proteins are too stable for a switch off strain to be effective.

The total protein extract before immunoprecipitation and the immunoprecipitate were run simultaneously on a 10% SDS PAGE protein gel. The IP was 10x more concentrated than the extract. A Western blot of the anti-HA IP was first probed with anti-HA antibodies. This showed that orp1-HAp is more abundant than cdc18-3HAp (Fig. 4.16 A, top panel lanes 2 and 3). Only a small amount of the cdc18-3HAp is precipitated. This could be due to the instability of cdc18p or decreased efficiency of the IP. No band is recognised by anti-HA antibodies in the 81-cdc18 S/O-JK lanes as would be expected (Fig. 4.16 A, top panel, lanes 1 and 4). The anti-cdc18p Western confirms this result as the total extracts from each strain contain wildtype cdc18p (Fig. 4.16 A, middle panel, lanes 1 to 3) but only the 81-
cdc18 S/O-wt cells contain cdc18-3HAp (Fig. 4.16 A, middle panel, lanes 2 and 5). These blots were next probed with the cdc21p antibody (Fig. 4.16 A, bottom panel). There is an abundance of cdc21p in the total extracts and a band of the correct molecular weight is also recognised in the pellet, however this is not enhanced in the 81-cdc18 S/O-wt IP compared to the cdc18 S/O-JK IP. This band could be either a cross-reacting band of the same molecular weight as cdc21p or a small amount of cdc21p could be precipitated in a non-specific manner with the beads.

The Western blot of the anti-cdc21p immunoprecipitates was first probed with the anti-cdc21p antibody. This shows that cdc21p is present in all of the total extracts as expected (Fig. 4.16 B, top panel, lanes 1 to 3) and is immunoprecipitated very efficiently when the antibody is present (Fig. 4.16 B, top panel, lanes 4 and 5 compared with 6). The blot was next probed with the anti-HA antibody. This demonstrated that the cdc18-3HAp is present in the total extracts with the 81-cdc18 S/O-wt but not the 81-cdc18 S/O-JK cells (Fig. 4.16 B, bottom panel, lanes 2 and 3 compared with 1). However, no cdc18-3HAp was seen in the anti-cdc21p IP (Fig. 4.16 B, bottom panel, lanes 4 to 6). This confirmed the result with the anti-HA IP that cdc21p and cdc18-3HAp can not be immunoprecipitated from exponentially growing cells.

A Hydroxyurea block and release was performed to synchronise the cells and look for an interaction at the cell cycle stage when the cdc18p was thought to load the MCM complex onto chromatin. This should maximise the chance of seeing an interaction. The immunoprecipitations were performed both when cells were blocked at the start of S phase in HU and at two timepoints after the release. The 81-cdc18 S/O-JK and 81-cdc18 S/O-wt strains were grown to exponential phase at 32°C. Thiamine was then added to the 81-cdc18 S/O-wt cells to deplete them of untagged wildtype cdc18 protein. This was to ensure that only the tagged protein was available to interact with cdc21p to maximise the chance of seeing an interaction. HU was added 30 minutes after the thiamine and cells accumulated at the block point at the beginning of S phase (Fig. 4.17 A). The first timepoint was taken after cells had been in HU for 3 hours. Cells were released from the block by washing three times in minimal media (81-cdc18 S/O-JK), or minimal media containing thiamine (81-cdc18 S/O-wt), resuspended in the same media and
incubated for 100 minutes at 32°C. The second and third timepoints were taken 80 minutes and 100 minutes after release from the HU block. FACS samples were taken in the block and every 20 minutes during the release (Fig. 4.17 A). Both strains completed the first S phase 40-60 minutes after release and a 4C shoulder was seen at 100 minutes in the \textit{81-cdc18 S/O-wt} strain indicating that the second S phase had been initiated. This corresponds to the peak of septation for both strains (Fig. 4.17 B). The percent of binucleate cells peaks at 80-100 minutes (Fig. 4.17 C). These results indicate that in the majority of cells, mitosis is completed by approximately 80 minutes and the following S phase is initiated by 100 minutes. Therefore, the 80 and 100 minute timepoints cover the window between the end of mitosis and start of S phase when cdc18p and cdc21p would be expected to interact. Cells from the HU timepoint should allow the interaction to be seen if it persists after initiation of DNA replication.

The immunoprecipitations were performed using cells from an HU block, 80 and 100 minutes after release, as previously described. The anti-HA antibody was able to immunoprecipitate cdc18-3HAp in all three timepoints as an enriched band is seen with both the anti-HA and anti-cdc18p westerns (Fig. 4.17 D a, b and c, top and middle panel, lane 4). The cdc21p band seen in the pellets is not enriched in the \textit{81-cdc18 S/O-wt} immunoprecipitates (Fig. 4.17 D, a, b and c, bottom panels, lanes 3 and 4). The cdc21p IP gave a similar result (Fig. 4.17 E). The cdc21p was immunoprecipitated very efficiently at each timepoint when the antibody was present but not when it was absent (Fig. 4.17 E, a, b and c lanes 4 and 5 compared with lane 6). However, the cdc18-3HA protein was not immunoprecipitated with the cdc21p antibody (Fig. 4.17 E, a, b and c, lanes 4 to 6). I was therefore still unable to IP cdc18p and cdc21p despite synchronising the cell cultures and performing the immunoprecipitations when there was the greatest chance of detecting an interaction. I was unable to use this as an assay for examining the defects of the cdc18p NTP binding and hydrolysis mutants.

An alternative to investigating an interaction between cdc18p and cdc21p could be to examine whether the mutants were able to load cdc21p onto chromatin. It has been reported recently that in fission yeast cdc18p is required for chromatin association of cdc21p (Kearsey et al., 2000; Nishitani et al., 2000). I crossed a strain
in which the cdc21p has been tagged with GFP at its C-terminus, with 81-cdc18 S/O-JK, 81-cdc18 S/O-wt, 81-cdc18 S/O-WA and 81-cdc18 S/O-WB. This would allow me to use the method developed by Kearsey et al. to investigate whether cdc21-GFPp was still able to bind chromatin when wildtype cdc18p was depleted but cdc18-WA-3HAp or cdc18-WB-3HAp were expressed. However, the 81-cdc18 S/O cells were again unable to form colonies following germination so I was unable to perform the experiment. I have therefore been unable to provide further insight into the biochemical defects of the cdc18p NTP binding and hydrolysis mutant.
Discussion

Cdc18p has a number of structural motifs that have implications both for the function and the regulation of the protein. To investigate the effect these motifs have on cdc18p function, a site-directed mutagenesis of several domains was performed. The mutants were expressed in a strain in which the wildtype cdc18 was under the control of a repressible promoter. The promoter was switched off to deplete cells of wildtype cdc18 protein and cells were examined to investigate whether they were still able to maintain growth. Only two mutants were unable to sustain normal growth. These were the NTP binding (Walker A) and hydrolysis (Walker B) mutants and it is these that will be discussed in more detail.

The Walker A mutant appeared to be completely non-functional and acted as though no cdc18p was present in the cells. The 81-cdc18 S/O-WA cells arrested with the same phenotype and kinetics as the 81-cdc18 S/O-JK strain in which only the vector was integrated (Fig. 4.5, 4.7 and 4.13). This is consistent with results obtained with the cdc18-NTPp mutant (Chapter 2), which was also mutated in the NTP binding domain. I discovered that this mutant was unable to initiate re-replication when overexpressed in wildtype cells, however it was capable of blocking mitosis (Fig. 2.2). As the N-terminus of cdc18p was able to interact with cdc2p (Fig. 2.4) and did not require the checkpoint rad and hus genes to block mitosis (Fig. 2.7) I constructed a mutant in which the N-terminus of the cdc18-NTPp mutant was deleted, thus irradicating the dominant cell cycle effect. When overexpressed this mutant was unable to either initiate re-replication or the checkpoint (Fig. 2.8), indicating that the NTP binding motif was essential for both functions. Results with the 81-cdc18 S/O-WA strain lead to the same conclusion but are more convincing as the mutant protein is expressed at endogenous levels, it is the only copy of cdc18p present in the cells and there is only a single amino acid change, not two amino acid substitutions and a 149 amino acid deletion.

From this work it was known that the cdc18-WA-3HA protein was non-functional in that it was unable to initiate either DNA replication or the checkpoint, however I did not know which aspect of protein function was defective. To understand the
biochemical nature of the mutant there were several questions to answer. These related to the interaction of the mutant protein with DNA and the MCM proteins. I hypothesised that the cdc18-WA-3HA protein would not bind to chromatin as it has been suggested that binding of ATP to some proteins stabilizes them in a conformation in which they have a high affinity for chromatin (Fujita et al., 1997; Story and Steitz, 1992). This hypothesis could be tested in one of two ways. A subcellular fractionation protocol to enrich for the chromatin component could be used (Lygerou and Nurse, 1999; Nishitani et al., 2000). Alternatively a Chromatin Association (CHIP) assay could be performed in which proteins bound to DNA are cross-linked with formaldehyde, DNA is sheared and antibodies to the protein of interest are used to immunoprecipitate the protein/DNA complex. The cross-linking is reversed and PCR is used to amplify specific DNA sequences, in this case origin DNA. Unfortunately, due to time constraints, these experiments were not performed.

I would also like to have determined whether the cdc18-WA-3HA protein was able to interact with the MCM proteins. As the mechanism of loading of MCMs onto chromatin by cdc18p is unknown, it is possible that the Walker A mutant would be able to bind the MCM complex but not DNA. However I was unable to detect an interaction by immunoprecipitation between wildtype cdc18p and the Mcm4p homologue, cdc21p so I did not perform any immunoprecipitation experiments with the mutant. There are several possibilities as to why an interaction between the two proteins was not seen. The first relates to the stability of cdc18p. As cdc18p is a very unstable protein much of the protein could have been degraded by the end of the experiment. This would greatly decrease the chance of seeing an interaction. Another possibility is that the interaction between cdc18p and cdc21p is transient. They may interact solely for the loading step so are not present as a complex on the DNA. I tried to maximise the chance of seeing a transient interaction by blocking the cells at the beginning of S phase using HU, then releasing cells to undergo a relatively synchronous cell cycle. Immunoprecipitations were performed from cells judged to be in G1, G1/S and early S phase. However, I was still unable to detect an interaction (Fig. 4.17). To stabilize a brief interaction it may be possible to chemically cross-link the proteins before performing the immunoprecipitation. This has proved moderately
successful when immunoprecipitating HsCdc6p with either HsMcm3p or HsMcm7p (Fujita et al., 1999). However, it is suggested that most of the HsMCM complexes are loaded onto chromatin away from sites of HsCdc6p binding so only a small proportion of the HsMCMs are complexed with HsCdc6p. To expand on this hypothesis it is possible that Cdc18p in fission yeast facilitates binding of the MCM proteins to chromatin but not through a direct interaction. It is possible that when cdc18p binds to the chromatin it induces either a conformational change to the DNA which allows MCM binding, or it results in the exposure of another protein that is able to interact with the MCM complex. The final alternative is that cdc18p binds to a MCM complex that does not contain cdc21p. There is evidence that the MCM proteins form a range of sub-complexes that possess different functions, in both fission yeast and higher eukaryotes (Adachi et al., 1997; Ishimi, 1997; Ishimi et al., 1998; Pasion and Forsburg, 1999; Sherman and Forsburg, 1998; Sherman et al., 1998). However, Sherman et al. were also unable to immunoprecipitate cdc18p and MCM subunits despite trying with mcm2p, 4p and 6p (Sherman et al., 1998). This suggests that cdc18p may not load the MCMs through a direct interaction.

To investigate whether the cdc18-WA-3HA protein could load the MCM proteins, either the subcellular fractionation chromatin assay mentioned previously or an in situ chromatin assay could be used (Kearsey et al., 2000). The in situ chromatin assay uses a GFP-tagged cdc21p which is detected by GFP fluorescence after the cell is permeabilized by enzymatic digestion and washed with a non-ionic detergent. Only proteins that are bound to chromatin will remain in the nucleus after this procedure. As discussed earlier, I attempted to construct strains in which the nmt81-cdc18, triple-HA tagged mutants and cdc21-GFP were all present. However this was unsuccessful due to the fact that the 81-cdc18 S/O strains did not form colonies after germination. I believe that this is due to cdc18 protein levels being too low to allow cells to re-enter the mitotic cell cycle. This could be addressed by expressing cdc18 on a multicopy plasmid to increase protein levels while the two strains mate. The plasmid could then be lost after colony formation by growing in non-selective media. Alternatively a new strain could be constructed in which the wildtype cdc18 is placed under the medium strength nmt41 promoter. The protein level may be slightly higher than the wildtype but if only one integrated copy is
present there should be sufficient cdc18p to allow the 41-cdc18 S/O strain to germinate but not sufficient to induce re-replication.

The Walker B mutant has a different phenotype to the Walker A mutant. An exponentially growing population of cells initially arrests with a 2C DNA content on depletion of wildtype cdc18p but cells later enter mitosis (Fig. 4.8 and Fig. 4.13). This is different to the cdc18-K46 temperature sensitive mutant which also arrests with a 2C DNA content and the same kinetics as 81-cdc18 S/O-WB after a shift to the restrictive temperature indicating that they have the same block point. However the 81-cdc18 S/O-WB cells accumulate abnormal nuclei at a much earlier stage than cdc18-K46 suggesting that they are unable to maintain the checkpoint arrest (Fig. 4.14).

The HU block and release experiment indicates that the 81-cdc18 S/O-WB cells are delayed on entry into S phase (Fig. 4.15). There could be several explanations for this, depending on what the function of ATP hydrolysis is. Replication initiation may occur at a slower rate if the cdc18-WB-3HA protein is unable to hydrolyse ATP. Alternatively only a subset of origins may fire or some of the components of the pre-replicative complex may not be loaded onto chromatin. To investigate which of these options may be correct a number of techniques could be used. The chromatin assay and in situ chromatin assay would allow us to determine whether the cdc18-WB-3HA protein and cdc21p are able to bind to chromatin and could give an indication as to whether less protein is loaded. A more detailed analysis to determine which proteins were able to bind to origin DNA and whether initiation proteins were loaded only onto a subset of origins could be achieved using the CHIP assay. This would give an indication as to whether fewer origins assembled the pre-replicative complex that would be required to allow S phase initiation. However, to determine unequivocally whether only a subset of origins fired, 2-dimensional agarose gel electrophoresis would have to be performed on a range of origins (Brewer and Fangman, 1987). This is a technique that allows the separation of DNA fragments according to two parameters, first size and second topology. The DNA molecules are detected by hybridization and the presence of a bubble arc indicates that an origin fired within the detected fragment.
Chapter 4 Mutational analysis of cdc18

The most puzzling phenotype of the $81^{-}$-cdc18 $S/O$ strain is that it appears unable to maintain a checkpoint signal to inhibit mitosis. Unfortunately, I have been unable to establish whether the $81^{-}$-cdc18 $S/O$-WB cells are able to initiate a checkpoint which later becomes defective, or whether the checkpoint signal is never sent, due to an inability to construct an $81^{-}$-cdc18 $S/O$-WB checkpoint defective strain. An alternative way of investigating whether a checkpoint signal is initiated would be to look for activation of either the chk1p or cds1p kinases implicated in the damage and replication checkpoint respectively. Activation of chk1p is detected as a bandshift that occurs in response to damaging agents and is thought to be due to autophosphorylation (Walworth and Bernards, 1996). Cds1p activation is detected by examining the kinase activity using Myelin Basic Protein (MBP) as a substrate (Lindsay et al., 1998). The level of mik1p could also be investigated as it has been shown to accumulate in response to stalled replication (Boddy et al., 1998; Christensen et al., 2000).

If a checkpoint signal is not sent in the Walker B mutant it is possible that the cell elongation seen is caused by a G1 delay prior to replication initiation as a different set of controls operates to inhibit cdc2p at this stage. However, exponentially growing cells do not accumulate with a 1C DNA content after depletion of wildtype cdc18p. Lack of a checkpoint signal would mean that the DNA was not recognised as incompletely replicated by the checkpoint rad proteins which could indicate a direct link between cdc18p function and the checkpoint signal. In support of this, a cut5/rad4 mutant allele, rad4-116, is able to initiate DNA replication but is deficient in the checkpoint (McFarlane et al., 1997). The implication of these results could lead to the hypothesis that a complex on the DNA is involved in sending a checkpoint signal, rather than replication structures. The checkpoint could be initiated by a complex on the DNA that is either altered or absent when the cdc18-WB-3HAp is expressed. Alternatively ATP hydrolysis may result in the removal of cdc18p from the chromatin and it may be a lack of cdc18p that is required to send a checkpoint signal. A careful characterization of which proteins are bound to chromatin when both the cdc18-WA-3HAp and cdc18-WB-3HAp are expressed may facilitate our understanding of how the checkpoint signal is sent.
There is still much to understand about the function of cdc18p and the analysis performed so far has suggested a way to proceed rather than solved any problems. Cdc18p function and regulation will be discussed in more detail in the next chapter, particularly in relation to results from other organisms.
Figure 4.1

Characterization of the \textit{81-cdc18 S/O} strain

Thiamine was added to an exponentially growing \textit{81-cdc18 S/O} culture and the phenotype of the cells was followed for 4 hours by FACS analysis (A) and DAPI staining (B) Bar 15 μm. Protein levels were determined by Western blotting with anti-cdc18p antibody (C top panel) and anti-α-tubulin antibody (C bottom panel). 50μg of protein was loaded per lane.
Figure 4.2

A 5.2kb genomic fragment of cdc18 (gcdc18) complements the 81-cdc18 S/O strain both when integrated and when expressed from a multicopy plasmid

The 81-cdc18 S/O strain was transformed with pJK148, pJK148-gcdc18 and pIRT2-gcdc18. Thiamine was added to exponentially growing cultures and the ability to complement the switch off phenotype was examined for 10 hours by FACS analysis (A).
Figure 4.2 B and C

Cell number was followed for 10 hours after the addition of thiamine (B). Cells were fixed and stained with DAPI (C). Bar 15μm.
Figure 4.3

cdc18 DNA and protein sequences and motifs

- Walker A box (WA)
- Walker B box (WB)
- Putative nuclear localization signal (N1)
- Putative nuclear localization signal (N2)
- Putative cdc2p consensus phosphorylation site (P1-6)
**Figure 4.4**

pJK148 and pJK148-gcdc18-3HA and mutants are all integrated at the *leu1* locus in the *81-cdc18 S/O* strain

Map to demonstrate the altered band pattern on integration of pJK148-gcdc18-3HA and mutants (A).
Figure 4.4 B

Southern blots of the integrated strains probed with either \textit{leu}1 (top panel) or \textit{cdc}18 (bottom panel) (B).
Figure 4.5

The vector pJK148 does not complement the 81-cdc18 S/O strain when integrated at the leu1 locus

The 81-cdc18 S/O strain containing the pJK148 vector integrated at the leu1 locus (81-cdc18 S/O-JK) was grown exponentially before the cultures were split and thiamine was added to half. Cells were then followed for 10 hours by cell number (A) and FACS analysis (B).
Figure 4.5 C and D

DAPI staining was performed both before thiamine was added and 1, 5 and 10 hours after the addition of thiamine (C). Bar 15µm. Protein levels were determined by Western blotting both before the addition of thiamine and 1 hour after. 50µg of protein was loaded per lane. Blots were probed with anti-cdc18p antibody (top panel), anti-HA antibody (middle panel) and anti-α-tubulin antibody (bottom panel) (D).
Figure 4.6

pJK148-gcdc18-3HA does complement the 81-cdc18 S/O strain when integrated at the leu1 locus

The 81-cdc18 S/O strain containing pJK148-gcdc18-3HA integrated at the leu1 locus (81-cdc18 S/O-wt) was grown exponentially before the cultures were split and thiamine was added to half. Cells were followed for 10 hours by cell number (A) and FACS analysis (B).
Figure 4.6 C and D

DAPI staining was performed both before thiamine was added and 1, 5 and 10 hours after the addition of thiamine (C). Bar 15μm. Protein levels were determined by Western blotting both before the addition of thiamine and 1 hour after. 50μg of protein was loaded per lane. Blots were probed with anti-cdc18 antibody (top panel), anti-HA antibody (middle panel) and anti-α-tubulin antibody (bottom panel) (D).
Figure 4.7

pJK148-gcdc18-3HA WA does not complement the 81-cdc18 S/O strain when integrated at the leu1 locus

The 81-cdc18 S/O strain containing pJK148-gcdc18-3HA WA integrated at the leu1 locus (81-cdc18 S/O-WA) was grown exponentially before the cultures were split and thiamine was added to half. Cells were then followed for 10 hours by cell number (A) and FACS analysis (B).
Figure 4.7 C and D

DAPI staining was performed both before thiamine was added and 1, 5 and 10 hours after the addition of thiamine (C). Bar 15μm. Protein levels were determined by Western blotting both before the addition of thiamine and 1 hour after. 150μg of protein was loaded per lane. Blots were probed with anti-cdc18p antibody (top panel), anti-HA antibody (middle panel) and anti-α-tubulin antibody (bottom panel) (D).
Figure 4.8

pJK148-gcdc18-3HA WB does not complement the 81-cdc18 S/O strain when integrated at the leu1 locus

The 81-cdc18 S/O strain containing pJK148-gcdc18-3HA WB integrated at the leu1 locus (81-cdc18 S/O-WB) was grown exponentially before the cultures were split and thiamine was added to half. Cells were then followed for 10 hours by cell number (A) and FACS analysis (B).
Figure 4.8 C and D

DAPI staining was performed both before thiamine was added and 1, 5 and 10 hours after the addition of thiamine (C). Bar 15μm. Protein levels were determined by Western blotting both before the addition of thiamine and 1 hour after. 150μg of protein was loaded per lane. Blots were probed with anti-cdc18p antibody (top panel), anti-HA antibody (middle panel) and anti-α-tubulin antibody (bottom panel) (D).
Figure 4.9

pJK148-gcdc18-3HA NLS1 does complement the 81-cdc18 S/O strain when integrated at the leu1 locus

The 81-cdc18 S/O strain containing pJK148-gcdc18-3HA NLS1 integrated at the leu1 locus (81-cdc18 S/O-N1) was grown exponentially before the cultures were split and thiamine was added to half. Cells were then followed for 10 hours by cell number (A) and FACS analysis (B).
Figure 4.9 C and D

DAPI staining was performed both before thiamine was added and 1, 5 and 10 hours after the addition of thiamine (C). Bar 15µm. Protein levels were determined by Western blotting both before the addition of thiamine and 1 hour after. 50µg of protein was loaded per lane. Blots were probed with anti-cdc18p antibody (top panel), anti-HA antibody (middle panel) and anti-α-tubulin antibody (bottom panel) (D).
**Figure 4.10**

pJK148-gcdc18-3HA NLS2 does complement the 81-cdc18 S/O strain when integrated at the *leu1* locus

The 81-cdc18 S/O strain containing pJK148-gcdc18-3HA NLS2 integrated at the *leu1* locus (81-cdc18 S/O-N2) was grown exponentially before the cultures were split and thiamine was added to half. Cells were then followed for 10 hours by cell number (A) and FACS analysis (B).
Figure 4.10 C and D

DAPI staining was performed both before thiamine was added and 1, 5 and 10 hours after the addition of thiamine (C). Bar 15μm. Protein levels were determined by Western blotting both before the addition of thiamine and 1 hour after. 150μg of protein was loaded per lane. Blots were probed with anti-cdc18p antibody (top panel), anti-HA antibody (middle panel) and anti-α-tubulin antibody (bottom panel) (D).
Figure 4.11
gcdc18-3HA-N1+N2 does complement the 81-cdc18 S/O strain when integrated at the leu1 locus

The 81-cdc18 S/O strain containing pJK148-gcdc18-3HA-N1+N2 integrated at the leu1 locus (81-cdc18 S/O-N1+N2) was grown exponentially before the cultures were split and thiamine was added to half. Cells were then followed for 10 hours by cell number (A) and FACS analysis (B).
DAPI staining was performed both before thiamine was added and 1, 5 and 10 hours after the addition of thiamine (C). Bar 15μm. Protein levels were determined by Western blotting both before the addition of thiamine and 1 hour after. 150μg of protein was loaded per lane. Blots were probed with anti-cdc18p antibody (top panel), anti-HA antibody (middle panel) and anti-α-tubulin antibody (bottom panel) (D).
Figure 4.12
gcdc18-3HA-P1-6 does complement the 81-cdc18 S/O strain when integrated at the leu1 locus

The 81-cdc18 S/O strain containing pJK148-gcdc18-3HA-P1-6 integrated at the leu1 locus (81-cdc18 S/O-P1-6) was grown exponentially before the cultures were split and thiamine was added to half. Cells were then followed for 10 hours by cell number (A) and FACS analysis (B).
Figure 4.12 C and D

DAPI staining was performed both before thiamine was added and 1, 5 and 10 hours after the addition of thiamine (C). Bar 15μm. Protein levels were determined by Western blotting both before the addition of thiamine and 1 hour after. 50μg of protein was loaded per lane. Blots were probed with anti-cdc18p antibody (top panel), anti-HA antibody (middle panel) and anti-α-tubulin antibody (bottom panel) (D).
Figure 4.13


Cells fixed in 70% ethanol from the experiments described in Figures 4.5, 4.7 and 4.8 were rehydrated in water. They were then stained with either calcofluor to determine the septation index (A) or with DAPI to follow the accumulation of abnormal nuclei (B). 200 cells were counted for each timepoint.
The *cdc18-K46* mutant arrests with similar kinetics as *81-cdc18 S/O-WB* but with a different terminal phenotype.

The *81-cdc18 S/O-WB* and *cdc18-K46* strains were grown at 25°C to exponential phase. The cultures were then shifted to 36°C and thiamine was added to *81-cdc18 S/O-WB*. Cells were then followed for 8 hours by FACS analysis (A) and cell number (B).
Figure 4.14 C and D

Cells were rehydrated and stained with calcofluor to determine septation index (C) or DAPI to determine the percentage of cells with abnormal nuclei (D). 200 cells were scored for each timepoint.
Figure 4.15

The 81-cdc18 S/O-WB strain completes S phase after release from an HU block then initiates a second round of replication after a 60 minute delay.

The 81-cdc18 S/O-JK, 81-cdc18 S/O-wt and 81-cdc18 S/O-WB strains were grown to exponential phase. HU was then added to the cultures to block the cells at the beginning of S phase. Thiamine was added after 3 hours to switch off the wild type cdc18 and cells were released 30 minutes later. Samples were taken for FACS analysis every 20 minutes for a further 3 hours.
Figure 4.15 B and C

Cells were rehydrated and stained with calcofluor to determine septation index (B) and DAPI to determine the percent of binucleate cells (C). 200 cells were scored for each timepoint.
Figure 4.16

An interaction between cdc18p and cdc21p can not be detected in exponentially growing cells

81-cdc18 S/O-JK, 81-cdc18-wt and orp1-HA cells were grown exponentially. Protein extracts were made and immunoprecipitations were performed with either an α-HA antibody (A), or an α-cdc21 antibody (B).
Figure 4.17

An interaction between cdc18p and cdc21p can not be detected in an HU block, or 80 and 100 minutes after release

The 81-cdc18 S/O-JK and 81-cdc18 S/O-wt strains were grown to exponential phase. Thiamine was added to 81-cdc18 S/O-wt then HU was added 30 minutes later to block the cells at the beginning of S phase. Cells were released from the block after 3.5 hours and samples were taken every 20 minutes for FACS analysis (A). 200 cells were counted for septation index (B) and number of binucleates (C).
Figure 4.17 D

Protein extracts were made from cells in the HU block (a), and 80 (b) and 100 minutes (c) after the release. Immunoprecipitations were performed with an α-HA antibody.
Figure 4.17 E

Protein extracts were made from cells in the HU block (a), and 80 (b) and 100 minutes (c) after the release. Immunoprecipitations were performed with an α-cdc21 antibody.
Chapter 5

General Discussion

Introduction

In this chapter I discuss my results in a wider context. I will compare how cdc18p is regulated in *Schizosaccharomyces pombe* with other organisms and discuss the consequences that regulation has on restricting S phase to only once per cell cycle. I will go on to discuss the function of cdc18p in both DNA replication and the checkpoint that ensures S phase is completed before mitosis is initiated. Finally I will discuss the requirement for the NTP binding and hydrolysis motifs and compare the effect of mutating these in fission yeast and other organisms.
5.1 Regulation of cdc18p

*CDC18* is an essential gene, required for both initiation of DNA replication and the checkpoint that inhibits mitosis until S phase has been successfully completed. Deletion of the gene results in cells entering mitosis without previously replicating their DNA. This results in a *cut* phenotype as the DNA can not be properly segregated into the two daughter cells. Overexpression of *cdc18* causes re-replication of the DNA as *cdc18* is able to block mitosis and to re-initiate DNA replication (Kelly et al., 1993; Nishitani and Nurse, 1995). It is therefore imperative that *cdc18* protein levels are carefully controlled throughout the cell cycle. In fission yeast, this is done in two ways: transcription and proteolysis.

The S phase transcriptional machinery is composed of *cdc10p*, *res1p*, *res2p* and *rep2p* (Aves et al., 1985; Baum et al., 1997; Caligiuri and Beach, 1993; Miyamoto et al., 1994; Nakashima et al., 1995; Tanaka et al., 1992; Zhu et al., 1994b). Targets of this complex are expressed periodically in the cell cycle and include *cdt1*, *cdc22* and *cig2* in addition to *cdc18*. It has been shown that *cdc18* transcription is activated at the onset of mitosis and continues until cells pass into S phase (Baum et al., 1998; Kelly et al., 1993). The protein does not, however, accumulate until cells exit from mitosis (Baum et al., 1998). This is due to the second type of regulation, proteolysis.

It has been known for some time that *cdc18p* is highly unstable (Muzi-Falconi et al., 1996) and that phosphorylation by the *cdc2* protein kinase directly targets it for degradation (Baum et al., 1998; Jallepalli et al., 1997). The phosphorylated protein is specifically recognised by *pop1p* and *pop2p/sud1p*, proteins that form part of the SCF (Skp1-cullin-F-box) complex. The ubiquitination of *cdc18p* by this complex results in its subsequent degradation via the 26S proteasome (Jallepalli et al., 1998; Kominami et al., 1998a; Kominami and Toda, 1997; Wolf et al., 1999b).

*Cdc18p* mutated in all six *cdc2* consensus phosphorylation sites (*cdc18-P1-6p*) is more stable than the wildtype protein, as would be expected if phosphorylation targets it for degradation (Baum et al., 1998; Jallepalli et al., 1997). It is also known that *pop1p* and *pop2p/sud1p* mutants result in the accumulation of both *cdc18p* and *rum1p* which allows an additional round of replication (Jallepalli et al., 1998;
Kominami et al., 1998a; Kominami and Toda, 1997; Wolf et al., 1999b). It was not known however, whether \textit{cdc18-P1-6} expressed from the endogenous \textit{cdc18} promoter would disturb the normal cell cycle progression that leads from G1 to S phase to G2 and would permit an additional round of replication due to its G2 stability, or would complement a lack of \textit{cdc18} function. I showed that the \textit{cdc18-P1-6p} mutant was able to fully complement the 81-\textit{cdc18} S/O strain when thiamine was added to deplete the cells of wildtype protein (Fig. 4.12). Cells maintain a 2C DNA content which shows that a diploid population does not accumulate (Fig. 4.12 B). These results indicate that phosphorylation mediated degradation of \textit{cdc18p} is only one of the redundant controls that restrict DNA replication to once per cell cycle and is not sufficient in itself to disrupt normal cell cycle progression. Another factor such as the presence of \textit{rum1p} may also be required before re-initiation of S phase can occur.

This conclusion is somewhat contradictory to results obtained by Wolf and co-workers (Wolf et al., 1999a). They found that titration of the proteolytic machinery by overproduction of \textit{ScCdc6p} resulted in the stabilization of \textit{cdc18p} so it accumulates to a level that is sufficient to induce an additional round of replication, even in the absence of \textit{rum1p}. The experiments I performed and described in Chapter 3 showed that overproduction of \textit{ScCdc6p} induces re-replication without a subsequent increase in the level of \textit{cdc18} protein (Fig. 3.7). This suggests that re-replication is induced due to an intrinsic function of \textit{Cdc6p}, not just through interfering with proteolysis (Chapter 3).

The \textit{cdc2p} consensus phosphorylation sites are found in the N-terminus of the protein. An N-terminal truncation of \textit{cdc18p} that results in five out of the six \textit{cdc2p} consensus phosphorylation sites being deleted is also more stable than the wildtype protein (Baum et al., 1998). The \textit{cdc18-150-577p} mutant is able to induce both re-replication and the checkpoint when overexpressed (Fig. 2.2) and is therefore able to perform both of the essential functions of \textit{cdc18p}. However it is unable to complement a \textit{cdc18-K46} temperature sensitive mutant when expressed at the restrictive temperature (Chapter 2). This suggests that the N-terminus of \textit{cdc18p} is required to perform a function that is essential for cell viability which is separate from its requirement for degradation of the protein in S phase.
One possible function for the N-terminal region of cdc18p could be the physical removal of cdc18p from origins. There is evidence in *Xenopus laevis* that Cdc6p must be removed from origins before initiation can occur and that this removal may require cdk2 activity (Hua and Newport, 1998). It has also been suggested from chromatin binding studies using synchronous budding yeast cultures that the majority of Cdc6p is unloaded from origins before firing (Weinreich et al., 1999). Chromatin assays in fission yeast indicate that cdc18p is probably not removed from origins prior to S phase initiation (Nishitani et al., 2000). However depletion of cdc18p from chromatin during S phase could still be necessary to signal that DNA replication has been completed, thus ensuring cell viability.

Cdc2p interacts strongly with the N-terminus of cdc18p (Fig. 2.4), perhaps more strongly than would be expected for a normal kinase/substrate interaction. It is possible that an essential first step is the binding of cdc2p to cdc18p thus removing it from origins; phosphorylation and degradation may be a non-essential later step. Some support for this hypothesis comes from the fact that intact cdc2p consensus phosphorylation sites are not required for a cdc2p/cdc18p interaction (Brown et al., 1997). This could explain why the cdc18-P1-6p mutant is fully functional, as it could still be removed from chromatin via a cdc2p/cdc18p interaction, despite not being phosphorylated and degraded. To investigate this hypothesis it would be necessary to see whether cdc18-150-577p remained bound to chromatin in G2 cells.

Cell cycle regulated expression of cdc18p is common to both fission and budding yeasts. *ScCDC6* is transcribed in G1 (Piatti et al., 1995; Zhou and Jong, 1990; Zwerschke et al., 1994) and is degraded on entry into S phase after Cdc28p/Clb phosphorylation (Elsasser et al., 1999). The human Cdc6p is regulated somewhat differently. *CDC6* is expressed selectively in proliferating but not quiescent mammalian cells and this regulated transcription is dependent on E2F (Hateboer et al., 1998; Ohtani et al., 1998; Yan et al., 1998). The mRNA levels have been shown to peak at the G1/S boundary but the protein levels remain constant throughout the cell cycle (Saha et al., 1998). Controls used by mammalian cells to prevent re-initiation of DNA replication in G2 do not therefore include degradation of Cdc6p. Human cells have evolved a different mechanism to ensure that Cdc6p is removed from the vicinity of chromatin after S phase initiation. Cdc6p has been shown to be
nuclear in G1 but is then selectively excluded from the nucleus after S phase initiation (Saha et al., 1998). Intriguingly, the subcellular localization of Cdc6p is regulated by phosphorylation. It has been shown that phosphorylation of Cdc6p on three N-terminal serine residues by Cdk2/Cyclin A results in the translocation of Cdc6p from the nucleus to the cytoplasm (Jiang et al., 1999b; Otzen Petersen et al., 1999). Phosphorylation is therefore essential for the local depletion of Cdc6/18p from the nucleus after S phase initiation. However, whether this is done by degradation or re-localization is dependent on the organism.

As phosphorylation of human Cdc6p affected localization it was possible that a similar re-localization occurred in response to phosphorylation in fission yeast and it was only after cdc18p had been transported to the cytoplasm that it could be ubiquitinated and degraded. Some support for this hypothesis came from the fact that mutation of two basic residues K57 and R58 inhibited the nuclear localization of HsCdc6p and mutation of S54 which is one of the sites phosphorylated by Cdk2 also partially inhibits nuclear localization (Takei et al., 1999). This could suggest a general mechanism whereby phosphorylation of Cdc6/18p by Cdk2/cdc2p next to a nuclear localization signal could prevent its nuclear accumulation. The two putative bipartite nuclear localization signals in cdc18p are both close to cdc2p consensus phosphorylation sites (Fig. 4.3). NLS1 is adjacent to P2 and contains P3 within it and NLS2 contains P4. In mutating the nuclear localization signals of cdc18p I hoped to see a lack of complementation of the 81-cdc18 S/O strain when the wildtype cdc18p was depleted by the addition of thiamine. However, as that was not the case and even cdc18p mutated in both nuclear localization signals was able to complement the depletion of wildtype cdc18p (Fig. 4.9-4.11) I did not pursue a link between phosphorylation and localization. In fact there is evidence to suggest that proteolysis occurs in the nucleus rather than the cytoplasm as the 26S proteasome has been localized to the nuclear periphery near the inner side of the nuclear envelope (Wilkinson et al., 1998).

As the nuclear localization signals of cdc18p were not essential for cdc18p function, it indicates that either the mutagenesis did not inactivate the NLS, that I was not mutating the correct motif, or that cdc18p enters the nucleus through binding to other nuclear bound proteins. It is known that a similar mutation of the cytokine...
interleukin-5 bipartite NLS did prevent nuclear localization (Jans et al., 1997) and that there are no other obvious NLSs in the protein. I must therefore conclude that cdc18p enters the nucleus by binding to other proteins. This is supported by the fact that mutation of the only putative bipartite nuclear localization signal of HsCdc6p does not alter its nuclear localization (Saha et al., 1998) and that recombinant XICdc6p can not cross the nuclear membrane despite the presence of a putative bipartite NLS (Coleman et al., 1996). An obvious candidate for the protein that transports cdc18p into the nucleus would be cdt1p which forms a complex with cdc18p, acts co-operatively in a DNA re-replication assay and possesses a putative NLS (Hofmann and Beach, 1994; Nishitani et al., 2000). An interesting point is that only ScCdc6p has been conclusively shown to have a functional NLS (Elsasser et al., 1999; Jong et al., 1996) and this organism appears not to possess a cdt1 homologue. However, as cdc18p is able to bind chromatin in the absence of cdt1p function (Nishitani et al., 2000), it indicates that other proteins must also be involved in the nuclear localization of cdc18p.

5.2 Function of cdc18p

Cdc18p has been shown to be required for the loading of the MCM complex onto chromatin prior to DNA replication in a number of organisms. This allows formation of the pre-replicative complex (Aparicio et al., 1997; Coleman et al., 1996; Donovan et al., 1997; Liang and Stillman, 1997; Nishitani et al., 2000; Stoeber et al., 1998; Tanaka et al., 1997). The precise mechanism is somewhat less clear and several groups have worked to understand how cdc18p performs this role. A common approach has been to perform site-directed mutagenesis on the Walker A motif, required for NTP binding, and Walker B motif required for NTP hydrolysis (Guenther et al., 1997; Story and Steitz, 1992; Walker et al., 1982). This could lead to identification of a stepwise mechanism for MCM loading.

However, the results of these analyses have been difficult to interpret due to experiments being performed in:
1) different organisms
2) using different amino acid substitutions
3) using different assays to assess function

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I will discuss the findings from other groups when first, the Walker A motif is mutated and second, the Walker B motif is mutated, relating their results to my own. I will then attempt to come to some conclusions as to how cdcl18p acts to load MCMs onto origins.

A mutation of the conserved lysine residue to glutamate in the Walker A motif (GxxGxGKT) of *Saccharomyces cerevisiae* Cdc6p has been shown to produce a non-functional protein that is unable to complement the cdc6-1 temperature sensitive mutant at the restrictive temperature of 37°C (Perkins and Diffley, 1998). Genomic footprinting shows that the Cdc6-K114Ep mutant is unable to generate any aspect of the pre-replicative footprint normally visualised in G1. These results are consistent with my own, which also involved a K→E mutation of the Walker A motif. The 81-cdc18 S/O-WA strain was unable to complement a depletion of the wildtype protein (Fig. 4.7). The cells arrested and accumulated abnormal nuclei with similar kinetics to the 81-cdc18 S/O-JK strain which possessed no cdcl18p after switching off expression of wildtype *cdcl8* by the addition of thiamine (Fig. 4.13).

Another group mutated the same lysine residue to three amino acids, all of which produced different phenotypes when expressed from the endogenous *CDC6* promoter (Weinreich et al., 1999). A substitution to arginine, which changed the configuration of the positive charge, resulted in a functional protein as there is no effect on cell growth and S phase progression. However, a mutation to the neutral amino acid alanine results in a temperature sensitive phenotype and a substitution to glutamate, a negatively charged amino acid, is lethal. This stresses the importance of the exact amino acid substitution made when interpreting experiments performed with mutated proteins. Interestingly, the phenotype of the Cdc6-K114Ep mutant in budding yeast is somewhat different to that obtained with the same mutation in fission yeast cdcl18p (Chapter 4). After depletion of wildtype Cdc6p, cells producing Cdc6-K114Ep accumulate with a 1C DNA content as they do not initiate S phase. Cells then arrest and do not undergo an aberrant mitosis as would occur in the complete absence of Cdc6p or after depletion of the wildtype cdcl18 protein in the 81-cdc18 S/O-WA strain (Fig. 4.7). When the N-terminus of the Cdc6-K114E mutant protein is deleted, cells also accumulate with a 1C DNA content but then undergo mitosis in the absence of DNA replication. The N-
terminus of Cdc6p is therefore required to inhibit premature entry into mitosis. It has been shown that the Cdc6-K114Ep is capable of binding to chromatin but is unable to load MCMs (Weinreich et al., 1999). I must therefore assume that the presence of the Cdc6-K114Ep on chromatin, perhaps with other initiation proteins but without the MCM complex, is sufficient to initiate a checkpoint signal and consequently that the ability to bind chromatin is located in the N-terminus of the protein. Given this, it is important to know whether the cdcl8 Walker A mutant is able to bind to chromatin in fission yeast. Both outcomes would be informative and tell us something about how the two yeasts differ. If the cdcl8-WA-3HAp does bind to chromatin it indicates that the checkpoint signal is initiated differently in budding and fission yeast. However, if the protein is not bound it indicates that the mechanism for chromatin binding is different in the two organisms. It is somewhat surprising that the N-terminus is required to send the checkpoint signal in budding yeast as my work (Chapter 2) demonstrated that the C-terminus alone was sufficient to induce both replication and the checkpoint to prevent mitosis (Fig. 2.2, 2.7 and 2.11).

The lysine residue of the Walker A motif of budding yeast Cdc6p was mutated to five different amino acids by another group (Wang et al., 1999). Again, a range of phenotypes were obtained depending on the precise mutation made. Lysine mutated to leucine (a neutral amino acid), glutamine (resulting in the substitution of an amide) or arginine (a positively charged amino acid) produced a functional protein that was capable of complementing the cdc6-l mutation at the restrictive temperature. However a lysine to proline (causing a general disruption) or glutamate (a negatively charged amino acid) mutation resulted in a protein that was unable to complement the cdc6-l mutation at the restrictive temperature. Further analysis was performed on the Cdc6-K114Ep as it appeared to have the most severe phenotype. It was found that the mutant was unable to stably bind chromatin and effectively load Mcm5p. The defect was therefore due to an inability to form functional pre-replicative complexes. This result is somewhat different to results obtained by Weinreich et al. who found that Cdc6-K114Ep bound to origins with the same kinetics as the wildtype protein. Wang et al. claim that the mutant is able to bind to chromatin but not as stably as wildtype Cdc6p so the results are not totally inconsistent.
It was also shown that Cdc6p could interact with Orc1p in vitro and in vivo (Wang et al., 1999) and that the in vivo interaction was compromised when the Cdc6-K114Ep was expressed. This could explain why the mutant protein appears to have a problem binding to chromatin. Recent data from experiments using purified budding yeast proteins in an in vitro assay confirms the Orc1p/Cdc6p interaction but states that the presence of functional ARS DNA is required for the interaction (Mizushima et al., 2000). The apparent instability of the Orc1p/Cdc6-K114Ep interaction could therefore be attributed to the lack of chromatin in an immunoprecipitation. The interaction may not be detected as there is no ARS DNA present to stabilize the weak interaction.

Experiments with baculovirus purified human Cdc6 proteins has led to a better characterization of ATP binding and hydrolysis (Herbig et al., 1999). A lysine to alanine mutation in the Walker A motif, K208A, produces a protein that has a much reduced ATP binding activity and barely detectable ATPase activity. It was previously shown that human Cdc6p was able to bind to Orc1p (Saha et al., 1998) and Herbig et al. confirm this result, adding to it by reporting that the Cdc6-K208A protein is still able to bind to Orc1p. Microinjection of the Cdc6-K208A mutant protein into HeLa-S3 cells blocks DNA synthesis indicating that the mutant is defective in S phase entry.

A study of the Walker A and B motifs of cdc18p has previously been performed in Schizosaccharomyces pombe (DeRyckere et al., 1999). They state that a lysine to alanine mutation of the Walker A motif displays a mixed phenotype when the wildtype protein is depleted. Some cells block with a DNA content of 1C and <1C, as is found with our experiments (Fig. 4.7 B), however, a proportion of the cells also block with a 2C DNA content. The cell cycle block point has been shown to be dependent on the cell cycle stage at which cells are depleted of wildtype cdc18p. If cells are blocked at the beginning of S phase with HU and are then released from the block after wildtype cdc18p has been depleted, cells will arrest with a 1C and <1C DNA content. However, if cells are blocked in G2 using a cdc25-22 temperature sensitive mutant and released in the absence of wildtype cdc18p, cells will block after the next S phase with a 2C DNA content. As this result is different
both from mine and from those obtained in other organisms, I presume that this specific mutation results in a partially functional protein. The specific amino acid substitution made has been shown to be critical for whether the protein is functional (Wang et al., 1999; Weinreich et al., 1999) and a K→A mutation in the Walker A motif of budding yeast Cdc6p has been shown to produce a temperature sensitive mutant (Weinreich et al., 1999) providing further evidence that the mutant protein could be partially active.

To summarise, cells mutated in the Walker A motif of Cdc6/18p are defective in initiating DNA replication. This occurs in both budding and fission yeast and in human cells. My work also indicates that cells are unable to initiate a checkpoint signal (Fig. 4.7). This result is different to that obtained in budding yeast which states that the same mutant is able to maintain a checkpoint in the absence of replication (Weinreich et al., 1999). This could be due to a difference in the ability to bind the ORC complex and chromatin. Alternatively it could be related to the different nature of the checkpoint signal. In fission yeast, the ability to initiate replication is not generally separated from the ability to initiate a checkpoint. A lack of replication therefore tends to be coupled to a deficient checkpoint and an aberrant mitosis. However, in budding yeast this may not always be the case as destruction of members of the MCM complex prior to S phase using the heat-inducible Degron causes cells to arrest with a 1C DNA content without entering mitosis (Labib et al., 2000). This result provides us with an example where the replication and checkpoint functions are separated. Another difference between the S phase checkpoint in the two yeasts is the checkpoint target. The checkpoint signal results in the inhibitory phosphorylation of the tyrosine-15 residue of cdc2p in fission yeast. The situation is a little more complicated in budding yeast and there are thought to be two pathways, a Mec1p/Rad53p pathway that operates in early S phase and a Mec1p/Pds1p pathway that operates later, both pathways ultimately act to inhibit spindle elongation (reviewed in Clarke and Giminez-Abian, 2000).

A mutation in the Walker B motif of Saccharomyces cerevisiae CDC6 was first isolated as a dominant negative mutant when overexpressed. It was found to be a glutamate to glycine (E→G) substitution of the DExD motif. The Cdc6-E224Gp
does not complement the \textit{cdc6-1} mutant when expressed at the restrictive temperature and cells block in S phase with a 1C-2C DNA content (Perkins and Diffley, 1998). These results are similar to those obtained by myself (Fig. 4.8) with the same amino acid substitution. A DE$\rightarrow$AA mutation of the Walker B motif of fission yeast \textit{cdc18p} also produced cells which arrested with a 1C-2C DNA content after depletion of the wildtype protein (DeRyckere et al., 1999).

Further characterization of the Cdc6-E224Gp mutant showed that it allowed formation of a G1 specific footprint by genomic footprinting but that this was not the same footprint as formed by the pre-replicative complex. Chromatin assays showed that this was due to a lack of MCMs bound to origins and explains why the mutant protein was unable to complement the \textit{cdc6-1} mutant at 37°C (Perkins and Diffley, 1998). I presume that binding of the Cdc6-E224Gp mutant to chromatin allows binding of other proteins as Cdc6p binding alone has been shown not to affect the pattern of DNaseI protection at ARS1 (Mizushima et al., 2000). This is interesting as the Cdc6-K114E mutant protein has been shown to bind DNA (Weinreich et al., 1999) but not generate a G1 specific footprint (Perkins and Diffley, 1998) implying that it is unable to recruit other proteins to origins. However, it is still capable of sending a checkpoint signal (Weinreich et al., 1999) indicating that the presence of Cdc6p on chromatin could be the sole requirement for initiation of a signal to block mitosis.

Experiments using human baculovirus purified proteins demonstrated that an E$\rightarrow$Q substitution in the Walker B box of Cdc6p had no effect on the ATP binding ability of the protein or the ability to interact with Orc1p but resulted in a clear decrease in the ATPase activity (Herbig et al., 1999). Microinjection of the Cdc6-E285Q mutant protein into HeLa-S3 cells led to a reduction of DNA synthesis indicating that S phase progression must be either slowed or halted.

ATP hydrolysis appears to be required for normal S phase progression. Without it some replication can occur but it is not complete. The indication is that replication is either occurring at a slower rate or at only a subset of origins. My data would support this hypothesis as a HU block and release experiment with the \textit{cdc18-WB-3HA} protein demonstrated that DNA replication initiation was delayed and S
phase progression appeared slow compared to that induced by the wildtype cdcl8 protein (Fig. 4.15). The obvious possibility for the limiting factor would be loading of the MCM complex onto chromatin. As some DNA replication occurs when the Walker B mutants are expressed in the absence of wildtype protein function I would presume that some MCM proteins must be bound to chromatin as a recent paper shows that cells lacking a single member of the MCM complex fail to initiate DNA replication (Labib et al., 2000). I therefore hypothesise that the MCM proteins are bound to DNA when the Cdc6-E224G protein is expressed in budding yeast but it is below detectable levels when assessed using a chromatin assay (Perkins and Diffley, 1998).

The mechanism for cdcl8p dependent loading of the MCM complex onto chromatin is unknown. I have been unable to detect an interaction between cdc21p (mcm4p) and cdcl8p (Fig. 4.16 and 4.17) and others have also tried unsuccessfully to immunoprecipitate cdcl8p with members of the MCM complex (Sherman et al., 1998). This suggests that cdcl8p does not load the MCM proteins by binding to the complex and bringing it to chromatin. However, a recent report has suggested a possible mechanism for how this role is performed. An in vitro system was developed to look at the origin specific interaction between Cdc6p and the ORC complex in Saccharomyces cerevisiae (Mizushima et al., 2000). Baculovirus purified ORC proteins (Bell et al., 1995) and Escherichia coli purified Cdc6p were shown to interact but require a functional ARS to do so, as mutations in the A and B1 elements of ARS1 abolishes the interaction. Two Cdc6p mutants were also purified, both of which have previously been characterized (Weinreich et al., 1999). The Walker A mutant, Cdc6-K114Ep, was described earlier and the Walker B mutant, Cdc6-DE223-224AAP, produced a completely functional protein that had little effect on cell growth allowing the authors to conclude that an intact Walker B motif is not essential for Cdc6p activity. The fact that this mutation produces a functional protein is not too surprising as it is known that the specific mutation made can have a great effect on the ability of the protein to rescue depletion of the wildtype protein. However it is worth remembering that the same mutation in Schizosaccharomyces pombe was defective as cells arrested with a 1C-2C DNA content (DeRyckere et al., 1999). Despite the fact that that the earlier report stated that the Walker B mutant was functional in vivo and the Walker A mutant non-
functional (Weinreich et al., 1999), the two Cdc6 mutant proteins were shown to act identically to each other and differently to the wildtype protein in the latter report (Mizushima et al., 2000). This throws doubt on the validity of the results obtained using this assay but still provides us with an interesting hypothesis worth further analysis.

Mizushima et al. propose that the ATPase activity of Cdc6p is required to prevent non-specific binding of ORC to chromatin. Lack of this activity due to either expression of a mutant protein or presence of a non-hydrolyzable analogue of ATP (ATP-γ-S) allows ORC to bind along the chromatin. This non-specific chromatin binding is not due to an inability of the Cdc6 mutant protein to bind ORC as these interact as strongly with ORC as the wildtype Cdc6 protein. Partial protease digestion indicates that wildtype Cdc6p but not Cdc6-K114Ep causes a conformational change in ORC that increases the binding specificity of ORC to DNA. Cdc6p would therefore specify binding of ORC to functional origins by inducing a conformational change which may then allow MCMs to bind to chromatin. This model provides a mechanism for how Cdc6p can allow loading of the MCM complex without a direct interaction. However, the problem of whether Cdc6-DE223-224AAP acts like the wildtype protein or the Walker A mutant and issues relating to how ORC proteins are specified to origins of both sister chromatids after replication when Cdc6p has been degraded need to be resolved.

The mutational analysis of the Walker A and Walker B motifs of both fission yeast cdc18p, and budding yeast and human Cdc6p has provided some insight into the mechanism that allows initiation of DNA replication. However, it also provides some insight into the checkpoint role of Cdc6/18p, as has been mentioned previously. In fission yeast, DNA replication and the S phase checkpoint are not generally separated. If replication is inhibited, the checkpoint signal is not sent and cells enter an aberrant mitosis. The Walker A mutant is no exception as expression of cdc18-WA-3HAp in the absence of wildtype cdc18p results in cells that are unable to initiate DNA replication or the S phase checkpoint and so enter an aberrant mitosis (Fig. 4.7). However, this is not the case in budding yeast. The Cdc6-K114Ep does not initiate DNA replication but the S phase checkpoint is activated so cells arrest with a 1C DNA content (Weinreich et al., 1999). This
reflects a fundamental difference between the two yeasts and possible explanations for this were discussed earlier in the chapter. Interestingly the intra-S phase checkpoint which is required to prevent late origins from firing when the progression of replication forks from early origins has been blocked by HU, is thought to involve a Mec1p/Rad53p pathway which inhibits late origin firing via a signal initiated by the stalled replication forks (Santocanale and Diffley, 1998). This is slightly at odds with the idea that it is a Cdc6p containing complex that initiates a checkpoint signal and not the presence of replication intermediates. This suggests that a diverse range of events can send the S phase checkpoint signal.

The phenotype of the cdcl8-WB-3HAp mutant has been discussed in some detail in Chapter 4. The protein is capable of initiating replication albeit with some delay (Fig. 4.15). S phase progression could also be slow and these phenotypes are not dissimilar from those found in the budding yeast and human Cdc6p Walker B motif mutants (Herbig et al., 1999; Perkins and Diffley, 1998). One thing that is currently unclear is whether the Walker B mutant is capable of initiating a checkpoint signal. Cells accumulate abnormal nuclei with a few hours delay compared to the null and Walker A mutants (Fig. 4.13 B) but at a much earlier stage than the cdcl8-K46 mutant at the restrictive temperature (Fig. 4.14 D). There is therefore an indication that the cells are capable of undergoing an initial cell cycle arrest which can not be maintained allowing cells to enter an aberrant mitosis. As cells arrest with a 2C DNA content it would be surprising if they were unable to initiate a checkpoint. This could indicate a direct link between cdcl8p function and the checkpoint (discussed in Chapter 4).

Another possibility is that the cells progress slowly through the initial events in mitosis without having properly left S phase and G2. This is the case with the cdcl3-117 mutant when shifted to the restrictive temperature as it arrests with a granular nuclear structure, often with three condensed chromosomes. Many cells are septated but the septa are aberrant and irregular in structure. However the cells possess cytoplasmic microtubules rather than short mitotic spindles which enables them to continue growing (Hagan et al., 1988). The cdc2p/cdcl3p kinase is sufficiently active to bring about some but not all aspects of mitosis. This is similar to the phenotype observed when thiamine is added to the 81-cdc18 S/O-WB strain.
and could account for why the cells grow so long despite appearing to enter mitosis. It could also explain why there appears to be a time lag when comparing the accumulation of abnormal nuclei with the increase in septation index (Fig. 4.14 C and D) as the cdc18-WB-3HAp is only able to partially activate a checkpoint so some events of mitosis proceed but others are inhibited.

Alternatively, it is possible that a critical number of active replication initiation complexes are required to establish the checkpoint signal. This hypothesis could certainly fit with the proposed defect of the cdc18-WB-3HAp which may initiate replication from only a subset of origins and could account for the three phenotypes, delay in initiation, slow progression through S phase and lack of checkpoint function.

Summary

Over the last four years much progress has been made in the DNA replication field, particularly in relation to the role played by cdc18p. Homologues in higher eukaryotes, such as *Xenopus* and humans, have been identified and a clear functional role has been defined for cdc18p, which appears to be common to all homologues. The work presented in this thesis has contributed to our current knowledge of cdc18p, particularly in relation to the checkpoint function (Chapter 2). I have performed a comparative study of the *cdc18* homologues to assess whether they can complement loss of *cdc18* function in fission yeast (Chapter 3) and have discussed the differences between results obtained by myself and other investigators. Finally, I have produced an experimental system that uses mutated copies of *cdc18* to improve understanding of cdc18p regulation and function (Chapter 4). With some modification, this system could be used in the future to produce a detailed stepwise analysis of cdc18p function in the formation of the pre-replicative complex and generation of the S phase checkpoint signal.
Chapter 6

Materials and Methods

6.1 Fission yeast physiology and genetics

6.1.1 Nomenclature
The genetic nomenclature of *Schizosaccharomyces pombe* is reviewed in (Kohli and Nurse, 1995). Fission yeast gene names consist of three italicised lower-case letters and a number, e.g. *cdc18*. Mutant alleles of genes are denoted by a hyphen and a number, or a combination of letters and numbers, e.g. *cdc18-K46*. Alleles conferring temperature sensitivity can be marked with a superscript, e.g. *cdc18-K46^*. The wild type allele can be designated with a superscript plus when the context requires specification of the wild type allele. Phenotypes are denoted by a non-italicised three-letter abbreviation corresponding to a gene name e.g. *cdc*. Gene deletions are indicated by the gene name followed by a “Δ”, e.g. *cdc18Δ*. Integrated DNA is indicated with a “::”. A gene deletion marked with an integrated auxotrophic marker is indicated with a “Δ::marker”, e.g. *mik1Δ::ura4^*. Gene products are denoted by the lower-case, non-italicised gene name followed by “p” for protein, e.g. *cdc18p*. Truncated and mutated gene products carry a non-italicised tag denoting the alteration, for example *cdc18-150-577p* and *cdc18-1-577 (NTP)p*. Due to the complicated nature of certain strains and plasmids, a shorthand version may be used. These will be explained fully, both in the strain list and plasmid explanation and in the results chapters as they are first described.

*Saccharomyces cerevisiae* genes are designated with three italicised upper-case letters e.g. *CDC6*. Recessive mutants alleles are in lower case. Budding yeast gene products are denoted by the non-italicised gene name with the first letter in upper case and a “p” at the end, e.g. *Cdc6p*. The nomenclature used for mammalian genes is identical to that used for budding yeast. Due to the use of homologous
genes of the same name in Chapter 3, the gene name will be prefixed with the initials of the organism, e.g. the *Saccharomyces cerevisiae* gene CDC6 will be called ScCDC6.

### 6.1.2 Strain growth and maintenance

Techniques used to grow and maintain fission yeast strains, to store and revive frozen cultures, to check phenotypes and to perform and analyse crosses by tetrad dissection or random spore analysis were performed as previously described (MacNeill and Fantes, 1993; Moreno and Nurse, 1994) and will not be further described.

#### Table 6.1

**Fission yeast strains previously constructed**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Strain location</th>
</tr>
</thead>
<tbody>
<tr>
<td>h* leu1-32</td>
<td>Our collection</td>
</tr>
<tr>
<td>h* leu1-32 ura4-D18</td>
<td>Our collection</td>
</tr>
<tr>
<td>h* leu1-32 ura4-D18 cdc18-K46</td>
<td>Our collection</td>
</tr>
<tr>
<td>h* leu1-32 ura4-D18 rad1-1</td>
<td>T. Carr</td>
</tr>
<tr>
<td>h* leu1-32 rad9-192</td>
<td>T. Carr</td>
</tr>
<tr>
<td>h* leu1-32 rad3-136</td>
<td>Our collection</td>
</tr>
<tr>
<td>h* leu1-32 rad17-h21</td>
<td>Our collection</td>
</tr>
<tr>
<td>h* leu1-32 hus1-14</td>
<td>Our collection</td>
</tr>
<tr>
<td>h* leu1-32 ade6-M210 cdc2-3w</td>
<td>Our collection</td>
</tr>
<tr>
<td>h* leu1-32 cut5-580</td>
<td>Our collection</td>
</tr>
<tr>
<td>h* leu1-32 mik1A::ura4+ wee1-50</td>
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<tr>
<td>h50 leu1-32 ade6-M210 rum1A:: ura4+ ura4-D18</td>
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</tr>
<tr>
<td>h* leu1-32 cdc2-3w cdc25A:: ura4+ ura4-D18</td>
<td>Our collection</td>
</tr>
<tr>
<td>h*/h* leu1-32/leu1-32 ade6-M210/ade6-M216</td>
<td>Our collection</td>
</tr>
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### Table 6.2

**Fission yeast strains newly constructed**

<table>
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<tr>
<th>Genotype</th>
<th>Strain shorthand</th>
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<tbody>
<tr>
<td>h' cdc18:: nmt81-cdc18 leu1-32 ade6-M210</td>
<td>81-cdc18 S/O</td>
</tr>
<tr>
<td>h' cdc18:: nmt81-cdc18 leu1-32 pJK148 ade6-M210</td>
<td>81-cdc18 S/O pJK148</td>
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<tr>
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<td>81-cdc18 S/O pJK148-gcdc18</td>
</tr>
<tr>
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<td>81-cdc18 S/O pIRT2-gcdc18</td>
</tr>
<tr>
<td>h' cdc18:: nmt81-cdc18 leu1-32:: pJK148 ade6-M210</td>
<td>81-cdc18 S/O-JK</td>
</tr>
<tr>
<td>h' cdc18:: nmt81-cdc18 leu1-32:: pJK148-gcdc18-3HA ade6-M210</td>
<td>81-cdc18 S/O-wt</td>
</tr>
<tr>
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<td>81-cdc18 S/O-WA</td>
</tr>
<tr>
<td>h' cdc18:: nmt81-cdc18 leu1-32:: pJK148-gcdc18-3HA-WB ade6-M210</td>
<td>81-cdc18 S/O-WB</td>
</tr>
<tr>
<td>h' cdc18:: nmt81-cdc18 leu1-32:: pJK148-gcdc18-3HA-N1 ade6-M210</td>
<td>81-cdc18 S/O-N1</td>
</tr>
<tr>
<td>h' cdc18:: nmt81-cdc18 leu1-32:: pJK148-gcdc18-3HA-N2 ade6-M210</td>
<td>81-cdc18 S/O-N2</td>
</tr>
<tr>
<td>h' cdc18:: nmt81-cdc18 leu1-32:: pJK148-gcdc18-3HA-N2 ade6-M210</td>
<td>81-cdc18 S/O-N1+N2</td>
</tr>
<tr>
<td>h' cdc18:: nmt81-cdc18 leu1-32:: pJK148-gcdc18-3HA-P1-6 ade6-M210</td>
<td>81-cdc18 S/O-P1-6</td>
</tr>
</tbody>
</table>

#### 6.1.3 Strain construction

The 81-cdc18 S/O strain was made by using a homologous integration technique (Bahler et al., 1998) to replace the endogenous promoter with that of the nmt81 promoter. PCR was used to amplify a cassette containing the kanamycin resistance gene and the nmt81 promoter flanked by sequence homologous to cdc18 promoter. The PCR product was then transformed into the h'/h' leu1-32/leu1-32 ade6-M210/ade6-M216 diploid strain. Presence of the cassette was selected for by resistance to G418 and was confirmed by colony PCR. The diploid was then
sporulated and tetrads were pulled to confirm a 2:2 segregation. The other strains were made by transforming 81-cdc18 S/O with other plasmids, as described below.

6.1.4 Transformation and integration of plasmids

Cells were transformed with plasmid DNA by either electroporation (using a Flowgen "Cellject" at 1500V, 40μF, 132Ω, as described previously (Prentice, 1992)), the lithium acetate method (Moreno et al., 1991; Okazaki et al., 1990) a modified lithium acetate method (Bahler et al., 1998) or by protoplasting (Moreno et al., 1991). Plasmids are maintained by providing a nutritional marker such as LEU2 or ura4, which is missing in the auxotrophic strain used for transformation.

Integrants were made using the pJK148 vector that does not contain an ars and is thought to integrate preferentially at the leu1 locus. Southern blotting was used to check integration.

6.1.5 Induction and repression of gene expression from the nmt promoter

The nmt promoter is derived from the nmt1* gene which is required for thiamine biosynthesis and is repressed in the presence of thiamine (Maundrell, 1990). Mutations in the TATA box of the promoter gave rise to modified versions, which allowed three different expression levels (Basi et al., 1993; Forsburg, 1993). The Rep3X vector contains the wildtype, strong nmt1 promoter. Rep41 and Rep42 drive an intermediate level of expression and the Rep81 and Rep82 vectors are used for the lowest expression levels. To induce expression from the nmt promoter, cultures were grown exponentially in minimal medium containing thiamine (5μg/ml) before being washed three times with medium lacking thiamine and subsequently grown in the absence of thiamine throughout the timecourse. To repress expression from the nmt promoter, thiamine was added back to the culture growing exponentially in minimal medium.

6.1.6 Physiological experiments with Hydroxyurea

Hydroxyurea (HU) is a drug that acts to inhibit the enzyme ribonucleotide reductase, and hence results in a depletion of nucleotides and cell cycle arrest in early S phase. Addition of HU, at a concentration of 11mM, to an exponentially growing population of cells can therefore be used to synchronise a cell culture.
Cells were released from the arrest after 3.5 hours at 32°C by washing three times in minimal medium. The culture then proceeds relatively synchronously through the next cell cycle.

6.1.7 Flow cytometric analysis
Between $2 \times 10^6$ and $2 \times 10^7$ cells were fixed in 70% ethanol. Samples can be stored at 4°C indefinitely. Cells were washed in 3ml 50mM Na$_3$Citrate before being resuspended in 0.5ml 50mM Na$_3$Citrate, 0.1mg RNase and incubated at 37°C for 2 hours. 0.5ml Na$_3$Citrate, 2μg/ml propidium iodide was then added and cells were sonicated for 45 seconds at setting 6 (Soniprep 150 sonicator, MSE). Analysis was carried out with a Becton Dickinson FACScan as previously described (Sazer and Sherwood, 1990).

6.1.8 Cell number determination
Cell number was determined by either counting using a haemocytometer, or with a Coulter Counter. The haemocytometer is a specialised microscope slide with an engraved grid. Once a special coverslip is placed on top of the slide it creates a region of known volume (0.1mm$^3$). A few microlitres of culture can then be pipetted under the coverslip and the number of cells in the grid counted. This number multiplied by 10000 gives the number of cells/ml.

For more accurate cell counting the Coulter counter was used. Cells were fixed by adding 0.8ml formol saline (0.9% saline, 3.7% formaldehyde) to 0.2ml cell culture. Cells can be stored at 4 °C for several weeks. Before processing for cell number, 19ml of ISOTON solution was added and cells were sonicated as above. Cell number was counted using a Coulter Counter.

6.2 Molecular Biology Techniques

6.2.1 General techniques
The following techniques were performed essentially as described (Sambrook et al., 1989): preparation of competent bacteria, transformation of bacteria with DNA, restriction enzyme digest of DNA, gel electrophoresis of DNA, phosphatase
treatment of vector DNA to remove 5′ terminal phosphate groups and ligation of DNA fragments. Both small (miniprep) and large (maxiprep) scale preparation of DNA from bacteria was done using Qiagen columns (Qiagen).

6.2.2 DNA preparation
5x10⁸ cells were broken using glass bead lysis, in 2% triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-HCl (pH8.0), 1mM EDTA in the presence of 1:1 v/v phenol:chloroform:isoamyl alcohol (GIBCO-BRL). DNA was precipitated by the addition of NH₄OAc to 0.3M and 2.5 volumes of ethanol.

6.2.3 Southern blotting
2µg DNA was digested for 3 hours before being run on a 0.8% agarose gel. The DNA was transferred to GeneScreen Plus (NEN) hybridisation membrane using a PosiBlot 30-30 Pressure Blotter (Stratagene). The DNA was cross-linked to the membrane using an UV Stratalinker (Stratagene). The membrane was pre-hybridised for 15 minutes at 68°C in QuikHyb (Stratagene) before the probe was added and hybridisation continued for 1 hour. The membrane was washed three times in 0.1X SSC 0.1% SDS at 65°C before being exposed to Fuji autoradiography film.

6.2.4 DNA probes
Probes for Southern blotting were prepared by random oligo priming with α(³²P) dATP using a Prime It II kit (Stratagene). Probes used were a cdc18 fragment from Rep3X-cdc18 (SalI/BamH1 digest) and a leu1 fragment from pJK148 (XhoI/Cla1 digest).

6.2.5 Construction of plasmids
The first five plasmids constructed were based on Rep3X-cdc18 (Nishitani & Nurse, 1995). To construct plasmid cdc18-1-141, a TAG stop codon and a BamH1 site were introduced after the codon for amino acid 141. The fragment was cut out using a SalI/BamH1 digest and was cloned into Rep3X. Plasmid cdc18-150-577 was constructed by introducing a SalI site and ATG start codon before the codon for amino acid 150 followed by a SalI/BamH1 digest and ligation into Rep3X. Plasmid cdc18-150-577 (T374A) was constructed by introducing a mutation in
cdc18-150-577 at amino acid 374 using a BioRad mutagenesis kit. ACT was mutated to GCG thus changing the amino acid from T to A. Plasmid cdc18-NTP was constructed by mutating amino acids 204 and 205 from GGA AAG to GCG GCC, thus altering the codons from GK to AA, using a BioRad mutagenesis kit. Plasmid cdc18-150-577-NTP was based on plasmid cdc18-NTP and the cloning was performed as for plasmid cdc18-150-577. Plasmids HA cdc18-1-141 and HA cdc18-150-577 were based on pRHA41, a gift from T. Carr. The fragments were cloned downstream of the Rep41 promoter and HA tag using a Sal1/BamH1 digest.

HsCdc6 was subcloned from pBSKS⁺ (a gift from A. Dutta) to pUC118 using a BamH1/Kpn1 digest. It was then cloned into both pRep41 and pRep81 on a Sal1 digest. A HinDIII digest confirmed orientation. XlCdc6 was subcloned from pVL1393 (a gift from T. Coleman) to pLitmus38 on an EcoR1/EcoRV digest. It was then cloned in to both pRep41 and pRep81 using a Nde1/Sal1 digest. ScCDC6 was subcloned from pLD3 (a gift from J. Diffley) to pRep41 and pRep81 using a BamH1 digest. Orientation was confirmed using a Pst1 digest.

The 5.2kb genomic cdc18 fragment (gcdc18) was subcloned from pIRT2-gcdc18 to pJK148 using a Sac1/Sal1 digest. pJK148-gcdc18 was tagged with 3HA by introducing a Not1 site in place of the cdc18 STOP codon using the QuikChange site-directed mutagenesis kit (Stratagene). The 3HA tag was then cloned from pTZ18R-3HAram1 using a Not1 digest. Orientation was confirmed by sequencing the area.

pJK148-gcdc18-3HA-WA (gcdc18-WA), pJK148-gcdc18-3HA-WB (gcdc18-WB), pJK148-gcdc18-3HA-N1 (gcdc18-N1), pJK148-gcdc18-3HA-N2 (gcdc18-N2), pJK148-gcdc18-3HA-N1+N2 (gcdc18-N1+N2) and pJK148-gcdc18-3HA-P1-6 (gcdc18-P1-6) were all made using the QuikChange site-directed mutagenesis kit (Stratagene). All constructs were sequenced over the entire gene to check that the mutation was correct and that no additional mutations had been acquired. WA-codon 205 was mutated from AAG to GAG thus changing the amino acid from K to E. WB-codon 287 was mutated from GAA to GGA thus changing the amino acid from E to G.
N1-codons 49-51 were mutated from AGA AAA CGT to GCA GCA GCT thus changing the amino acids from RKR to AAA.
N2-codons 91 and 92 were mutated from AAG AAA to GCG GCA thus changing the amino acids from KK to AA.
P1-codon 10 was mutated from ACA to GCA thus changing the amino acid from T to A.
P2-codon 46 was mutated from ACA to GCA thus changing the amino acid from T to A.
P3-codon 60 was mutated from ACA to GCA thus changing the amino acid from T to A.
P4-codon 104 was mutated from ACC to GCC thus changing the amino acid from T to A.
P5-codon 134 was mutated from ACA to GCA thus changing the amino acid from T to A.
P6-codon 374 was mutated from ACT to GCT thus changing the amino acid from T to A.

6.2.6 DNA sequencing
Sequence analysis was carried out using the dideoxynucleotide method (Sanger et al., 1977). The sequencing reaction was performed on either 50 ng of purified PCR product or 0.4 μg of double stranded DNA made using the Qiagen miniprep kit. 3.2 pmoles of each primer were used and the termination ready reaction mix (A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, dITP, dATP dCTP dTTP, Tris-HCl [pH 9.0], MgCl₂, thermal stable pyrophosphatase, and AmpliTaq DNA polymerase, FS). Samples were run on a 4.8% acrylamide gel and detected using an ABI Prism 377 DNA sequencer.

6.2.7 Cell Extract Preparation
Cell extracts were prepared in several different ways:

Figure 2.4 B
Total boiled extracts were prepared from 2×10⁸ cells. Cells were washed in STOP buffer (150 mM NaCl, 50 mM NaF, 10 mM EDTA, 1 mM NaN₃ [pH 8.0]), resuspended in 150 μl of RIPA buffer (10 mM NaPO₄, 2 mM EDTA, 150 mM NaCl,
Chapter 6 Materials and Methods

50mM NaF, 0.1mM sodium orthovanadate, 1mM PMSF, 4μg/ml leupeptin, 1% triton and 0.1% SDS) and boiled for 5 minutes. Cells were broken using glass beads then extracts were recovered by washing with 250μl of RIPA buffer. The protein concentration of the extracts was determined by the BCA assay kit (Sigma) after boiling 10 μl of extract for 5 minutes in 2x sample buffer (160mM Tris [pH 6.8], 20% glycerol and 4% SDS). The rest of the extract was added to 5x sample buffer (400mM Tris [pH 6.8], 50% glycerol, 10% SDS, 500mM DTT and 0.02% bromophenol blue) and boiled for 5 minutes.

**Figure 2.2, 2.3 and 2.6**

Soluble protein extracts were prepared as described previously (Moreno et al., 1991). Extracts were prepared from frozen or fresh cells, broken in 20 μl of HB buffer (60mM β-glycerophosphate, 25mM MOPS [pH 7.2], 0.1mM sodium orthovanadate, 15mM p-nitrophenylphosphate, 15mM MgCl2, 15mM EGTA, 1mM DTT, 1mM PMSF, 20μg/ml aprotinin, 20μg/ml leupeptin and 1% triton) with glass beads before being washed with another 500μl of HB buffer. The extract was recovered and protein determination was performed using 2μl of extract and the BCA assay kit. The rest of extract was boiled in sample buffer.

**Figure 2.4 A**

For suc1p bead precipitation, 1x10^9 cells were protoplasted and lysed with HB buffer as described by Grallert and Nurse (1996). Extracts were spun at 15 000rpm for 15 minutes and the supernatant was used for the precipitation.

**Figures 3.7, 4.1 and 4.5-4.11**

2x10^8 cells were washed in STOP buffer then resuspended in 150μl of HB buffer (60mM β-glycerophosphate, 25mM MOPS [pH 7.2], 15mM MgCl2, 15mM EGTA and 1% triton) before being boiled for 5 minutes. Cells were then either frozen at -70°C before extracts were made at a later time (Figure 4.1), or were immediately broken with 1ml of glass beads in the FastPrep cell breaker (Bio101). The extract was then recovered protein determination was performed and extract was boiled in sample buffer.

**Figures 4.16 and 4.17 D and E**

1x10^8 cells were washed once in STOP buffer before being resuspended in 150μl of HB buffer (60mM β-glycerophosphate, 25mM MOPS [pH 7.2], 0.1mM sodium orthovanadate, 15mM p-nitrophenylphosphate, 15mM MgCl2, 15mM EGTA, 1mM...
DTT, 1mM PMSF, 20µg/ml aprotinin, 20µg/ml leupeptin and 1% triton) and broken with 1ml of glass beads in the FastPrep cell breaker. The extract was then spun for 5 minutes at 13 000rpm. Protein determination was then performed.

6.2.8 Western Blotting

Protein extracts were run on SDS-polyacrylamide gels (Laemmli, 1970). The protein was then transferred to Immobilon membrane (Millipore) and detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody and an ECL kit (Amersham) or Super Signal (Pierce).

Table 6.2
Antibodies used for Western blotting and immunoprecipitation

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-cdc18p polyclonal (H. Nishitani)</td>
<td>1:2500 when overexpressed</td>
</tr>
<tr>
<td>anti-cdc18p polyclonal (H. Nishitani)</td>
<td>1:1000 wildtype expression</td>
</tr>
<tr>
<td>anti-C-terminus cdc18p polyclonal (H. Nishitani)</td>
<td>1:2500</td>
</tr>
<tr>
<td>anti-rum1p polyclonal (J. Correa-Bordes)</td>
<td>1:500</td>
</tr>
<tr>
<td>anti-cdc2p PSTAIR antibody</td>
<td>1:5000</td>
</tr>
<tr>
<td>anti-HA 12CA5 monoclonal (ICRF)</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-HA 16B12 monoclonal (Babco)</td>
<td>5µl/IP</td>
</tr>
<tr>
<td>anti-cdc21p polyclonal (Z. Lygerou)</td>
<td>1:1000</td>
</tr>
<tr>
<td>anti-α-tubulin monoclonal (Sigma)</td>
<td>1:10000</td>
</tr>
<tr>
<td>anti-rabbit-HRP (Amersham)</td>
<td>1:2500</td>
</tr>
<tr>
<td>anti-mouse-HRP (Amersham)</td>
<td>1:2500</td>
</tr>
</tbody>
</table>

6.2.9 Suc1p bead precipitation

Extracts were prepared as described above. 10µl of suc1p beads or BSA control beads were added to the extracts and rotated at 4°C for 1 hour. The precipitated complexes were washed 3 times in HB buffer and were boiled in 5x sample buffer for 5 minutes at 100°C. Western blots were performed as described above, and probed with anti-HA 12CA5 antibody and anti-cdc2p PSTAIR antibody.
6.2.10 Immunoprecipitations

Immunoprecipitations were performed on 5mg of soluble protein in a volume of 0.5ml. Either no antibody or 5μl of antibody (16B12 or cdc21) was added to the extracts and they were rotated at 4°C for 1 hour. 60μl of 1:1 bead suspension in HB buffer (Protein G beads-16B12 or Protein A beads-cdc21) was then added to the extracts and they were rotated for a further hour at 4°C. The beads were then washed 3 times in HB buffer and were boiled in 2x sample buffer for 5 minutes at 100°C.

6.2.11 Histone kinase assays

1μl of rabbit polyclonal anti-cdc13p antibody SP4 was added to 400μg of protein and incubated on ice for 1 hour. The immunocomplexes were precipitated with protein A-Sepharose beads (Pharmacia) for 30 minutes at 4°C before being washed 3 times in HB buffer. The beads were then resuspended in 20 μl of reaction buffer containing 1mg/ml H1 histone, 200μM ATP and 40μCi/ml [γ-32P]ATP and were incubated at 30°C for 20 minutes. The reactions were stopped by the addition of 5μl of 5x sample buffer before boiling for 3 minutes at 100°C. Samples were run on a 12% SDS-polyacrylamide gel. Phosphorylated histone H1 was detected by autoradiography.

6.2.12 Visualisation of nuclei by DAPI staining

Cells were fixed in 70% ethanol and stored at 4°C. Cells were then rehydrated in water, heat fixed to a slide and mounted in 1μg/ml DAPI, 1mg/ml para-phenylenediamine, in 50% glycerol. Photographs were taken using the Hamamatsu camera.

6.2.13 Visualisation of septa by calcofluor staining

Ethanol fixed cells were rehydrated as described above. Cells were left to air dry on slides before being stained with calcofluor diluted 1000x in water. Cells were then scored for septation using the fluorescent microscope.
Bibliography


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