Control of S-phase transcription in fission yeast.

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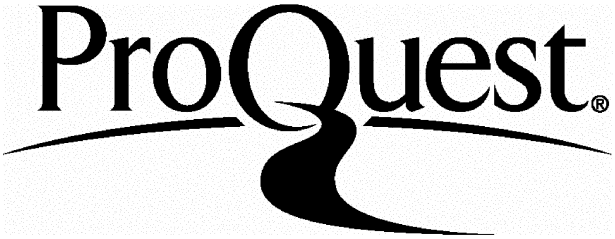

DEDICATION

I would like to dedicate this thesis to:

Zaida, who never let me win at chess.

My mother and father for giving me heterozygosity:
my mother who can find inspiration in chaos, who gave me Art, and my father who can distil order from his bright imaginings, who gave me Science.

Josh, for taking the opposite path, and for asking such difficult questions (can the cell cycle be made to go backwards?)
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ABSTRACT

In all eukaryotes the periodic transcription of S-phase genes is essential for DNA replication. In fission yeast, S-phase transcription is driven by a transcriptional complex, composed of cdc10p and res1p. This thesis investigates the control of this *cdc10* dependent S-phase transcription, in the fission yeast mitotic cell cycle.

Previously, S-phase transcription in budding and fission yeasts were ~~was~~ thought to be analogous, and activated late in G1 by cyclin dependent kinase (CDK) activity. However, it was found that *cdc10* dependent transcription, in contrast to its counterpart in budding yeast, is active early in G1 and independently of CDK activity. In fact, it is first activated in metaphase of the previous cell cycle, in cells with condensed chromatin and high levels of the mitotic kinase. This is surprising because in all eukaryotes, transcription in general is thought to be inhibited in mitotic chromatin.

The observation that S-phase transcription begins in mitosis also seems to contradict an important feature of cell cycle control, the mutually exclusive nature of M and S-phase processes. However, although *cdc18*, the critical target of *cdc10*, is expressed in mitosis, the gene product, cdc18p, is unstable in the presence of CDK activity, and cannot accumulate. On exit from mitosis, as the cdc2p kinase activity falls, cdc18p rapidly accumulates. In G1, cdc18p helps prepare cells for DNA replication. In this way, mitotic transcription of *cdc18* and regulated proteolysis, together, couple the exit from mitosis with the simultaneous setting up of the subsequent S-phase.

The re-assessment of the timing of *cdc10* dependent transcription also revealed that the presence of the cdc10p bandshift complex, DSC1, through the cell cycle, correlates with inactive transcription. Previously this complex was thought to represent the active transcriptional complex. Evidence was also found that res2p is both a component of this cdc10p complex, and required for the repression of S-phase transcription in G2 cells. It is therefore possible that the periodicity of S-phase transcription in fission yeast is brought about by both transcriptional activation and repression, mediated by alternating cdc10p complexes.

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CHAPTER 1

Introduction

The Origin of Cells

All living things are constructed from cells. Before the evolution of cells, it is likely that our ancestors were single self-replicating "aperiodic crystals" scavenging from the primordial soup. These were perhaps RNA like molecules which combined information and the ability to catalyse their own replication (Cech, 1987; Lahav, 1993; von Ahsen and Schroeder, 1993; James and Ellington, 1995). Modern day genes and genomes encode enzymes which carry out all the organisms life functions. However, if the information contained within a primitive replicator was used to design catalysts to aid its self-propagation, these accessory molecules are at risk of being lost by diffusion. This explains, in part, the importance of cells, the characteristic feature of which is their semi-permeable plasma membrane, which functions as a selective barrier separating the inside from the outside (Cavalier, 1987). In a cellular environment, the information contained within a replicator can be used to direct the synthesis of enzymes (Lohse and Szostak, 1996) which maintain and replicate the entire cell. Therefore, once cells had arisen during evolution, a transition could occur enabling the gradual separation of information content and biological activity. This allows information carrying molecules to take on a more limited function, as vessels of information, a role which is served better by DNA than RNA. (DNA being less chemically active and therefore less labile than RNA). As this occurs, all the information carrying molecules contained within a cell can begin to take on a group identity and purpose, each specialising in the control of a specific process. In this way, intrinsically "selfish genes" can be co-opted into the generation of greater and greater cellular complexity. It is only at this stage in evolution, with the development of sophisticated cells, that the distinction between life and chemistry becomes apparent.

The Cell Cycle

The series of events by which a cell replicates itself is called the cell cycle. In the simplest case, for one cell to become two, all of the cellular components

must be first duplicated and then segregated equally into two daughter cells. Simple cellular constituents are amassed during general growth and partitioned by cell division at the middle of the cell, which cuts the cell into two approximately equal parts. In contrast, the information carried in the DNA is present in only one or two copies per cell and must be duplicated and accurately segregated once per round of cell division, to ensure that each daughter cell receives its full genetic complement. This process can be regarded as the primary purpose of a cell. The duplication and segregation of other low copy cellular constituents, e.g. centrosomes, must also be precisely regulated. This is not easily achieved and requires an ordered cell division cycle. For this reason, the essence or logic of the cell cycle is the same for prokaryotes and eukaryotes, although the strategies used differ. In prokaryotes the problem is circumvented by linking the decision to initiate a round of replication with segregation (Jacob *et al*, 1963; Kornberg and Baker, 1992), using a single origin of replication that also acts as a segregation marker (reviewed in Wheeler and Shapiro, 1997). It has been speculated that this was also the case in the primordial eukaryote, where DNA replication and mitosis may have occured simultaneously, with the replication initiation complex also acting as a kinetochore (Wuarin, 1996).

The Eukaryotic Cell Cycle

Eukaryotes in contrast to prokaryotes, store their nuclear information in several linear chromosomes. If a cell is to produce viable progeny at division, each replicated chromosome must be separated from its sister molecule. This requires a process which can identify each pair of molecules at a single site, the centromere, and physically pull them apart, one into each daughter cell. This is elegantly achieved by the mitotic apparatus. The advent of this division machinery relaxes the constraints on the cell's genetic information load, by enabling the simultaneous segregation of multiple chromosomes and also by allowing the use of multiple origins of replication per DNA molecule. However, in order to ensure genomic stability through cell division a link must be established between the replication and separation of the genome.

A. Timers.

Eukaryotes use a cell cycle "clock" to order alternate rounds of discrete cell cycle events in time: DNA replication (S-phase) and segregation (M-phase or

mitosis), followed by cytokinesis. These events are often separated by gap phases G1 and G2, during which growth occurs, so that the whole cycle reads as G1, S, G2, M, before repeating.

B. Checkpoints.

In certain cases a cell cycle clock may be sufficient to order the cycle. However, if cell cycle progression is blocked in some way, additional mechanisms are required to stop the cell cycle clock. For this reason, eukaryotes have evolved "checkpoints" to improve the fidelity of the cell division cycle (Hartwell and Weinert, 1989); Elledge, 1996). Essentially, checkpoints monitor cell cycle events, e.g. S-phase and mitosis and delay the initiation of the next cell cycle event until the previous one is complete. Cells also use additional checkpoints to assess DNA damage (Enoch and Nurse, 1991; D'Urso and Nurse, 1995), mitotic spindle defects and attachment of the chromosomes to the mitotic apparatus (Murray, 1994). In the event of a problem, the checkpoint arrests the cell cycle until the defect is rectified. The cell cycle machinery incorporates both timers ("clocks") and checkpoint controls to ensure that the cell cycle is a high-fidelity process (Murray, 1992).

The strict order of the cell cycle is altered in certain circumstances. During meiosis cells undergo two consecutive rounds of division without an intervening S-phase (Yamamoto, 1997). In addition certain spore forming protozoa, like the malaria parasite and certain plants and algae can replicate by undergoing multiple rounds of DNA replication, followed by fission to completion (Goff and Coleman, 1990; Nagl *et al*, 1985). Also, in many higher eukaryotes, specific terminally differentiated cells undergo endoreduplication, whereby the DNA goes through multiple rounds of S-phase without nuclear division. For instance, *Drosophila* nurse cells increase their ploidy in this way, to enable them to dramatically increase their capacity for protein synthesis (Spradling, 1993). These examples serve to demonstrate that in the eukaryotic cycle, S-phase and M-phase are coupled together by molecular circuitry which is not intrinsic to the events themselves. This adds flexibility to the cell cycle, so that the order of S-phase and M-phase can be altered according to circumstance or by mutation. In fact, the sole function of the cell cycle machinery is to create a unidirectional molecular logic, which then orders the sequential events of the cell cycle.

Finally, the whole nuclear cycle is coordinated with cellular growth so that the cell is able to approximately double its less complex constituents between each round of division (Fantes, 1977; Johnston *et al*, 1977). In yeast, growth and entry into the cell cycle are limited primarily by nutrient availability. However, in multicellular organisms, cells are generally bathed in a nutrient rich environment. In this case, regulation of cell multiplication is imposed by ensuring that cell growth and proliferation are dependent on intercellular signals and the prior history of the cell (Norbury and Nurse, 1992). Both proliferative and anti-proliferative signals are transduced to the cell cycle machinery to coordinate cell behaviour. Genetic alterations in these signalling pathways can lead to autonomous cell proliferation and cancer (Sherr, 1996).

Much of the progress in cell cycle research has been driven by genetic studies in yeast, where genes controlling the cell cycle were isolated by identifying mutations that uncouple tightly linked cell cycle events (reviewed in Nurse, 1990). Initially, conditional mutations were found that uncoupled growth from the cell cycle, either by arresting the cell cycle with continued growth, resulting in an increase in cell mass (Hartwell *et al.*, 1970; Nurse *et al*, 1976), or by accelerating the cycle, which reduces the cell size at division (Nurse, 1975). These phenotypes arise because wildtype yeast cells, in a given media, grow at close to their maximal rate. Later, mutations were identified which perturb the cell cycle order itself, enabling multiple rounds of S-phase in the absence of mitosis (Broek *et al*, 1991; Moreno and Nurse, 1994) or causing cells to enter mitosis prematurely from G1 of the cycle (Hirano *et al.*, 1986). Finally checkpoint functions were studied by the isolation of mutations that allow the cell cycle to continue when S-phase or M-phase are inhibited (Hartwell and Weinert, 1989; Enoch and Nurse, 1991). Multiple screens in budding and fission yeasts identified many of the same key cell cycle regulators, and much of this machinery has been shown to be conserved during evolution and is used in all eukaryotes (Nurse, 1990). I will now turn to discuss the central components controlling passage through the the cell cycle, cyclin dependent kinases or CDKs.

CDKs: master regulators of the cell cycle.

Entry into S-phase and mitosis in eukaryotes is controlled by the activation of cyclin dependent kinases (CDKs) (Morgan, 1995). CDKs phosphorylate protein substrates at serine or threonine residues, within a motif containing the residues S/T, P, (X), K/R or K/R, S/T, P. This protein motif was first identified from the analysis of phosphorylation sites within the histone H1 (Langan, 1978; Langan *et al.*, 1989). In fact, H1 kinase activity was shown to regulate cell cycle progression in *Physarum*, almost 15 years prior to the discovery of CDKs (Bradbury *et al.*, 1974a; Bradbury *et al.*, 1974b; Inglis *et al.*, 1976). The activity of these protein kinases is regulated by a number of mechanisms which serve to integrate information about growth, checkpoints, cell cycle stage etc. (Nurse, 1990). When all the necessary criteria are met, the CDK becomes active and phosphorylates a number of target proteins (some of which have been identified, see Nigg, 1993), which then coordinate the appropriate physical event, either S-phase or mitosis.

In yeasts, a single CDK core enzyme, encoded by *cdc2* in fission yeast (Nurse and Bissett, 1981) and its homologue *CDC28* in budding yeast (Beach *et al.*, 1982), bind to cyclin partners to activate both S-phase and Mitosis (Nigg, 1995). In higher eukaryotes, Cdc2p initiates mitosis and Cdk4p/6p followed by Cdk2p act sequentially to control progression through G1 and the onset of DNA replication (Sherr, 1995).

In general CDKs are subject to several controls which limit their activity, substrate specificity and cellular localisation. Recently, the complexity of mechanisms governing the biochemical activity of CDKs has become apparent, reflecting the biological importance of their timely activation and inactivation during the cell cycle. These include control over cyclin accumulation, inhibitory phosphorylation and the presence of CDK inhibitors, CKIs (Sherr and Roberts, 1995; Nigg, 1995; Morgan, 1995). These mechanisms are shown in the diagram in Figure 1.1.

The complex mechanisms controlling CDK activity.

A) The role of cyclins in the control of CDK activity.

The primary means of controlling the activation of a CDK, is by the availability of a cyclin partner (Morgan, 1995). Both subunits of the CDK/cyclin complex are essential for biochemical activity, and each is likely

to play a role in defining the substrate specificity of the complex (Peeper *et al*, 1993). Cyclins were initially identified as molecules whose abundance oscillated once per cell cycle during the early divisions of sea urchin embryos (Evans *et al.*, 1983), hence their name. Subsequently, mitotic cyclins were shown to form an active protein kinase complex, together with $p34^{cdc2}$ which is sufficient to drive oocytes into M-phase (meiosis) (Dunphy *et al*, 1988; Gautier, et al., 1988; Labbé *et al*, 1989; Minshull *et al*, 1989). In all eukaryotes, CDK activity (as measured using H1 as a substrate) peaks during mitosis, disappears suddenly in early G1, and steadily increases through G2 until passage through the following mitosis (reviewed in Nurse, 1990). This oscillation usually follows changes in cyclin levels, and the dramatic destruction of cyclins at mitosis is the major irreversible step in the CDK cycle which may impart it with directionality (Murray *et al*, 1989). The association of cyclin partners and CDK subunits is somewhat promiscuous, and in yeasts the single CDK subunit associates with several cyclin partners through the cell cycle, in a stage specific fashion, to bring about ordered cell cycle events (Nasmyth, 1993). All eukaryotes use multiple cyclins which, despite their limited homology, fall into distinct classes that correspond to the timing of their accumulation in the cell cycle (Sherr, 1993); G1 cyclins, which include D-type cyclins in "higher" eukaryotes and CLNs in budding yeast, and "mitotic" or B-type cyclins, which accumulate from S-phase onwards until their rapid degradation at the metaphase anaphase transition (King *et al*, 1996). In *S. pombe*, the major mitotic cyclin is cdc13p (Booher and Beach, 1988; Solomon *et al.*, 1988; Fisher and Nurse, 1995; Fisher and Nurse, 1996); cig1p and cig2p, the other known B-type cyclins, play more subtle roles in driving cell cycle progression (Bueno *et al*, 1991; Bueno and Russell, 1993a; Bueno and Russell, 1993b; Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994; Fisher and Nurse, 1995).

B) Control of CDK activity by inhibitory phosphorylation.

CDK activity is inhibited by phosphorylation of the CDK core at the ATP binding cleft, on Y15 (together with T14 in some CDKs) (Gould and Nurse, 1989). In fission yeast, dephosphorylation at Y15 is the rate limiting step governing entry into mitosis, and in certain circumstances it may be rate-limiting in the control of cell cycle transitions in higher eukaryotes (Krek and Nigg, 1991; Kumagai and Dunphy, 1992; Nurse, 1990). In *S. cerevisiae*, phosphorylation of this residue is used, primarily, to allow cells to adapt to a prolonged mitotic arrest, induced by the spindle checkpoint, allowing them

to re-enter G1 (Minshull *et al.*, 1996). In all cases, the level of inhibitory phosphorylation is controlled by the relative opposing activities of a conserved protein kinase, *wee1,* and a phosphatase, *cdc25* (Russell and Nurse, 1986; Russell and Nurse, 1987; Parker *et al*, 1991; Featherstone and Russell, 1991; Meijer *et al.*, 1991).

C) CDK inhibitors

Finally, cyclin dependent kinase inhibitors (CKIs) have been identified which inhibit the activity of CDK/cyclin complexes, *in vivo* and *in vitro,* at nM concentrations (Sherr and Roberts, 1995). Rum1p is the major CKI identified in *S. pombe*. In *vitro,* rum1p inhibits the activity of the cdc13p/cdc2p protein kinase complex specifically (Correa-Bordes and Nurse, 1995), and *in vivo,* it may also act to promote the degradation of cdc13p (Correa-Bordes and Nurse, 1997). Rum1p accumulates in G1 cells where its ability to inactivate cdc13p/cdc2p is essential for the expansion of the G1 phase of the cycle (Martin-Castellanos *et al*, 1996; Moreno and Nurse, 1994). Sic1p in *S. cerevisiae* (Mendenhall *et al*, 1987; Schwob, *et al*, 1994; Donovan *et al*, 1994) and *roughhex* in *Drosophila* (Gonczy *et al*, 1994) may be functionally homologous to rum1p.

Mitosis and S-phase

In the next section I will consider the molecular mechanisms by which the major cell cycle events, mitosis and S-phase, are brought about.

The decision to enter mitosis is mediated by CDK activity.

Mitosis is the most visually dramatic event of the cell cycle. At the onset of mitosis, chromosomes condense and a spindle is constructed from microtubules nucleating at the 2 (recently duplicated) spindle pole bodies, to form a bipolar array. Chromosomes attach to the spindle via kinetochores, and line up at the metaphase plate. Then, at the onset of anaphase, sister chromatids rapidly segregate away from each other. Cytokinesis follows, dividing the cell into two. In higher eukaryotes the nuclear membrane breaks down during mitosis and there is a complete cessation of transcription (Weisenberger and Scheer, 1995; Segil *et al.*, 1996; Martinez-Balbas *et al.*, 1995) . In fission yeast, the chromatin visibly condenses,

although to a less dramatic extent than in higher eukaryotes. The nuclear membrane remains intact, as is the case for many fungi.

Mitosis was the first cell cycle event to be understood at a molecular level. Studies in *Xenopus* and starfish oocytes identified a biochemical activity, MPF (maturation promoting factor), which could initiate the onset of mitosis (Kirschner *et al*, 1985; Gautier, et al., 1988; Labbé *et al*, 1989a; Labbé *et al*, 1989b; Dunphy, et al., 1988). Simultaneously, genetic studies in fission yeast (described earlier) identified several molecules whose activities were required and rate-limiting for mitosis. In both cases, a cdc2p/cyclin B protein kinase complex was identified as the rate-limiting mitotic activator (Lohka, 1989).

In fission yeast, cdc2p and a B-type cyclin, cdc13p, initiate mitosis after an inhibitory phosphate on Y15 in the ATP binding cleft, is removed by the action of the *cdc25* phosphatase (Gautier *et al*, 1991; Dunphy and Kumagai, 1991; Millar *et al*, 1991; Strausfeld *et al*, 1991) and the inactivation of the *wee1* protein kinase (Parker *et al*, 1991; Featherstone and Russell, 1991; Meijer, et al., 1991). After cdc2p/cdc13p has driven cells into mitosis, at the metaphase-anaphase transition, the B-type cyclins are rapidly degraded (King *et al*, 1996), as is cut2p, a protein that may function to tie sister chromatids together (Funabiki *et al*, 1996). Cyclin degradation is required for exit from mitosis, and entry into G1. This specific proteolysis is coordinated by the activation of the cyclosome (Sudakin *et al*, 1995), alternatively called the APC (or anaphase promoting complex) (King *et al*, 1995). This is a ubiquitin conjugating enzyme that catalyses the addition of ubiquitin moieties to target proteins, directing them for proteolysis (King *et al*, 1996). In *Xenopus* extracts, the cyclosome is activated, with some temporal lag (Felix *et al*, 1989), by high mitotic Cdc2p/cyclin B activity itself (Sudakin *et al*, 1995). Therefore the activation of Cdc2p/cyclin B, which brings about mitosis, may simultaneously initiate its own destruction. This limits mitosis to a discrete event, culminating in G1, which is characterised by low levels of B-type cyclin, and resets the cell cycle to its null or ground state. Work in budding yeast (Amon *et al*, 1994) and more recently in fission yeast (Correa-Bordes and Nurse, 1997) shows that the cyclosome remains active through G1, preventing the re-accumulation of cyclin B.

Entry into mitosis also initiates septation, which occurs with some delay, after the completion of anaphase (reviewed in Simanis, 1995). This event, which lies genetically downstream of the mitotic kinase, may be controlled by activation of *polo* kinase (Sunkel and Glover, 1988; Llamazares *et al*, 1991), encoded by *plo1* in fission yeast (Ohkura *et al.*, 1995). In mutants, the separation of regions of the centromeric DNA by the mitotic spindle, is sufficient to initiate cytokinesis (Funabiki *et al*, 1993), so this event may constitute the cue for septation.

Control of DNA replication.

Research towards an understanding of DNA replication has closely followed a framework first laid down 30 years ago, as a model to explain DNA replication in bacteria (Jacob *et al*, 1963). Like mitosis, DNA replication is controlled at the initiation step. DNA replication begins at specific sites in the eukaryotic genome, termed origins of replication. The trigger initiating S-phase, is likely to be local unwinding of the DNA duplex at origins, catalyzed by an unidentified activity. This allows DNA polymerases access to a single stranded template; a necessary prerequisite for RNA priming and DNA synthesis (Kornberg and Baker, 1992). In yeast, origins of replication have been defined by isolating sequences that enable the autonomous replication of episomal DNA, and many of these sequences have subsequently been shown to initiate DNA synthesis in their native chromosomal site (Brewer and Fangman, 1987; Huberman *et al*, 1987; Fangman and Brewer, 1991). In budding yeast, a short origin consensus sequence has been identified (Marahrens and Stillman, 1992), while in fission yeast (and probably in higher eukaryotes), origins appear to be large relatively undefined sequences rich in A and T (De Pamphilis, 1996). This characteristic composition may aid denaturation of the DNA double helix.

The origin recognition complex or ORC.

In *S. cerevisiae*, the identification of a discrete conserved origin enabled the biochemical characterisation of an "origin recognition complex" or ORC, composed of 6 co-purifying polypeptides (Bell and Stillman, 1992) (for a review see (Stillman, 1996). This complex binds to origins *in vitro*, in the presence of ATP (Bell and Stillman, 1992). Analogous ORC complexes have now been isolated from several eukaryotes (Carpenter *et al*, 1996; Rowles *et al*, , 1996). Although, in all cases, 6 ORC subunits appear to associate in a complex, the amino acid sequences of many of the ORC subunits (especially

that of ORC6) have diverged significantly during evolution, and in several cases it has been shown that the equivalent ORCs cannot function in different organisms (Ehrenhofer *et al*, 1995; Gavin *et al.*, 1995). This may be explained in part by the difference in character between origins in different eukaryotes. However, in both budding (Bell *et al*, 1993; Micklem *et al*, 1993; Liang *et al*, 1995; Fox *et al*, 1995) and fission yeasts (Grallert, 1996; Leatherwood *et al*, 1996; Muzi-Falconi, 1995) and in a *Xenopus in vitro* replication assay (Carpenter *et al*, 1996; Rowles *et al*, , 1996; Romanowski *et al*, 1996), ORC subunits have been shown to be essential for DNA replication. Therefore, they are likely to fulfil the same function in divergent organisms.

The ability of the ORC complex to bind origin sequences *in vitro*, was first shown to be relevant to its function *in vivo*, by the use of genomic footprinting. The pattern of protection of the purified ORC complex bound to DNA *in vitro* was shown to be essentially identical to the minimal footprint seen at the origin *in vivo* (Diffley and Cocker, 1992). The minimal *in vivo* footprint persists throughout the cell cycle, but is extended during the period from the end of mitosis until late G1 (Diffley *et al*, 1994). The ORC complex is therefore thought to mark origins of replication, and to recruit additional molecules in a cell cycle dependent manner, to form a "pre-replicative complex" which gives rise to the additional protection observed prior to S-phase. This structure then orchestrates initiation (Stillman, 1996). However, the complex corresponding to the "pre-replicative complex" is also observed at inactive origins (Santocanale and Diffley, 1996), suggesting that either, additional undetected factors bind to distinguish functional and non-functional origins, and/or that the choice of origin is regulated at a different level of organisation. ORC also has a genetically separable, and therefore apparently unrelated function in transcriptional silencing (Ehrenhofer *et al*, 1995; Fox *et al*, 1995; Bell *et al*, 1995; Kelly *et al*, 1994). This may be brought about by the recruitment of a different assembly of factors by Orc1p (which has homology to Sir3p, a component of a multi-purpose transcriptional silencing complex). For this reason, it has been proposed that the ORC plays a more general role in organising the structure of chromatin throughout the cell cycle (personal communication M. Botchan).

MCM proteins.

An additional family of 6 proteins important for DNA replication, MCMs, were first identified in *S. cerevisiae* as genes required for the "Maintenance of a Mini-Chromosome" (Chong, 1996). This original phenotype suggested that mutations in these genes cause a defect in DNA replication (Moir *et al*, 1982; Maine *et al*, 1984). This has subsequently been demonstrated *in vivo*, (Yan *et al*, 1991; Gibson *et al*, 1990; Hennessy *et al*, 1991) and *in vitro* replication assays (Madine *et al*, 1995a; Coleman, 1996). The 6 MCM proteins are conserved across eukaryotes, and all contain a nucleotide binding motif which resembles that found in DNA dependent ATPases, including in the initiator protein, DnaA, from bacteria (Koonin, 1993).

In *Xenopus* extracts, MCMs are present as a complex (Chong *et al*, 1995) and are only competent to bind DNA after passage through mitosis (Chong *et al*, 1995; Kubota *et al*, 1995; Coleman, 1996). This G1 association of MCMs with the DNA, is inhibited by high levels of CDK activity (Hua *et al*, 1997; Mahbubani *et al*, 1997). MCMs are then progressively lost from the chromatin during S-phase (Chong *et al*, 1995; Kubota *et al*, 1995; Coleman, 1996). In *S. cerevisiae*, Cdc46p, an MCM component, was shown to shuttle between the cytoplasm and nucleus with cell cycle progression (Hennessy *et al*, 1990) and to associate with the DNA during the period from early G1 until S-phase (Donovan *et al*, 1997). Changes in the cellular localisation of MCMs have since been seen in other organisms (Kimura, 1994; Todorov *et al*, 1995; Madine *et al*, 1995b; Coue *et al*, 1996). Control over the ability of MCM proteins to enter the nucleus and bind chromatin may play a crucial role in defining the cell cycle state of the origin (Chong, 1996).

One outstanding feature of the MCM proteins, is that they are 10 fold more abundant than ORCs in the cell. Therefore, it is possible that on recruitment to the DNA, MCMs spread to surrounding DNA regions extending the complex. This may explain the steady depletion of chromatin associated MCMs with passage through S-phase, as MCMs are lost from the replicated portions of the genome. Human MCMs have been shown to associate with Pol α-primase (Kimura, 1994), so it is also possible that MCMs move with the replication fork itself.

cdc18/CDC6

Another conserved gene function, known as *CDC6* in *S. cerevisiae* and *cdc18* in *S. pombe*, is essential for DNA replication in yeast (Hartwell, 1976; Nasmyth and Nurse, 1981; Bueno and Russell, 1992; Hogan and Koshland, 1992; Kelly *et al*, 1993). In the absence of *cdc18/CDC6* cells cannot enter S-phase, and, as a consequence, enter mitosis from G1 of the cycle (Kelly *et al*, 1993; Piatti *et al*, 1995). The *Xenopus* homologue has also been shown to be essential for DNA replication in an *in vitro* assay. *CDC6* and *cdc18* share homology, and have a limited sequence similarity to *ORC1*, (which includes the nucleotide binding motif) (Muzi-Falconi, 1995; Bell *et al*, 1995; Hori *et al*, 1996; Grallert, 1996). Both *CDC6* and *cdc18* genes are transcribed periodically during the cell cycle and are targets of the S-phase transcriptional machinery (see below) (Zhou and Jong, 1990; Zwerschke *et al*, 1994; Piatti *et al*, 1995; Kelly *et al*, 1993). In both yeasts, the timing of this expression coincides approximately with the time at which origins are competent to replicate. Importantly, in *S. pombe*, when *cdc18* is ectopically expressed at high levels in G2 cells, it is able to induce continuous DNA synthesis in the absence of mitosis (Nishitani and Nurse, 1995; Muzi-Falconi *et al*, 1996). Interestingly, this may not represent complete and distinct rounds of S-phase. *cdc18* is the only intrinsic element of the initiation complex which has been shown to have such an activity. This over-replication is likely to differ from other types of induced re-replication (see below), in that it can occur even in the presence of the protein synthesis inhibitor cyclohexamide. This suggests that the presence or absence of cdc18p alone may be sufficient to define the state of an origin (Nishitani and Nurse, 1995). For these reasons, cdc18p and Cdc6p may be the critical regulators of S-phase, and the restriction of their periodic synthesis to G1 may confine DNA replication to this portion of the cell cycle.

Several disparate pieces of data link *CDC6* and *cdc18* function to the pre-initiation complex (Liang *et al*, 1995). Firstly, in *S. cerevisiae*, the extended ORC footprint requires the presence of Cdc6p and is labile in a $cdc6^{ts}$ mutant (Cocker *et al*, 1996; Detweiler and Li, 1997). In fission yeast, cdc18p has been shown to form a complex with orp2p and cdc2p, and in another study with orp1p (a fission yeast ORC subunit; Leatherwood *et al*, 1996; Grallert and Sipiczki, 1991). In addition, the chromatin association of MCMs was shown to be dependent on *CDC6* in *S. cerevisiae* (Donovan *et*

al, 1997). In *Xenopus*, a homologue of Cdc6p/cdc18p has been shown to load MCM proteins onto DNA already bound by ORCs, in G1 extracts (Coleman, 1996). Although after the loss of Cdc6p from the chromatin at G1/S, MCMs are likely to remain bound (Donovan *et al*, 1997). Furthermore, both Cdc6p and cdc18p contain multiple putative cdc2p consensus phosphorylation sites, and both proteins associate with cdc2p/Cdc28p (Leatherwood *et al*, 1996; Elsasser *et al*, 1996; Brown *et al*, 1997). This suggests that Cdc6p/cdc18p may be one of the important S-phase targets of cdc2p.

cdt1 and cut5.

In *S. pombe*, another gene, *cdt1*, is periodically transcribed by the S-phase transcriptional machinery (Hofmann and Beach, 1994). Like *cdc18*, *cdt1* is required for S-phase, and in its absence cells enter mitosis from G1. It may also enhance the capacity for cdc18p induced re-replication (Hideo Nishitani personal communication). Therefore, cdt1p in association with cdc18p may be involved in setting up and/or maintaining the "initiation complex" in preparation for DNA replication. However, *cdt1* has no *S. cerevisiae* homologue, and no overt similarity to any sequence in the data base. In addition, another *S. pombe* gene, *cut5* (identical to *rad4* (Fenech *et al*, 1991)), has a similar phenotype allowing mitosis to occur from G1 of the cycle (Saka *et al*, 1994; Saka and Yanagida, 1993). However, *cut5* expression appears to be constitutive through the cell cycle and is not affected by mutations which inactivate S-phase transcription. At present the function of *cut5* is unclear.

A model showing a contemporary view of the mechanisms controlling the initiation of DNA replication.

Data in this field are rapidly accumulating, augmenting the work described above, and a picture is gradually falling into place which explains many of the mysteries surrounding the control of DNA replication. A model of the current view of DNA replication is presented in Figure 1.2. In this scheme ORCs are considered to mark the sites of origins of replication throughout the cycle. In G1, Cdc6p/cdc18p is thought to load MCMs, possibly together with several other interacting proteins, onto the ORC landing pad. Presumably, at the onset of S-phase, one or more of the components are phosphorylated by CDK/cyclin B activity, it having re-accumulated in G1, to trigger their ATP dependent DNA helicase activity and the initiation of S-

phase. However, despite the consensus of opinion on many of these points, the enzymatic functions of all the proteins bound at the origin remain unknown.

Controlling global cell cycle order.

As discussed above, cell cycle controls maintain the order of S-phase and mitosis in the eukaryotic mitotic cycle. These controls ensure that for S-phase to occur, two sets of criteria must be fulfilled. Firstly, that origins of replication are only competent to re-initiate replication after passage through mitosis (Blow and Laskey, 1988). This limits S-phase to one round per cell cycle. Secondly, DNA replication only occurs after cells have reached a sufficient size. This control is mediated by a decision point in G1, called START in yeast (Nurse and Thuriaux, 1977; Nurse *et al*, 1976; Nurse, 1975) and the Restriction point in higher eukaryotes, at which time they become committed to the completion of a full mitotic cycle (Hartwell *et al*, 1974; Nurse and Bissett, 1981). A similar size control over DNA replication may also exist in bacteria (Boye *et al*, 1996).

Ordering S-phase and Mitosis.

Insights into the process by which passage through mitosis relieves the G2 block over re-replication, were gained by the identification of mutations in fission yeast, which uncouple S-phase from M-phase, resulting in endoreduplication (Moreno and Nurse, 1994). It was shown that removal of G2 CDK activity by use of temperature sensitive alleles of cdc2p, removal of cdc13p or overexpression of rum1p (a potent inhibitor of cdc2p/cdc13p) in G2 of the cycle, can induce fission yeast to undergo multiple rounds of S-phase without an intervening mitosis (Broek *et al*, 1991; Moreno and Nurse, 1994; Hayles *et al*, 1994). Each subsequent re-replicative S-phase requires some level of CDK activity and the transcription of S-phase genes (Fisher and Nurse, 1996; Moreno and Nurse, 1994). Thus, endoreduplication only occurs if the inactivation of the cdc2/cyclin B CDK activity is transient. This suggests that the loss of CDK activity in a G2 cell causes re-replication by re-setting the cell cycle to a G1 state: characterized by low CDK/B-type cyclin activity and high levels of S-phase transcription.

Several lines of evidence suggest that the CDK activity present in G2 cells probably plays a similar role in all eukaryotes. Mammalian cells can be

induced to re-replicate by the use of generic protein kinase inhibitors (Usui *et al.*, 1991). In addition, it was recently shown that a mammalian *CDC2* deletion cell line, carrying *CDC2* expressed from a regulatable promoter, undergoes multiple rounds of S-phase after transcription of *CDC2* is switched off (Itzhaki *et al*, 1997). In *S. cerevisiae*, a transient inactivation of the Cdc28p/cyclin B kinase in G2, causes the reappearance of the extended pre-replicative footprint at origins of replication, and an additional round of S-phase (Dahmann *et al*, 1995). These observations imply that although the mitotic CDK activity in G2 is insufficient to bring about mitosis (because, in G2 in *S. pombe*, cdc2p is phosphorylated at a tyrosine residue in the catalytic cleft), it is used to define the G2 state. Cdc2p may act directly to inhibit the assembly of a pre-replicative complex at origins. This explanation can account for the mechanism by which completion of mitosis and degradation of B-type cyclins potentiate DNA replication. It also provides a simple way for cells to ensure that S-phase only occurs after passage through mitosis.

Another observation from *S. pombe* enables a further simplification of the model by which the CDK cycle orders the alternating rounds of S and M. In *S. pombe*, the major mitotic cyclin, cdc13p, is able to drive orderly cycles of S-phase and M-phase in the absence of any other known B-type cyclin (Fisher and Nurse, 1996). This is possible even though *cdc13* is constitutively transcribed and translated through the cell cycle (Correa-Bordes and Nurse, 1997), and is surprising given that *cig1* and *cig2*, the other B-type cyclins in fission yeast, are thought to be responsible for initiating S-phase in wildtype cells (Mondesert *et al*, 1996; Martin-Castellanos *et al*, 1996; Fisher and Nurse, 1996; Correa-Bordes and Nurse, 1997; reviewed in Fisher and Nurse, 1995). Firstly, this indicates that the different CDK/cyclin complexes involved in the control of S and M phases may have similar substrate specificities. Evolution may therefore have driven the diversification of cyclins, primarily to widen the flexibility of cell cycle control. However, more significantly this observation suggests a "quantitative model" for cell cycle control by CDKs, in which low levels of CDK/cyclin B activity are sufficient to trigger S-phase, and higher levels initiate mitosis (Stern, 1996). This model is consistent with the profile of cdc2p/cdc13p activity, which begins to accumulate in late G1 until it reaches a peak level at mitosis, and with the observation that S-phase requires a lower threshold of cdc2p protein kinase activity than M-phase. This type of regulation guarantees that once the cell cycle has been reset, S-phase always

precedes mitosis. It also explains how a single cyclin oscillation, driven by continuous synthesis and periodic degradation, can order the cell cycle.

Linking CDK control over re-replication to the action of proteins at the origin of replication.

In order to widen this model to encompass the function of CDK activity in preventing the reinitiation of S-phase from G2, we should consider the fact that DNA replication is only brought about by the sequential removal and re-accumulation of CDK activity (Su *et al*, 1995; Stern, 1996; Piatti *et al*, 1996; Nasmyth, 1996). To explain this two step process, an additional component in the system is required, which will act as a molecular memory of the temporal change in kinase level. It is possible that this reflects the sequential events at the origin of replication. Under conditions of low CDK activity, a pre-replicative complex will form at origins of replication. The subsequent increase in CDK activity then leads to the phosphorylation of components of this structure e.g. Cdc6p/cdc18p and MCMs. This may cause a structural change that initiates DNA replication, and which simultaneously blocks the ability to re-form the pre-replicative complex. If this scheme is accurate, the initiating event, for both S-phase and mitosis, destroys the capacity to re-establish the conditions necessary to repeat it. This feature is reminiscent of prokaryotic DNA synthesis in which the activity of DnaA, the initiator of DNA replication, is destroyed in the act of DNA unwinding, by the hydrolysis of an essential ATP molecule at the active site (Sekimizu *et al*, 1987; Mizushima *et al*, 1997). The ADP form of DnaA is then inactive and must be recycled to initiate a further round of replication.

A further prediction of this scheme is that the premature activation of the mitotic kinase may block assembly of the pre-replicative complex in G1 cells. In both *S. pombe* and *S. cerevisiae*, it has been shown that accumulation of the mitotic kinase in G1 cells, prior to the expression of *cdc18/CDC6* can drive cells directly into mitosis, bypassing S-phase (Hayles *et al*, 1994; Piatti *et al*, 1995). In a biochemical test of this model, in *Xenopus* extracts, Cdc2p/cyclin B activity has also been shown to inhibit the capacity to initiate DNA replication (Adachi and Laemmli, 1994; Hua *et al*, 1997).

In conclusion, B-type cyclin/CDK complexes are able to initiate both S and M-phases at different thresholds of activity, and are required to inhibit replication from G2 of the cycle. The cell cycle can therefore accurately be described as a CDK cycle, as shown in Figure 1.3.

Control of the Timing of S-phase.

During the G1/S portion of the cell cycle, yeast and mammalian cells are able to assess environmental cues and the internal cell state (e.g. cell size) in order to make an appropriate decision, either to commit themselves to S-phase and complete a round of mitosis, or to an alternative developmental fate e.g. terminal differentiation or meiosis (Woollard and Nurse, 1995; Sherr, 1994). Prior to a decision, cells can introduce a variable G1 delay, but once passed a point during G1, termed START, in yeast (Hartwell *et al*, 1974) and the Restriction point in mammalian cells (Reed, 1992), cells become refractory to other potential fates, and committed to the cell cycle. As yet, it is not clear exactly what mechanisms mediate this switch. However, the molecular mechanisms by which commitment to the mitotic cycle and the initiation of DNA replication are brought about in metazoan cells, budding yeast and fission yeast are known to share many features, including a requirement for coordinated expression of genes required for S-phase and CDK/cyclin B activity. The control of G1/S progression and S-phase transcription has been most thoroughly studied in the budding yeast, *Saccharomyces cerevisiae* (Johnston, 1992; Koch and Nasmyth, 1994; Breeden, 1996) and in mammalian cells (Slansky and Farnham, 1996).

Periodic transcription required for S-phase.

In budding yeast, periodic transcription of S-phase genes is mediated by two transcriptional complexes, *SWI4/SWI6* and *SWI6/MBP1*, which act through conserved promoter elements known as SCB (CACGAAA) and MCB (ACGCGT) sites respectively (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989; Taba *et al*, 1991; Lowndes *et al*, 1991; Dirick *et al*, 1992; Koch *et al*, 1993; for a recent review see Breeden, 1996). A DNA binding activity containing Swi6p and Mbp1p, termed DSC1 or MBF, that recognises MCB elements, is thought to be involved in transcriptional activation (Verma *et al*, 1991; Lowndes *et al*, 1992a; Dirick *et al*, 1992; Koch *et al*, 1993).

In mammalian cells, the E2Fp/DP1p transcriptional complex, although unrelated to *SWI4/SWI6/MBP1*, serves an analogous function.

In fission yeast, the S-phase transcriptional machinery is essential for passage through START and entry into S-phase (Nurse and Bissett, 1981). It is composed of cdc10p, res1p, res2p, and rep2p proteins (Aves *et al*, 1985; Lowndes *et al*, 1992b; Caligiuri and Beach, 1993; Tanaka *et al*, 1992; Miyamoto *et al*, 1994; Zhu *et al*, 1994; Nakashima *et al*, 1995). cdc10p, res1p and res2p have a high degree of homology to Swi4p, SWI6p and Mbp1p, and coordinate the expression of target genes (with homologues in *S. cerevisiae*), through the same MCB promoter elements used in *S. cerevisiae*. Target genes for the machinery include *cdc18, cdc22, cdt1* and possibly *cig2* (Kelly *et al*, 1993; Gordon and Fantes, 1986; Hofmann and Beach, 1994; Obara-Ishihara and Okayama, 1994). DSC1, an endogenous bandshift activity found in fission yeast cell extracts (Lowndes *et al*, 1992b), binds MCB sites and contains cdc10p and res1p (Lowndes *et al*, 1992b; Caligiuri and Beach, 1993). Res2p also interacts with cdc10p to form an alternative complex, which can bind specifically to MCB elements *in vitro* (Zhu *et al*, 1994). Genetic analyses suggest that cdc10p/res1p is the major transcriptional regulator during the mitotic cell cycle (Tanaka *et al*, 1992), and cdc10p/res2p during the meiotic cell cycle (Miyamoto *et al*, 1994). In addition, a role has been proposed for cdc10p in repression of transcription, based on the observation that a truncation of cdc10p causes elevated levels of transcription throughout the cell cycle (McInerny *et al*, 1995).

Control of S-phase transcription

Periodic expression in budding yeast is thought to be controlled primarily by oscillations in CDK activity through the cell cycle, although it is not fully understood how CDKs interact with the transcriptional complexes to regulate transcription. In *S. cerevisiae*, the *CLN3/CDC28* protein kinase is thought to become activated early in G1 (Tyers *et al.*, 1993; Dirick *et al.*, 1995) Stuart and Wittenberg, 1995; Levine *et al*, 1996). This leads to the activation of the two START transcriptional complexes Swi4p/Swi6p and Mbp1p/Swi6p (Andrews and Herskowitz, 1989; Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). Targets of this periodic transcription include enzymatic functions necessary to bring about DNA replication, and in addition, B-type cyclin partners of *CDC28*, which activate the onset of S-phase (Epstein and

Cross, 1992; Schwob *et al.*, 1994). Since deletions in *CLN3* delay both the onset of S-phase (Richardson *et al*, 1989; Cross, 1990) and the activation of the S-phase transcriptional machinery, and gain of function mutations shorten the G1 phase (Cross, 1988; Nash *et al*, 1988), the activation of Cln3p/Cdc28p kinase is viewed as the rate-limiting step governing G1/S progression. While, no direct interaction between Cln3p/Cdc28p and Swi4p/Swi6p or Mbp1p/Swi6p have been observed, Swi4p has been shown by immuno-precipitates to be associated with a CDK complex containing Clb2p, a B-type cyclin, and Cdc28p (Siegmund and Nasmyth, 1996). However, this may represent an inhibitory interaction, as Clbp/Cdc28p activity is thought to downregulate the transcriptional activity of this complex in G2 of the cycle (Koch *et al*, 1996).

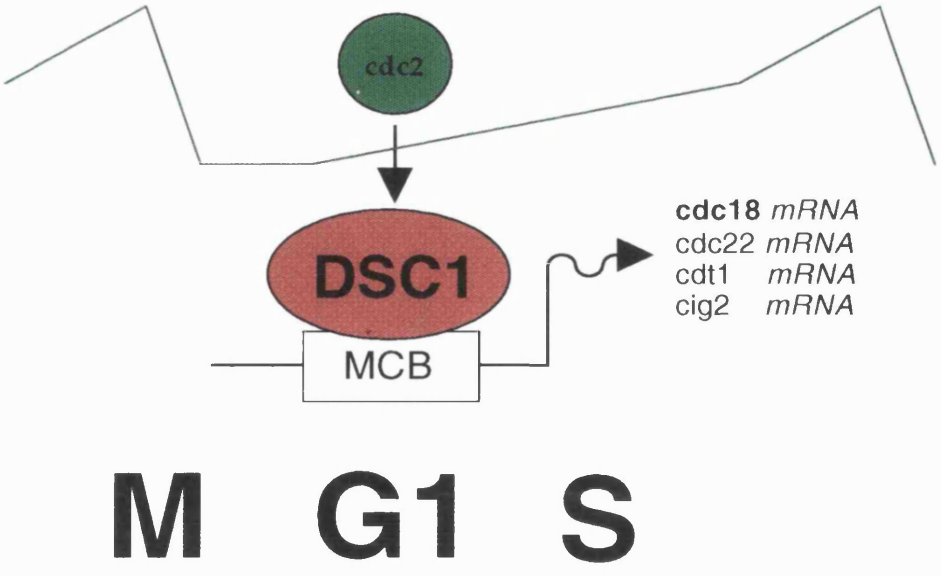
Work in mammalian cells has identified an analogous series of events which drive progression from a quiescent G0 state through G1, and into S-phase (Pardee, 1989). In these cells, Cdk4p(6)/cyclin D kinases accumulate early in G1 in response to stimulatory growth factors. When the level of G1 CDK activity reaches a threshold level, sufficient to overcome inhibition by the cyclin dependent kinase inhibitor, p27 (Kato, et al., 1994; Polyak, et al., 1994; Toyoshima and Hunter, 1994), it phosphorylates Rb protein, a repressor which restrains the activity of the E2Fp/DP1p transcriptional machinery (Sherr, 1995). Once free, E2Fp/DP1p is able to transcribe B-type cyclins, which are necessary for the onset of S-phase. Subsequently, during S-phase, the transcriptional complex is inactivated by Cdk2p/cyclinA activity (Xu *et al*, 1994; Krek *et al*, 1994). During *Drosophila* embryonic development, overexpression of either *E2F* or its target *cyclin E*, is sufficient to bring about S-phase in the absence of upstream events (Knoblich *et al*, 1994). In mammalian cells, the importance of these G1 controls is evident from the number of viral genes and oncogenes that deregulate these events, leading to the uncontrolled activation of E2Fp/DP1p (Hinds and Weinberg, 1994). Gene knockouts have been less informative about the function of these genes (Yamasaki *et al*, 1996; Field *et al*, 1996), possibly because of genetic redundancy in the system.

Differences between control of the cell cycle in *S. cerevisiae* and *S. pombe*.

In *S. pombe*, the mechanism controlling periodic transcription is not understood. The level of cdc10p through the cell cycle is constant, indicating that oscillations in cdc10p do not control the periodicity of transcription (Simanis and Nurse, 1989). In addition, the patterns of transcription of *res1*, *res2* and *rep2* do not give a simple explanation for the periodicity of S-phase transcription (Tanaka *et al*, 1992; Miyamoto *et al*, 1994; Nakashima *et al*, 1995). However, it has been suggested that DSC1, containing res1p and res2p, plays a role in transcriptional activation, and that the formation of DSC1 and the onset of *cdc10* dependent transcription are triggered in G1 by cdc2p activity (Reymond *et al*, 1993). This would be analogous to the model for G1 regulation in budding yeast, and is caricatured below. In this scheme, the DSC1 complex is activated in late G1, as cdc2p activity re-accumulates, (CDK activity is shown in green). The phosphorylated transcriptional complex then binds to MCB elements in promoters of several target genes to activate their transcription and the onset of S-phase.

Figure 1.5

A working model for the control of S-phase transcription in *S. pombe*.



However, there is little evidence for such a mechanism. Furthermore, the passage of G1 is regulated differently in budding and fission yeasts. (See Figure 1.4). For example, unlike *S. cerevisiae*, *S. pombe* only mates under nutrient poor conditions and, as a consequence, conjugation usually serves as a prelude to meiosis and sporulation (Yamamoto *et al.*, 1997) This explains why cells are normally haploid. Nevertheless, like *S. cerevisiae*, *S. pombe* uses the G1 phase of the cycle to decide between two alternate fates; either conjugation and meiosis, or entry into an additional round of the mitotic cell cycle. This requires regulated progression through G1.

Most eukaryotes, including budding yeast spend a considerable portion of the cell cycle in G1. In contrast, during exponential growth, fission yeast cells spend only 10% of the cycle in G1, growing predominantly in the G2 phase of the cycle (Nasmyth *et al*, 1979); they use a G2 size control to couple the cell cycle with cellular growth (Nurse, 1975). A cryptic G1 size control has been shown to exist (Nurse and Thuriaux, 1977; Nasmyth *et al*, 1979), but it is bypassed under nutrient rich conditions because cells enter G1 at a sufficient size to overcome this control, and enter S-phase almost immediately. The short G1 phase may be a biological consequence of *S. pombe*'s predominantly haploid existence, since in the G1 phase of the cycle, a haploid has no opportunity to use recombinational repair in the event of DNA damage. Under nitrogen starvation conditions, however, *S. pombe* cells enter mitosis prematurely. This uncovers the silent G1 size control, which expands the G1 phase, and after two divisions, in the absence of much additional growth, cells arrest in G1 (Fantes and Nurse, 1977). This arrest prepares cells for meiosis, and is accelerated by the presence of pheromone (Davey and Nielsen, 1994; Stern and Nurse, 1997; Yamamoto, et al., 1997). Thus, in light of these observations, it may be concluded that under conditions of rapid growth, *S. pombe* cells do not regulate their progression through G1, but that nutrient limitation invokes the controls required for decisions at START.

The unusual regulation of G1 in *S. pombe* is reflected in the molecular machinery it uses to control progression through G1. Firstly, in *S. pombe*, a functional homologue of the G1 cyclins, in particular *CLN3*, has not yet been identified. Although the *S. pombe* gene *puc1* has sequence similarity to *CLN3*, and is a potent activator of G1/S progression when ectopically

expressed in *S. cerevisiae* (Forsburg and Nurse, 1991), the deletion of *puc1* has little, if any, cell cycle phenotype in *S. pombe* (Forsburg and Nurse, 1994). Also, in contrast to other eukaryotes, periodic B-type cyclin transcription in *S. pombe,* is not a pre-requisite for cell cycle control; post-translational control over *cdc13* protein accumulation is sufficient to drive both S-phase and M-phases (Fisher and Nurse, 1996; Correa-Bordes and Nurse, 1997). (The periodic transcription of *cig2* may be important for subtle regulation of G1/S, and in controlling the efficiency of conjugation (Obara-Ishihara and Okayama, 1994)). Therefore, the short G1 in *S. pombe* may be accompanied by a paucity of molecular controls at START.

Introduction to the Data Chapters.

This thesis, investigates the control of the periodic S-phase transcriptional machinery in the fission yeast mitotic cell cycle. I have conducted a physiological analysis of this transcriptional control. This has been lacking primarily because it is not easy to assess the timing of events in the short *S. pombe* G1 phase. The model for the control of periodic S-phase transcription from *S. cerevisiae* has been widely accepted as a general paradigm, applicable to other eukaryotes, because it links the S-phase transcriptional control elegantly into the CDK cycle. Since, the components of the transcriptional machinery (including the promoter elements) are highly homologous in the two yeasts, *S. pombe* is frequently assumed to regulate S-phase transcription in the same way as *S. cerevisiae*. Nevertheless, after approximately 10 years of research in the field, there is, as yet, little molecular evidence for the direct activation of S-phase transcription by a G1 cyclin/CDK complex, in either *S. cerevisiae* or in *S. pombe*. Furthermore, the mechanism preventing S-phase transcription in G2 of the cycle is not known. For this reason, I considered a detailed study of *cdc10* dependent transcription to be a worthwhile project.

Chapter 2.
I begin by addressing whether the molecular events which lead to activation of S-phase transcription in late G1 and subsequently DNA replication in *S. pombe*, are similar to those described in *S. cerevisiae*. In particular, is cdc2p activity, in late G1, responsible for the binding of the cdc10p complex to the promoters of target genes, leading to the activation of *cdc10* dependent transcription. (See Figure 1.5 for working model).

Chapter 3.
In this section, I re-address the timing of S-phase transcription in the fission yeast cell cycle.

Chapter 4.
In this Chapter, I focus on the function of the periodic expression of *cdc18*, and investigate the timing of cdc18p accumulation through the cell cycle. Much of this work was done in collaboration with Hideo Nishitani.

Chapter 5.
I conclude my fission yeast work by presenting a limited physiological and biochemical analysis of the cdc10p complex responsible for the periodic transcription. The analysis of DSC1 was done in conjunction with Jérôme Wuarin in the lab.

Chapter 6.
Finally I explore whether there are reasons to re-assess the widely accepted version of the control of S-phase transcription by Cln3p/Cdc28p in *S. cerevisiae*.

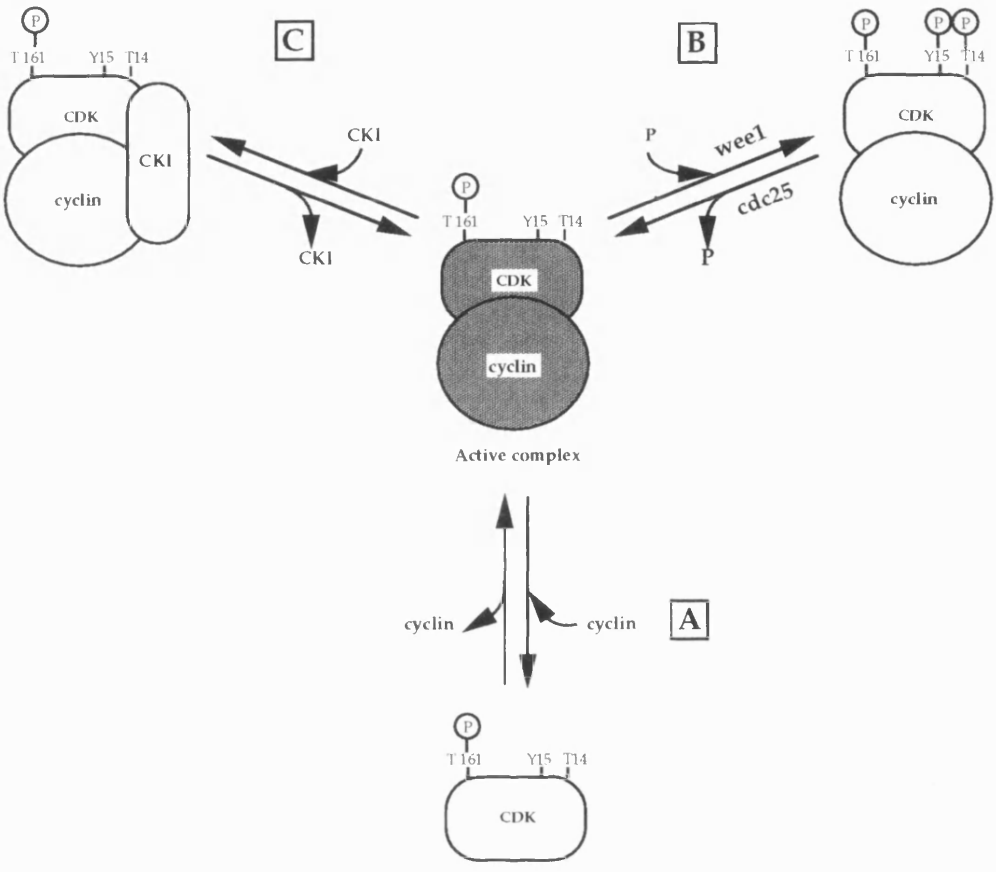


Figure 1.1

Principles of CDK regulation.

The active CDK is associated with a cyclin subunit and phosphorylated on a conserved threonine (161 in fission yeast cdc2p) by CAK (CDK acivating kinase) which is constitutively active through the cell cycle. Regulation of CDK activity involves three different mechanisms: **A.** availability of the cyclin subunit, regulated at transcriptional level and/or at the level of cyclin turnover. **B.** inhibitory phosphorylation on tyrosine 15 (and threonine 14) by a wee1p kinase and its reversal by the cdc25p dual specificity phosphatase, **C.** reversible binding of cyclin-dependent kinase inhibitors (CKIs).

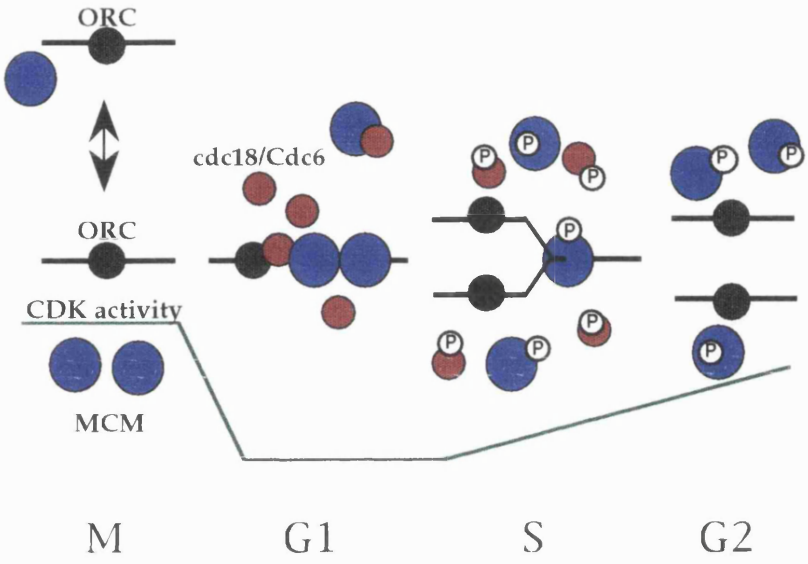


Figure 1.2

A model of the current view of the mechanisms controlling the initiation of DNA replication in eukaryotes.

In this scheme ORCs are considered to mark the sites of origins of replication throughout the cycle. In G1, in the presence of low CDK activity, Cdc6p/cdc18p is thought to load MCMs, possibly together with several other interacting proteins, onto the ORC landing pad. Presumably, at the onset of S-phase, one or more of the components are phosphorylated by CDK/cyclin B activity, it having re-accumulated in G1, to trigger their ATP dependent DNA helicase activity and the initiation of S-phase. MCM proteins may remain bound through S-phase. Phosphorylation probably inactivates Cdc6p/cdc18p probably inhibiting their capacity to bind DNA and/or MCMs.

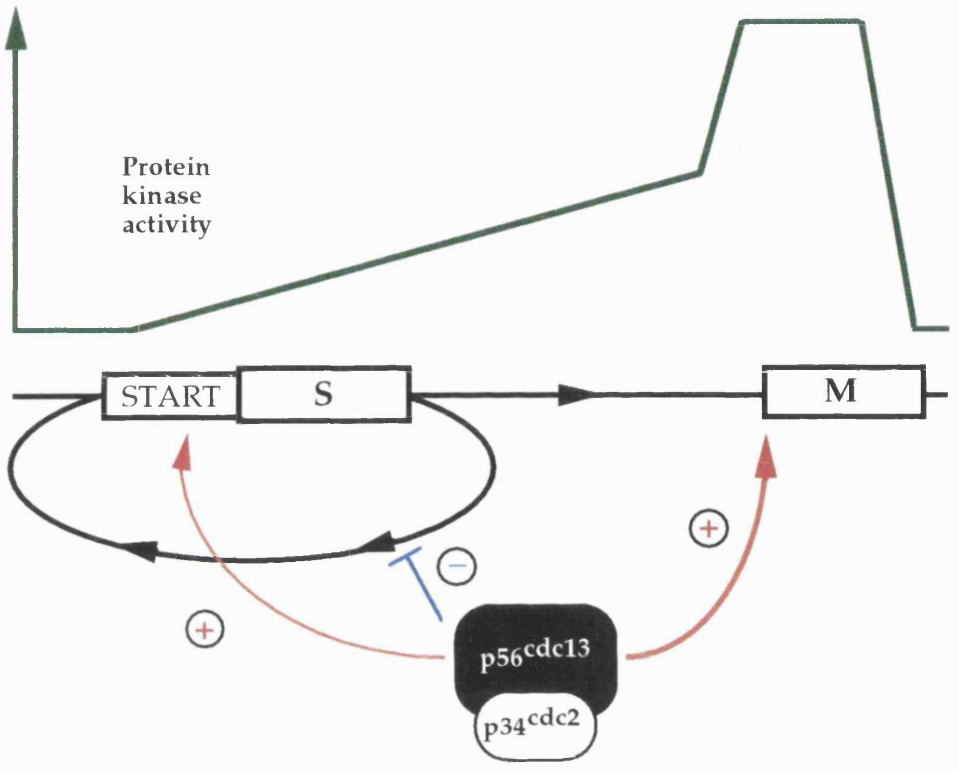


Figure 1.3

A single cdc2/cyclin complex can drive ordered rounds of S-phase and M-phase in fission yeast.

The cdc2p/cdc13p protein kinase activity initiatlly rises after START, and climbs during S-phase and G2 to a peak level in mitosis (green). This complex can fulfil three different functions: it is essential for the onset of mitosis, and low level cdc2p/cdc13p activity prevents re-replication from G2 and is sufficient to drive S-phase from G1.

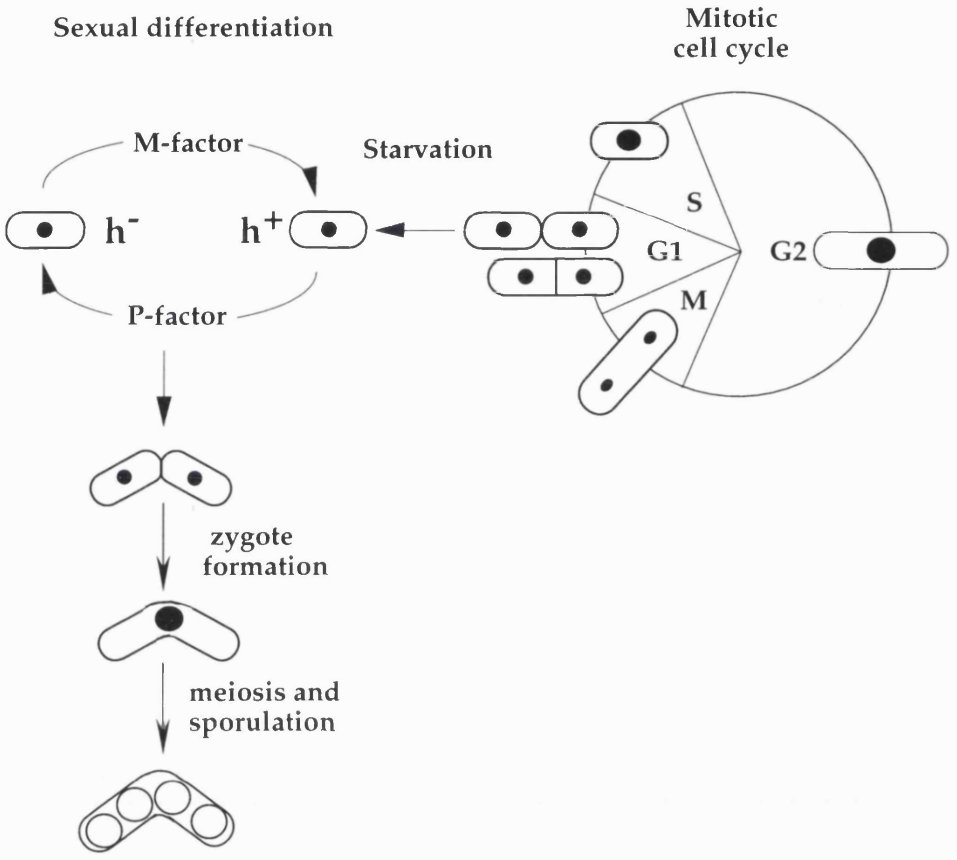


Figure 1.4

The fission yeast life cycle.

In the presence of sufficient nutrients, haploid fission yeast cells grow mitotically with a cell cycle in which the G2-phase comprises about 70% of the cycle, whereas G1, S and M-phases each take up 10% of the remaining portion of the cycle. Upon starvation, cells follow an alternative developmental fate and undergo sexual differentiation. h+ or P cells and h- or M cells conjugate in response to secreted mating type specific pheromone. P cells secrete P-factor and responds to M-factor while M cells produce M-factor and respond to P-factor. Meiosis and sporulation immediately follow the formation of the diploid zygote, resulting in an zygotic ascus with four haploid spores.

CHAPTER 2

The relationship between *cdc10* and *cdc2* gene functions.

Introduction

In this Chapter, I begin by analysing the relationship between CDK activity and the periodic transcription required for S-phase. This analysis was prompted by work in *S. cerevisiae* and mammalian cells which suggests that oscillations in CDK activity, and the exchange between G1 and G2 specific cyclin partners for Cdc28p, control the periodicity of S-phase transcription through the cell cycle. First of all I pose the question: in *S. pombe* are *cdc2* and its known cyclin partners required for the activation of *cdc10* dependent transcription in G1 cells? Periodicity requires both an on and an off switch, so this question can be extended to ask whether CDK activity controls the status of S-phase transcription in cells in S-phase and G2. Finally this leads me to several conclusions concerning the relationship between *cdc2* and *cdc10*, and the timing of their G1 function with respect to the onset of S-phase.

Cdc2p is not required for active *cdc18* transcription in G1 cells.

I began by investigating whether cdc2p is required in G1 for the activation of *cdc10* dependent "S-phase" transcription. In these experiments, *cdc10* dependent transcription was monitored by assessing the levels of several target transcripts. However, I have focused on the regulation of expression of a key target of *cdc10*, *cdc18* (Kelly, *et al.*, 1993). Firstly, I used $cdc2^{ts}$ mutations to perturb *cdc2* function in G1 cells. This requires the prior arrest of cells in G1, because mutations in *cdc2* arrest cells at both G1/S and G2/M at the restrictive temperature (Nurse and Bissett, 1981). I used nitrogen starvation to pre-synchronize a population of $cdc2^{ts}$ cells in G1. *cdc10* dependent transcription was monitored as cells proceeded towards S-phase after the re-addition of nitrogen, in the presence and absence of *cdc2* function (at the permissive and restrictive temperatures). The alleles of *cdc2* most severely compromised for progression through G1, *cdc2-M26* and *cdc2-33*, were used (MacNeill *et al.*, 1991; Broek, *et al.*, 1991). Similar results were obtained with both mutants.

In the first experiment (Figure 2.1), temperature sensitive *cdc2-M26* cells were synchronised in G1, by nitrogen starvation at 25°C and re-fed allowing them to resume growth and re-enter the mitotic cell cycle. As expected, after the addition of nitrogen, S-phase began within 3 hours at 25°C, as measured by FACS analysis (indicated by the arrow, FACS data not shown), but did not take place at the restrictive temperature, of 36.5°C. As a measure of *cdc10* dependent transcription at the two temperatures, RNA was isolated and Northern blotting used to assess levels of *cdc18* and *his3* messages. *cdc18* mRNA levels were low in nitrogen starved cells, but began to increase, in this experiment, 1.5h after the addition of nitrogen at 25°C; about 1 hour before the onset of S-phase (Figure 2.1a). This suggests that *cdc18* transcription is activated in small G1 cells some time before they reach the critical size required for the onset of S-phase (Nurse, 1975; Nurse and Thuriaux, 1977). A similar increase in *cdc18* transcript levels was observed at 36.5°C (Figure 2.1a), although since *cdc2* is functionally inactivated at this temperature, cells were unable to enter S-phase (see Figure 2.2c). Quantification, by phosphor-imager analysis, of *cdc18* mRNA levels with respect to those of *his3*, confirmed that there is little difference between transcription at 25°C and 36°C (data not shown).

These data suggest that transcription of *cdc10* targets is independent of *cdc2* activity in G1. It is possible however that transcription of S-phase genes is subject to both *cdc2* dependent and independent controls. To test this, I carried out an experiment to determine whether the levels of *cdc18* mRNA would increase further on release from the G1 *cdc2* block. I repeated the protocol from Figure 2.1a using the *cdc2-33* allele (Figure 2.1b). Cells were first arrested in G1, re-fed with nitrogen at 36.5°C for 3 hours, and then shifted to 25°C to allow them to enter S-phase. *cdc18* message levels were followed using *cdc2* mRNA as a control. After the shift to the permissive temperature there was no further increase in *cdc18* mRNA levels. This shows that the re-activation of *cdc2* in G1 does not augment the activity of *cdc10* dependent transcription. The levels of *cdc18* mRNA fell dramatically as cells passed through into S-phase and G2. (In similar experiments S-phase was complete after approximately 1 hour at 25°C).

In order to assess whether residual cdc2p kinase activity is likely to be present in $cdc2^{ts}$ cells in G1 at the restrictive temperature, the H1 histone kinase levels associated with cdc2p were determined in cdc2p immuno-precipitates from extracts of wildtype and *cdc2-M26* cells released from nitrogen starvation at 36.5°C (Figure 2.2a). In both cases, cells were able to grow (Figure 2.2c right hand panels), although *cdc2-M26* cells failed to enter S-phase (Figure 2.2c left hand panels). As in Figure 1a, *cdc18* transcripts steadily accumulated in the *cdc2-M26* strain at 36.5°C (Figure 2.2b). In the wildtype, *cdc18* mRNA levels rose transiently and then fell as most cells passed through into S-phase and G2. The H1 kinase assays were carried out at 36.5°C (Figure 2.2a) and quantified by phosphor-imager analysis. In the *cdc2-M26* strain, H1 kinase activity at the time when *cdc18* mRNA began to accumulate, was only 0.13% of that seen in the wildtype strain. In the control experiment, the H1 kinase activity peaked at around 3 hours, as cells passed from G1 into G2 (probably as a result of the accumulation of B-type cyclins late in G1 (Hayles, *et al.*, 1994; Fisher and Nurse, 1995; Fisher and Nurse, 1996), whereas *cdc18* message accumulated to maximal levels within 2 hours of the addition of nitrogen (Figure 2.2b right hand panel). Therefore, temporally, the peak of *cdc10* dependent transcription in the wildtype precedes the re-accumulation of the H1 kinase activity late in G1.

In conclusion, these first experiments demonstrate that *cdc10* is fully active in G1 in the absence of sufficient cdc2p activity to bring about S-phase. In

addition, the re-activation of cdc2p does not cause a further increase in *cdc10* activity. Since fission yeast cells arrested at the G1 *cdc2* block can conjugate, these cells remain pre-START (Nurse and Bissett, 1981). This observation is supported the finding that *cdc18* mRNA is able to accumulate while cells are still relatively small, early in G1 (Fantes and Nurse, 1977). In the wildtype, the increase in *cdc18* mRNA levels preceded the increase in cdc2p associated H1 kinase activity by an hour. These data indicate that the activation of *cdc10* dependent transcription is likely to precede the late G1 committment to the mitotic cycle. Therefore, although *cdc10* is often called the "START" transcriptional control e.g. (Zhu *et al.*, 1997), it is active before cells become committed to the mitotic cell cycle. In contrast, in *S. cerevisiae*, if cells are arrested pre-START using ts-alleles of *CDC28*, S-phase transcription is severely compromised [Peterson, 1985 ; Breeden and Nasmyth, 1987a ; Marini and Reed, 1992).

puc1 has a marked effect on passage through G1 in strains deleted for *cig1* and *cig2*, but this is not due to an effect on S-phase transcription.

In *S. cerevisiae*, Cln3p/Cdc28p activity is thought to activate S-phase transcription. In *S. pombe*, a cyclin homologous to *CLN3*, *puc1*, has been identified. Recently it was shown that in cell extracts, the puc1p/cdc2p complex is found physically associated with cdc10p. This CDK may therefore bind cdc10p and alter its activity, imparting it with cell cycle periodicity (Connolly *et al.*, 1997; Caligiuri *et al.*, 1997). This data was the first evidence that *puc1* is involved in mitotic cell cycle control, and suggested a functional similarity between the way the periodicity of this transcription is controlled in *S. pombe* and *S. cerevisiae*. However, the genetic evidence implies that *puc1* is not normally important for G1/S progression (Forsburg and Nurse, 1991; Forsburg and Nurse, 1994). In particular, while in *S. cerevisiae* the deletion of *CLN3* causes a significant S-phase delay (Cross, 1988; Nash, et al., 1988; Richardson, et al., 1989), disruption of the *puc1* gene has little or no mitotic phenotype. Also, the observations in Figures 2.1 & 2.2 show that in *S. pombe*, inactivating cdc2p in G1 cells, using $cdc2^{ts}$ alleles, has no effect on *cdc10* dependent transcription. Because cdc2p is the only identified cell cycle CDK in *S. pombe* and has been shown to bind puc1p (Forsburg and Nurse, 1994), this data suggests that *puc1* is unlikely to play a functionally homologous role to

CLN3. To resolve these conflicting observations, I decided to assess whether *puc1* has some effect on *cdc10* dependent transcription. To confirm that any effect due to the loss of *puc1* is not masked by the presence of other known cyclins acting in a redundant fashion, I used the strain *puc1Δ cig1Δ cig2Δ* (constructed and donated by S. Moreno), and assessed the activity of *cdc10* dependent transcription in a nitrogen starvation/release protocol (Figure 2.3). Early in G1, *S. pombe* is unable to accumulate cdc13p (Hayles, *et al.*, 1994), so in effect, until passage through late G1, the *puc1Δ cig1Δ cig2Δ* strain lacks all the known cdc2p cyclin partners implicated in cell cycle control.

Although *cig1Δ cig2Δ* cells have a relatively normal mitotic cell cycle (Fisher and Nurse, 1996), the additional removal of *puc1*, which by itself has no effect, causes a considerable extension of the G1 portion of the cycle in the triple deletion strain (Figure 2.3a top panel) and a corresponding increase in cell length. This is evident in the FACS analysis of exponentially growing cells. (Figure 2.3a lower panels, forward scatter is approximately proportional to cell volume). Wildtype cells starved of nitrogen undergo two rounds of division without much increase in mass, and as a consequence arrest in G1 as small cells. However, *puc1Δ cig1Δ cig2Δ* cells treated in the same way arrest in G1 without a concomitant reduction in mass. This implies that *puc1Δ cig1Δ cig2Δ* cells arrest in G1 of the first cycle following removal of nitrogen from the media. (This has also been observed by other groups, personal communication S. Moreno). This phenotype is not seen in strains deleted for *cig1Δ cig2Δ* (data not shown) (Fisher and Nurse, 1996; Caligiuri, *et al.*, 1997) and the deletion of *puc1* does not visibly alter the behaviour of *cig1* or *cig2* mutant cells (Fisher and Nurse, 1996). Thus, although *puc1* alone cannot bring about S-phase, it plays an important role in G1/S progression in mutants with compromised G1/S CDK activity. This observation emphasises the redundancy of cyclin functions in G1. Interestingly, although *puc1Δ cig1Δ cig2Δ* cells have more mass under starvation conditions than wildtype cells, after the re-addition of nitrogen they enter S-phase at approximately the same time as the wildtype; presumably after a similar increase in cell mass (Figure 2.3a). Therefore, it is unclear whether the cyclin deletion cells should be considered to be defective in entry into S-phase, or whether in the absence of *puc1*, *cig1* and *cig2*, the size control is adjusted, increasing the mass threshold necessary for passage through START.

In order to test whether *cdc10* dependent transcription is altered by the removal of puc1p activity, *cdc18* mRNA levels were measured by Northern analysis following the addition of NH_4Cl to *puc1Δ cig1Δ cig2Δ* cells arrested in G1 by nitrogen starvation. Despite the dramatic difference in size between the wildtype and the triple deletion strain it is clear that there is no detrimental effect of the cyclin deletions on *cdc10* dependent transcription. In fact, in the triple mutant, levels of *cdc18* mRNA in the starved state are elevated above those in the wildtype. This may reflect the difference in cell size in the two strains, which is likely to alter the physiology of the starved state. Puc1p is therefore unlikely to function in analogous fashion to Cln3p in *S. cerevisiae*, although in certain circumstances puc1p may play an alternative role in progression through G1. This experiment verifies the data obtained using ts-alleles of *cdc2*, and similar results were also obtained using *cig1Δ cig2Δ cdc13-117* cells released from nitrogen starvation at 36.5°C (data not shown) and with a *cig1Δ cig2Δ cdc13Δ* strain arrested in G1 (Figure 2.6a). Thus, although *cdc10* dependent transcription has not been analysed in a quadruple cyclin deletion strain, it is likely that all four of the *S. pombe* cyclins known to be involved in progression through the cell cycle, *puc1*, *cdc13*, *cig1* and *cig2* are dispensable for active S-phase transcription in G1 cells. In cyclin deletion experiments however, a possible caveat exists in that other unknown G1 cyclins may be involved in controlling passage through G1 into S-phase.

cdc10 is active in a pheromone induced cell cycle arrest.

As an additional test to assess the activity of *cdc10* in pre-START cells, *cdc10* dependent transcription was assayed by measuring the level of *cdc18* mRNA in cells entering a pheromone induced G1 arrest (Figure 2.4). This was done in the *cyr1Δ sxa2Δ* strain. The *cyr1* gene encodes adenylate cyclase, and the deletion mimics nitrogen starvation which is a prerequisite for arrest in G1. The Sxa2p pheromone protease must also be deleted to enable an efficient and prolonged pheromone signal (Imai and Yamamoto, 1994; Davey and Nielsen, 1994). Close to 100% of *cyr1Δsxa2Δ* cells are arrested in G1 6 hours after the addition of P-factor (FACS data not shown). *cdc18* mRNA accumulated in the arrest, probably reflecting the synchronization of cells as they all enter G1. This agrees with published data showing that *cdc18* is expressed in a P-factor induced arrest, and that this requires *cdc10* function (Stern and Nurse, 1997). These data show that, once again, *cdc10* dependent

transcription is active in cells arrested prior to START in G1. This contrasts dramatically with the situation in *S. cerevisiae,* where pheromone inhibits S-phase transcription, (reviewed in Hirsch and Cross, 1992)and see Figure 6.1). *rep1* transcript levels remained constant (with respect to *his3* mRNA, data not shown) but *rep2* expression increased in parallel with that of *cdc18*. The meiotic *cdc10* complex is thought to require the transcriptional induction of *rep1* and the inactivation of *rep2* transcription. Rep1p may then mediate the switch, from a mitotic, to a meiotic *cdc10* complex (Sugiyama *et al.*, 1994; Nakashima, *et al.*, 1995). For this reason, the observed changes in *rep1* and *rep2* transcript levels imply that the expression of *cdc18,* in the pheromone arrest, is probably still driven by a mitotic *cdc10* complex.

cdc2 is not required for *cdc10* activity prior to entry into a re-replicative S-phase.

In a second set of experiments, I studied the *cdc2* requirement for the activation of *cdc10* dependent transcription, in *cdc2-33* cells re-entering S-phase from G2 (Figure 2.5). *cdc10* function has been shown to be required for *rum1* induced endoreduplication (Moreno and Nurse, 1994) and re-replication as a result of the deletion of *cdc13* (Fisher and Nurse, 1996). Therefore, before the re-initiation of DNA synthesis, *cdc10* function is likely to be activated. Importantly, this experiment provides a different test from that of previous experiments, in which the activity of the *cdc10* complex was assayed after release from nitrogen starvation. Prolonged starvation may itself alter the transcriptional activity of *cdc10,* and it is conceivable that a G1 CDK is active prior to the G1 arrest. On the other hand, in cells induced to re-replicate, it is possible to test whether *cdc10* can be re-activated from the "off" or G2 state in the absence of *cdc2* function.

Cells in this experiment were first arrested in G2 at the restrictive temperature for *cdc2-33,* in the absence of nitrogen, and then subjected to a brief heat treatment at 49°C (Broek, *et al.*, 1991) (starvation helps cells to survive the heat shock). This procedure inactivates the G2 form of cdc2p and thereby allows cells to undergo an additional round of DNA synthesis. At the permissive temperature of 28.5°C, G2 cells re-replicated their DNA 4-5 hours after the re-addition of nitrogen (Figure 2.5b). (While the $cdc2^{ts}$ gene is able to function normally at 28.5°C, growth and recovery from heat

shock occur more rapidly at this temperature than at 25°C). However, when cells were incubated at 36.5°C, with functionally inactive *cdc2*, they failed to re-replicate. After the heat treatment, the levels of *cdc18* mRNA were very low, but *cdc18* mRNA re-accumulated to a peak level, sufficient to bring about S-phase, 4 hours after the addition of nitrogen at the permissive temperature. A similar increase in the level of *cdc18* transcript was seen at 28.5 and 36.5°C, i.e. in both the presence and absence of *cdc2* function (Figure 2.5a). This suggests that the re-activation of *cdc10*, prior to endoreduplication, does not require an active *cdc2* kinase.

Next, *cdc2-M26* and *cdc13-117* strains were subjected to the same protocol and *cdc10* dependent transcription assessed, before and after the heat shock (Figure 2.5c/d). *cdc2-M26* mutant cells were allowed to recover at both the permissive and the restrictive temperature. (As expected, cells were unable to enter S-phase at 36.5°C (Figure 2.5d)). 5 hours after recovery from the heat shock at 36.5°C, a portion of the *cdc2-M26* culture was shifted to the permissive temperature. Firstly, this confirmed that cells were primed for re-replication. This also allows me to test whether *cdc10* activity is altered following the restoration of *cdc2* function. The *cdc13-117* strain began to re-replicate with high efficiency, 5 hours after the heat shock at 36.5°C. In this strain, *cig1* and *cig2* cyclin/CDK complexes remain active after the treatment and can initiate DNA synthesis (Hayles, *et al.*, 1994) (Figure 2.5d: bottom panel). Therefore, this serves as a good control for the effects of temperature on *cdc10* dependent transcription during endoreduplication.

As in the previous experiment, *cdc10* was re-activated to a similar extent at the permissive and restrictive temperatures in the *cdc2-M26* mutant strain. In addition, shifting the culture recovering from the heat shock at 36.5°C, to 25°C, to re-activate the ts-*cdc2* function, did not lead to a further increase in S-phase transcription, although cells were now able to enter S-phase. In this case, *cdc18*, *cig2* and *cdt1* transcripts were monitored. All accumulated to a similar extent, at both the restrictive and permissive temperature after re-feeding, and to levels exceeding those observed before the heat shock. However, slight differences were observed in the responses of different *cdc10* targets and, interestingly, *cdt1* levels seemed to peak and then fall while in the *cdc2* block. It is unclear whether the differences between target genes reflects additional controls acting over their transcription. In the *cdc13-117* strain, 3 hours after release from the heat shock, (and prior to re-

replication), the expression of *cdc18, cdt1* and *cig2* was induced, to levels above those seen in G2 cells (compare Figure 2.5c, lanes 15, 18 and 19). In this experiment, *cdt1* mRNA accumulation slightly preceded that of *cdc18*. These data confirm the observation that *cdc10* is re-activated in cells induced to re-replicate by the selective loss of cdc2p/cdc13p activity. It is clear from the levels of *his3* mRNA in this experiment, that the treatment used to induce endoreduplication may affect levels of polII transcription in general.

In the experiments presented thus far, although the inactivation of *cdc2* function prevents cells entering S-phase, it cannot be ruled out that the residual activity of cdc2p at the restrictive temperature is sufficient to activate *cdc10* dependent transcription. However, this seems unlikely given the low levels of *in vitro* H1 kinase activity detectable at the restrictive temperature, and the similar results observed in the cyclin deletion strain. Therefore, it can be concluded, with some confidence, that prior to S-phase, *cdc10* dependent transcription is likely to be active, to a similar extent, in the presence or absence of *cdc2* function. Interestingly, a further implication of these experiments is that the complete inactivation of *cdc2* in G2 induces entry into a physiological G1 state with active *cdc10* dependent transcription. As *cdc10* target transcripts accumulate approximately 2 hours before the second discrete round of DNA replication, this G1 like phase may precede S-phase by some time. Once the cell cycle has been "re-set" the cell may have no choice but to re-enter the cell cycle from G1.

cig1, cig2 and cdc13 are required after *cdc10* function to bring about the onset of S-phase.

The experiments described above show that *cdc10* is active in G1 in the absence of *cdc2* function. A prediction of this finding is that *cdc2*, together with *cig1, cig2* and *cdc13* B-type cyclins, acts downstream of *cdc10* to bring about S-phase. To test this hypothesis I constructed a strain containing the temperature sensitive *cdc10-V50* mutation, deletions of *cig1* and *cig2*, while the remaining cyclin, *cdc13*, was placed under control of the thiamine repressible promoter (Fisher and Nurse, 1996) (Figure 2.6). This allows *cdc10* dependent transcription and CDK activity to be manipulated independently. As a control, the parent strain, *nmt-cdc13 cdc10-V50 cig1*$^{+}$*cig2*$^{+}$, containing the wildtype *cig1* and *cig2* genes was used. First, cells were shifted to the

restrictive temperature for 3 hours to inactivate *cdc10* and to arrest the majority of cells in G1. Then the control and experimental cultures were split in two, and *cdc13* was switched off in half of each culture by the addition of thiamine. After a further 1 hour at 36°C, cells were shifted back to 25°C, re-activating *cdc10* function in the presence or absence of the B-type cyclins. APC mediated proteolysis is active in early G1 cells, ensuring that cdc13p is rapidly degraded in the *cdc10* arrest. Therefore, after release from the block, in the presence of thiamine, cdc13p was reduced to a level of only 2% of that seen before the arrest (Figure 2.6b: quantified using a densitometer). (Hayles, *et al.*, 1994) . This level of cdc13p was insufficient to push cells into S-phase (Figure 2.6c). This experiment shows that at least one of the known B-type cyclins is required to drive the onset S-phase after the activation of *cdc10*.

Proof that these B-type cyclins are required for S-phase, after the activation of *cdc10,* requires the additional demonstration that *cdc10* is re-activated in these cells, in the absence of *cig1*, *cig2* and *cdc13*. I used *cdc18* mRNA levels as a measure of *cdc10* dependent transcription. The level of *cdc18* transcript increased to a maximum within 1.5h of the release from the *cdc10* block, to a similar level in the presence or absence of G1 cyclins (Figure 2.6a). It is possible that *cdc18* mRNA re-accumulation is slightly delayed in the absence of *cig1* and *cig2* genes. However, this experiment supports the idea that *cdc10* dependent transcription in G1 can occur independently of *cdc2*, and shows that *cig1*, *cig2* and *cdc13* are required for S-phase downstream of *cdc10* in G1.

I also looked at the accumulation of cdc18p. Interestingly, after release from the *cdc10* arrest, I observed vast differences in cdc18p levels between the control and experimental strains, despite the similar levels of *cdc18* mRNA. cdc18p levels were found to be low in exponential cells (when most cells are in G2 of the cycle), and in G1 cells in the absence of the *cdc10* function (Figure 2.6b). However, within 2h of shift to 25°C, cdc18p accumulated to a high level in the cyclin deletion strain; exceeding that seen in *cig1*$^+$*cig2*$^+$ cells which enter S-phase (Figure 2.6b). This observation confirms that after release from the block the level of *cdc10* activity in the cyclin deletion strain is probably sufficient for S-phase. Also, the elevated levels of cdc18p in the cyclin deletion strain hint at the possibility that CDK activity may promote the degradation of cdc18p. This possibility is explored further in Chapter 4

(see Figure 4.6). One possible conclusion from this experiment is that cdc2p/cyclinB complexes act late in G1 to bring about S-phase, by phosphorylating targets of *cdc10* such as cdc18p.

Next I assessed the residual cdc2p kinase activity in this experiment (Figure 2.6d). In all cases, CDK activity remained at relatively low levels in the *cdc10* block. This is due to the fact that *cdc10* is required for inactivation of the APC and/or for the degradation of rum1p (Hayles, *et al.*, 1994; Correa-Bordes and Nurse, 1997). Subsequently, CDK activity re-accumulated within 90 minutes of the shift to the permissive temperature in both the cyclin deletion strain expressing cdc13p and in the $cig1^+$ $cig2^+$ strain in the presence of thiamine (Figure 2.6d). However, after 90 minutes at 25°C, the level of cdc2p associated H1 kinase activity in the absence of the three B-type cyclins reached only 1.5% of that observed in the control population which continue to express *cdc13* from the *nmt* promoter and which are able to enter S-phase. The residual kinase may be due to puc1p, other unknown G1 cyclins, or the result of leaky expression of *cdc13* from the *nmt* promoter in its repressed state. In the $cig1^+$ $cig2^+$ strain, depleted of cdc13p, H1 kinase activity reached a maximum level of only 12% of that of cdc2p/cdc13p alone (in the *cig1Δcig2Δ* strain). This probably includes 1.5% residual H1kinase activity and is sufficient for S-phase. The absence of *cdc13* in this strain delayed S-phase by at least 30 minutes. It is usually assumed that *cig2* controls S-phase in the wildtype, but this experiment suggests, that in bringing about S-phase, the overall level of kinase activity may be more important than the nature of the cyclins involved. This is a prediction of the quantitative model for the control of the cell cycle by CDK activity.

I augmented these results by investigating the activity of *cdc10*-dependent transcription following release from the *cdc10* block, in cells expressing *cdc13*, in either the presence or absence of *cig1* and *cig2* cyclins (Figure 2.7). In both cultures cells enter S-phase after between one and 2 hours at 25°C (Figure 2.7c). *cdc18* mRNA was analysed and quantified with respect to a control (Figure 2.7b). As in Figure 2.6a, *cdc18* transcription was re-activated with a similar profile in the presence and absence of *cig1* and *cig2* . A slight elevation in the level of *cdc18* transcripts was observed in the exponential population of *cig1Δ cig2Δ* cells. This may result from a moderate extension of the G1 period in these cells. This experiment confirms the conclusion

that alterations in the level of CDK activity do not have a profound affect on the activity of *cdc10* dependent transcription.

It is possible that cdc2p/cdc13p activates *cdc10* prior to the $cdc10^{ts}$ block, and that the complex retains a molecular memory of this active state at the restrictive temperature. While this explanation is considered unlikely, because the cdc2p/cdc13p kinase cannot be assembled in a *cdc10* block, an experiment was done to test whether the inactive *cdc10* complex can reform and drive transcription at the permissive temperature without *de novo* synthesis of protein (Figure 2.7d). *cdc10-V50* cells were arrested in G1 at 36°C for 4 hours and cyclohexamide was then added as cells were shifted to the permissive temperature. In strains containing or lacking *cig1* and *cig2* genes, the addition of cyclohexamide caused a moderate increase in the level of control transcripts in proportion to rRNA levels. However, cyclohexamide prevented the re-accumulation of *cdc10* targets. In the cyclin deletion strain a smeared signal remained. Therefore, protein synthesis is required for the re-activation of *cdc10* dependent transcription following a *cdc10* ts-arrest. The simplest explanation for the requirement of protein synthesis is that cdc10p must be re-synthesized following the *cdc10* block. This reduces the likelihood of a scenario in which cdc10p remembers its prior activation by CDK mediated phosphorylation while in the block at the restrictive temperature.

In conclusion, these "cyclin switch off" experiments show that the B-type cyclins, which are responsible for the vast majority of the measurable H1 kinase activity in G1 cells, are dispensable for active S-phase transcription. We cannot exclude the possibility that other cyclins are present in these cells which can form a CDK complex capable of activating *cdc10* dependent transcription; although, if these hypothetical cyclins exist, they do not make a significant contribution to the overall cdc2p associated H1 kinase activity in G1 cells. These data also indicate that *cig1*, *cig2* and *cdc13* probably act after *cdc10* and after the accumulation of cdc18p, to bring about entry into S-phase.

The timing of the *cdc2* requirement in G1 for entry into S-phase.

The experiments presented so far in this Chapter show that *cdc2* function is not required in G1 for the activation of S-phase transcription, or for the

accumulation of cdc18p, one of the key targets of this machinery. This indicates that in *S. pombe*, CDK activity is required late in G1 for the onset of DNA replication. In order to get a better idea of the point at which the S-phase function of *cdc2* is complete, several additional experiments were conducted using *cdc2* ts-alleles arrested at the *cdc2* block point in G1 (Figure 2.8). In the first experiment, cells were released from nitrogen starvation into the G1 *cdc2* block, and after 3 hours at 36.5°C, the culture was shifted to the permissive temperature. At various time points after the release, aliquots of cells were returned to the restrictive temperature to determine the point at which *cdc2* completes its function (Figure 2.8a lower panel). Cells were then sampled by FACS analysis, 90 minutes after the initial release. Between 30 and 45 minutes at the permissive temperature were sufficient to allow all the cells to eventually pass through into G2 (Figure 2.8a lower panel). This correlates with the timing of the onset of S-phase (Figure 2.8a upper panel). These data suggest that although cells spend 2-3 hours in G1 upon release from nitrogen starvation, *cdc2* function probably acts close to the boundary of G1/S phase.

As shown in Figure 2.6b, cdc18p is able to accumulate in cells arrested in G1 in the absence of *cdc2* function. Therefore it is likely that these cells are poised at the boundary of S-phase, with all the machinery necessary for S-phase already assembled, waiting for the *cdc2* cue to initiate DNA replication. To test this hypothesis, I decided to see whether after the shift to the permissive temperature, cells at the *cdc2* block point require the synthesis of additional proteins to enter and complete S-phase (Figure 2.8b right hand panel). Addition of cyclohexamide to the culture, simultaneously with the release to the permissive temperature, prevented DNA replication (Figure 2.8b). Unfortunately, this outcome is not necessarily informative. It is likely that the denatured ts-cdc2p is unable to refold efficiently and it may be degraded in the block (Broek, *et al.*, 1991). Therefore this result probably reflects the need for *de novo* synthesis of cdc2p, rather than a requirement for the synthesis of additional functions downstream of *cdc2*. If cells are incubated at 25°C for 15 minutes prior to the addition of cyclohexamide, DNA synthesis does occur and cells pass into G2 (Figure 2.8b; left hand panel, last FACS profile). 15 minutes may constitute the time required to synthesize enough cdc2p to carry out its function. Interestingly, $cdc2^{ts}$ cells at the G2/M transition can enter mitosis after

shifting to 25°C in the presence of cyclohexamide; although the drug dramatically alters the kinetics of the process (Figure 3.4).

In the above experiment, I also monitored *cdc10* target transcript levels (Figure 2.9). As expected, *cdc18* and *cig2* message levels were already elevated in the *cdc2* G1 block and thus decreased after the release at 25°C, as cells completed S-phase. Upon release from the block, *cig2* mRNA levels fell away somewhat more slowly than those of *cdc18*. This result is likely to reflect a difference in the stability of *cig2* and *cdc18* transcripts. Interestingly though, the addition of cyclohexamide had an unexpected effect on the levels of *cdc10* target transcripts. Cyclohexamide induced a dramatic and rapid increase in the level of *cdc10* target transcripts, including *cdc18*, *cig2* and *cdt1* (data not shown), in cells released from the G1 *cdc2* block point. When standardised against *ura4* message levels, and quantified by phosphor-imager analysis, *cdc10* target transcripts were found to accumulate to levels 10 fold higher than those observed in G1 or S-phase cell cycle arrests. In contrast, the addition of cyclohexamide to cells in the *cdc2* block, at 36.5°C, only had a modest effect (Figure 2.9a : last lane). Therefore the increased levels of *cdc10* target transcripts are partially dependent on *cdc2* activity or on temperature. Cyclohexamide also caused a slight increase in the proportion of mRNA to rRNA as evident from the *ura4* signal, (all samples contain approximately 10µg of total RNA), as in Figure 2.7d. The treatment may therefore alter the overall transcription or stability of polII, polIII and polIII transcripts. However, of the mRNAs tested, *cdc10* target transcripts, including those of *cdc18*, *cdt1* and *cig2*, were the only ones whose levels were dramatically affected by the addition of cyclohexamide. Nevertheless, it is possible that these constitute an unrepresentative group of mRNAs, which are particularly unstable.

I carried out an additional experiment to assay whether this effect of cyclohexamide on *cdc10* target transcripts was specific to a particular stage of the cell cycle. To do this, cyclohexamide was added to *cdc10-V50* cells arrested in HU (for 4 hours at 25°C). *cdc10* dependent transcription is maximally active in these cells (Figure 2.9b left hand panel). As cyclohexamide was added, half the culture was simultaneously shifted to the restrictive temperature for *cdc10* (Figure 2.9b right hand panel). The addition of cyclohexamide did not induce an increase in *cdc18* transcript levels at 25°C. Also, *cdc18* transcripts rapidly disappeared in cyclohexamide

at 36°C, indicating that transcript stability was not dramatically altered by the addition of cyclohexamide. These data support the notion that the effect of cyclohexamide may reveal a specific cell cycle control over *cdc10*-dependent transcription. This peculiar observation could reflect the existance of an unstable inhibitor of the transcription complex which is rapidly degraded in G1 cells upon addition of cyclohexamide. A similar elevation in the level of *cdc10* target transcripts has been observed in one other situation, at low temperatures in the *cdc10-C4* strain. This mutant carries a truncated *cdc10* allele which encodes a protein lacking its C-terminal portion. Therefore, the putative inhibitor could bind the tail of cdc10p (McInerny, *et al.*, 1995). This portion of the protein has also been shown to interact with res1p and res2p partners (Sturm and Okayama, 1997; Zhu, *et al.*, 1997). In Chapter 5, evidence is presented which suggests that the activity of *cdc10* is controlled in part by res2p mediated inhibition of transcription (Figure 5.1). It is conceivable that an inhibitor of *cdc10* dependent transcription, such as res2p, could alter the activity of S-phase transcription, by interacting with the C-terminus of cdc10p. However, further experiments are needed to ascertain whether the effect of cyclohexamide on S-phase transcription reveals a specific physiological control over periodic transcription, or is simply an experimental artefact.

cdc10 function is required to maintain *cdc18* mRNA levels in early S-phase.

Next, I decided to extend the observation that *cdc2* function is not required for the onset of *cdc10* dependent transcription, and to investigate the control of *cdc10* dependent transcription during S-phase and in G2 cells. I first verified that *cdc10* dependent transcription was active in cells arrested in HU, at the onset of S-phase (Figure 2.10a). This was done by first arresting wildtype and *cdc10-129* cells in HU at 25°C and then shifting both cultures to 36°C for 30 minutes, to inactivate *cdc10*. (The continued presence of HU prevents cells from entering S-phase). While, at the permissive temperature, *cdc18* transcript levels were elevated in both wildtype and *cdc10-129* cells in HU (Figure 2.10a; lanes 2 and 5), the *cdc18* mRNA signal was essentially undetectable in *cdc10* cells at the restrictive temperature, (Figure 2.10a compare lanes 3 and 6). Thus, in S-phase, *cdc10* function is required continuously to maintain *cdc18* transcript levels. In a second experiment, (Figure 2.10b), wildtype cells were first arrested in HU and then

released, by washing cells free of the drug, into S-phase and G2 and *cdc18* mRNA was monitored. As expected, *cdc18* mRNA levels fell rapidly as cells passed through S-phase, remaining low in G2 cells.

cdc10 function is not required after HU for the completion of S-phase.

These results show that *cdc10* is active at the onset of S-phase. Nevertheless, *cdc10* function may not be required during S-phase. To determine whether active *cdc10* dependent transcription is required after HU for completion of DNA replication, the HU block and release experiment was repeated with a slight variation; *cdc10-V50* cells were arrested at 25°C in HU, shifted to 36°C for 30 minutes and then HU washed out at the restrictive temperature (Figure 2.10c). Strains carrying *cdc22-M45* and *cdc23-M36* mutations were used as controls. (These ts-mutations only impair but do not totally block progression through S-phase at the restrictive temperature). Approximately 30 minutes after washing free of HU, the majority of *cdc10-V50* and wildtype cells had completed S-phase, whereas in *cdc22* and *cdc23* mutant cells, passage through S-phase was significantly delayed. This suggests that *cdc10* is not required once cells enter early S-phase.

cdc2 function is not required in S-phase or in G2 for the appropriate control of periodic *cdc10* dependent transcription.

To investigate whether cdc2p plays a role in maintaining *cdc10* dependent transcription during S-phase, or in switching off this transcription at the end of S-phase, I monitored *cdc18* transcript levels, first in an HU block, with and without *cdc2* function, and secondly after release from HU into mutant blocks lacking mitotic CDK activity (Figures 2.11a and 2.11b). I utilised the CDK mutant strains *cdc2-33* and *cdc13-9*, which contain ts-mutations in cdc2p and cdc13p, the major cyclin partner in G2 and M cells. Cells were arrested at the G1/S boundary by the addition of HU, shifted to 36°C for 30 minutes and then washed free of HU using media at the restrictive temperature. In these strains, this treatment does not induce re-replication. *cdc18* mRNA levels remained high in the absence of *cdc2* function (Figure 2.11a lanes 1-3, data not shown for *cdc13-R9*). Therefore, active *cdc10* dependent transcription during S-phase does not require continued CDK activity. After 30 minutes at the restrictive temperature,

cells were washed with pre-warmed media to remove HU. Subsequently, *cdc18* transcript levels rapidly decreased in both strains (Figure 2.11a *cdc2-33* lanes 4 and 5, and *cdc13-9* lanes 3 and 4). The cdc2p associated H1 kinase activity was assessed in *cdc2-33* cells released from HU at 36.5°C, as cells entered G2. The level of H1 kinase activity was found to be only 0.75% of the level seen in wildtype cells in G2 (data not shown). Therefore, the moderate levels of *cdc2* kinase activity found in wildtype G2 cells are not required to keep *cdc10* dependent transcription switched off.

cdc2 function is not required after HU for completion of S-phase.

The HU block and release experiment was repeated using *cdc2-33* and *cdc2-M26* mutants. On released from the HU block, cells from both cultures were able to enter and complete S-phase, with similar kinetics to the wildtype (Figure 2.11b, compare to 2.10c). This suggests that, although *cdc2* function is essential for the onset of S-phase, it is not required for the completion of DNA replication once cells have reached the HU sensitive step.

The inactivation of *cdc10* dependent transcription in G2 cells occurs normally in cyclin deletion strains.

As with experiments earlier in the Chapter, the observation that *cdc10* activity in S-phase and G2 is unaffected by perturbations of a *cdc2* function in a $cdc2^{ts}$ mutant, was confirmed using cyclin deletion strains. Four strains were subjected to the HU block and release protocol: a *cig2Δ*, strain a *puc1Δ* strain, the triple cyclin deletion strain, *puc1Δ cig1Δ cig2Δ*, and a strain deleted for *cig1* and *cig2* and carrying a ts-*cdc13* allele, *cdc13-117* (Figure 3.12a). The first three strains were grown in HU, for 3.5 hours at 32°C, to arrest cells at the G1/S boundary. Close to 100% of cells arrested with a G1 FACS peak (data not shown). HU was then washed out in pre-warmed media, and cells followed as they completed DNA synthesis and entered G2. *cig1Δ cig2Δ cdc13-117* mutant cells were arrested at the beginning of S-phase in HU for 3.5h at 25°C (Figure 2.11a), shifted to 36°C to inactivate the ts-*cdc13* protein, and then released into S-phase and G2 at this temperature by washing cells free of HU. RNA was isolated and the levels of *cdc18* mRNA determined in a Northern Blot.

In these mutants, as in the previous experiments, there was very little observable effect of the reduction in CDK activity on *cdc10* dependent

transcription. Also, in the cyclin deletion cultures, the changes in the level of *cdc18* mRNA during the HU block and release were similar to those observed in wildtype cells in a similar experiment (Figure 2.10b).

Tyrosine phosphorylation of *cdc2* is not required in S-phase or in G2 for the appropriate control of periodic cdc10 dependent transcription.

Finally, an additional perturbation of *cdc2* function was used to test whether tyrosine phosphorylated *cdc2* is responsible for the down regulation of *cdc10* in G2. Cdc2p is tyrosine phosphorylated from late G1 until M phase (Hayles and Nurse, 1995). This closely parallels the period of inactive *cdc10* dependent transcription, (see Chapter 3). To investigate whether this plays a role in the control of *cdc10*, I subjected cells with defects in *cdc2* tyrosine phosphorylation, *wee1-50 and wee1-50 mik1Δ* mutants, (Nurse, 1990; Lundgren *et al.*, 1991), to a hydroxyurea block and release protocol (Figure 2.12b). Cells were arrested in HU, at 25°C, and shifted to 36°C to inactive the ts-*wee1* function. HU was then washed out, allowing cells to proceed through S-phase and into G2. These mutant cells then enter mitosis prematurely, as a result of the inability to phosphorylate cdc2p on tyrosine. RNA was therefore sampled 30 minutes after washing cells free of HU. Although, in this experiment, the RNA was not evenly loaded, *cdc10* dependent transcription can clearly be seen to be down regulated as cells enter G2. Interestingly, exponentially growing wee cells, e.g. *cdc2-3W* (Figure 2.12b, compare first and second lanes) and *wee1-50* cells growing at 36°C (see Figure 3.10), were found to express *cdc10* target transcripts at elevated levels. This is likely to result from the extended G1 period in wee mutant strains. However, if *cdc10* is first activated at the G1/S boundary, levels of *cdc10* target transcripts should be similar in wee and wildtype strains. The fact that they are not, implies that *cdc10* is active for a large portion of the G1 period, thereby corroborating an idea put forward earlier in the Chapter.

cdc10 dependent transcription is unlikely to be under the direct control of *pat1*.

pat1 maintains the mitotic character of the cell cycle. Pat1p does this primarily by phosphorylating and inactivating mei2p, the protein controlling entry into meiosis (Watanabe *et al.*, 1997). Recently is has been

suggested that *pat1* has additional roles in the mitotic cycle, and directly controls the choice between the mitotic and meiotic S-phase transcriptional machinery (Caligiuri, *et al.*, 1997). However, the effect of *pat1* mutations on *cdc10* dependent transcription has not been investigated. Therefore, I checked to see whether alterations in *pat1* activity affect *cdc10* dependent transcription in a relatively crude experiment (Figure 2.13). Firstly, the activity of *cdc10* dependent transcription was measured in exponentially growing wildtype cells, *pat1-114* ts-mutant cells and in cells deleted for *pat1* and *mei2* gene functions (Figure 2.13, lanes 1,3, and 5). In the latter strain, the removal of *mei2* prevents cells entering meiosis (Beach *et al.*, 1985; Shimoda *et al.*, 1985). This separates the effects of *pat1* function mediated by *mei2*, from other downstream effects. Cells were then shifted to 36°C for 30 minutes. This treatment briefly inactivates the ts-pat1p kinase in the *pat1-114* strain (Figure 2.13, lanes 2,4, and 6). This is probably insufficient time to induce the meiotic programme, therefore, any effects are likely to be a direct consequence of the action of pat1p on *cdc10*. Firstly, the levels of *cdc10* dependent transcription were the same in exponentially growing *pat1Δ* cells and *pat1*$^+$ cells. In this experiment, heat shock itself seemed to affect *cdc10* dependent transcription in all three strains. However, the levels of *cdc18* mRNA observed in both *pat1* mutant strains were identical to those of the wildtype before and after temperature shift. This suggests that *pat1* has no direct effect on *cdc10* activity. This result is not unexpected because the *pat1 mei2* double delete, despite its meiotic defect, is perfectly viable and shows no G1 peak by FACS (data not shown). The switch to the meiotic machinery, including downregulation of *rep2* expression and the up-regulation of *rep1* and then *res2* transcripts, requires starvation and conjugation. Therefore, if the mitotic transcriptional apparatus required active pat1p, the *pat1Δmei2Δ* mutant would be expected to exhibit defects in progression through G1 into S-phase. These are not observed and cells have a normal FACS profile (data not shown). Nevertheless, it is still possible that *mei2* and other elements of the meiotic program alter the *cdc10* dependent transcriptional machinery during normal conjugation and meiosis, and that in this way, *pat1* indirectly influences S-phase transcription.

Discussion

In this Chapter, I have presented a physiological analysis of the role of cdc2p in the regulation of the periodic expression of genes required for S-phase during the fission yeast mitotic cell cycle. Cdc2p protein kinase activity does not appear to be required for the activation of *cdc10* dependent transcription in G1, for the maintenance of this transcription during S-phase or for its repression, in G2 of the cell cycle.

I first showed that *cdc10* activity is independent of cdc2p using $cdc2^{ts}$ alleles in G1, S-phase and in G2. In these experiments, *cdc2* function at the restrictive temperature was unable to drive entry into S-phase and H1 kinase levels were reduced to less than 1% of the control level. However, this type of analysis is subject to the criticism that the barely detectable levels of residual CDK activity may be sufficient to activate *cdc10* dependent transcription. Another approach, which goes some way towards addressing this caveat, was to manipulate *cdc2* activity using strains deleted for various cyclin partners of cdc2p, and to observe the effects on *cdc10* dependent transcription. To do this, I used cells deleted for all of the cyclins known to be involved in cell cycle control, *cdc13*, *cig1*, *cig2* and *puc1*, in various combinations. These are the only cyclins implicated in controlling progression through the mitotic cycle in *S. pombe*. They also make up at least 98.5% of H1 kinase activity in G1 cells. Although these cyclins are required for both S-phase and mitosis, I was unable to observe a significant effect of their deletion on *cdc10* dependent transcription, in any portion of the cell cycle. I also obtained some evidence that even when *cdc10* is first inactivated, necessitating *de novo* protein synthesis in G1 before the resumption of transcription at the permissive temperature, *cig1*, *cig2* and *cdc13* are not required for renewed transcriptional activity. This implies that the protein (probably cdc10p) which is re-synthesized after the arrest, to re-activate transcription, is unlikely to have remembered an earlier signal mediated by these cyclins, such as CDK mediated phosphorylation. These experiments almost certainly rule out the involvement of *cig1*, *cig2* and *cdc13* in the control of *cdc10* dependent transcription. However, in experiments using the cyclin deletion strains it is possible that other unidentified cyclins may complex with cdc2p and activate *cdc10* dependent transcription. (An unidentified CDK could also be involved). I also showed

that mutations which prevent the phosphorylation of the regulatory tyrosine on *cdc2*, leading to its premature activation in G2 of the cell cycle, have no profound effects on the activity of *cdc10* dependent transcription.

This analysis shows the difficulty in ruling out an association between two biological functions. However, taking these findings together, it is possible to be relatively confident in the assertion that CDK activity in G1, S-phase or G2 does not control the activity of S-phase transcription. It is interesting to note that several groups, assuming that *cdc10* is controlled in an analogous way to S-phase transcription in *S. cerevisiae*, have reported the physical association of cdc10p and cdc2p. It is unclear what the functional consequences of this interaction are likely to be.

My observations in *S. pombe* contrast with those made in budding yeast. In *S. cerevisiae*, *CDC28* has been implicated in both activating S-phase transcription and repressing *SWI4/SWI6* dependent transcription (Tyers *et al.*, 1993; Dirick, *et al.*, 1995; Koch, *et al.*, 1996). In particular, temperature sensitive mutations in *CDC28* and the deletion of *CLN3* dramatically reduce the activity of S-phase transcription mediated by SW14p/SW16p and SWI6p/MBP1p (Breeden and Nasmyth, 1987a; Peterson *et al.*, 1985). Differences in the control of the homologous S-phase transcriptional machinery between the two yeasts also correspond to differences in the mechanism by which pheromone induces cell cycle arrest in the two organisms. The addition of pheromone to genetically sensitized *S. pombe* cells, inhibits *cdc2* activity causing G1 arrest, but, as shown, does not down-regulate *cdc10* activity (Stern and Nurse, 1997). In contrast, in *S. cerevisiae*, α-factor has a dramatic inhibitory effect on both G1 CDK activity and S-phase transcription (perhaps as a downstream effect of CDK inhibition) (Hirsch and Cross, 1992). Thus, CDKs may not have a universal function in controlling the activation of the periodic expression of genes required for S-phase.

In addressing the relationship between CDK activity and S-phase transcription, I also re-examined *puc1*, an *S. pombe CLN* type cyclin. Previous genetic experiments did not reveal a clear mitotic function for *puc1* (Forsburg and Nurse, 1991; Forsburg and Nurse, 1994). However, although the deletion of *puc1* had no apparent effect on the activity of S-phase transcription, I was able to identify a role for *puc1* in the progression

of *cig1Δ cig2Δ puc1Δ* cells from G1 into S-phase. These cells are viable and grow well, but are longer than wildtype *S. pombe* and spend a greater portion of the cell cycle in G1. Under starvation conditions, the deletion of *puc1*, in addition to *cig1* and *cig2*, has a more pronounced phenotype, and cells arrest in G1 in the first cycle following the removal of nitrogen from the media. Therefore, in rapidly growing cells, puc1p/cdc2p may act by contributing in a redundant fashion to the overall G1 kinase levels, and cells may invest more control in the activity of puc1p/cdc2p under starvation conditions. Perhaps in cases where *puc1* does play a role in progression through the mitotic cycle, puc1p/cdc2p may accelerate the inactivation of the APC. This is necessary to enable the accumulation of B-type cyclins required for S-phase. An analogous role has been proposed for the action of CLNs in *S. cerevisiae* (Amon *et al.*, 1994; Amon, 1997). Rather surprisingly, these experiments using the *cig1Δ cig2Δ puc1Δ* strain also show that in *S. pombe cdc13* alone, (in the absence of all other known mitotic cyclins), is able to drive an ordered cell cycle.

In this Chapter, I conducted experiments which address the timing of events from G1 through until S-phase. I observed active *cdc10* dependent transcription in several situations in cells which were effectively pre-START. Firstly, *cdc10* was active in relatively small G1 cells re-fed after nitrogen starvation (Fantes and Nurse, 1977). Secondly, *cdc10* dependent transcription was active in cells arrested pre-START at the G1 $cdc2^{ts}$ block (Nurse and Bissett, 1981) and is active in cells arrested by pheromone (Stern and Nurse, 1997). These observations suggest that the activation of *cdc10* dependent transcription and the accumulation of its major target, cdc18p, are not rate-limiting for the onset of S-phase. An additional interesting implication of the finding that *cdc10* is active early in G1 is that this enables *cdc10* dependent transcription in early G1 to provide the necessary gene products for a mitotic or meiotic S-phase, depending on the subsequent decision of cells to pass START. This possibility has already been suggested by genetic experiments (Miyamoto, *et al.*, 1994). This may not be so surprising as many aspects of DNA replication are likely to be fundamentally similar in both meiotic and mitotic S-phases. Therefore, the requirement for a control regulating the mitotic *vs* meiotic character of S-phase transcription may be less critical. This may explain why *pat1*, which is thought to be just such a switch, does not appear to directly alter the activity of *cdc10* dependent transcription.

I also explored more closely the temporal relationship between *cdc10* and *cdc2* functions in G1. In the wildtype, cdc2p protein kinase activity appeared to peak close to the onset of S-phase, more than an hour after *cdc10* target transcripts had reached their maximal level. This correlative analysis suggested that *cdc10* acts before *cdc2* function in G1. This view of the order of events is strengthened by the demonstration that B-type cyclins (cig1p, cig2p or cdc13p) are required after the activation of *cdc10* and the accumulation of its major target cdc18p, for S-phase to occur. This shows that cdc2p/B-type cyclin complexes act downstream of *cdc10* in G1. The fact that *cdc2* activity remains low in a $cdc10^{ts}$ block (Hayles, *et al.*, 1994) also supports this idea by showing that active *cdc10* function is a pre-requisite for the accumulation of CDK activity in G1. Some additional data was generated which narrows the window in time within which the wildtype *cdc2* function is likely to act. *cdc2* was shown to act close to the initiation of DNA replication itself. Also *cdc10* and *cdc2* were both found to be dispensable for the completion of DNA synthesis, after its initiation in HU (as in Naysmyth and Nurse, 1981). This implies that *cdc2* function is probably required right at the boundary of S-phase, after the accumulation of cdc18p, to commit cells to the mitotic cycle and to drive cells into S-phase; but is not required for the elongation phase of DNA synthesis. The G1 CDK activity in *S. pombe* may therefore be required to triggers origin firing by the phosphorylation of *cdc10* targets.

In this Chapter, I addressed the behaviour of *cdc10* dependent transcription in cells induced to re-replicate using ts-*cdc2* and ts-*cdc13* mutations. I found that *cdc10* was activated in cells re-entering S-phase from G2 prior to re-replication. This was already suggested to be the case by the observation that *cdc10* is required for *rum1* and *cdc13Δ* induced re-replication (Moreno and Nurse, 1994; Fisher and Nurse, 1996). In a $cdc2^{ts}$ mutant, the re-activation of *cdc10* dependent transcription occured irrespective of the temperature at which cells recovered from the heat shock. Interestingly, these data suggest that, while reducing the *cdc2* kinase activity in G2 by over 99% blocks mitosis without altering *cdc10* activity, a more complete inactivation of *cdc2* induces cells to re-replicate and re-activates *cdc10*. This suggests that there is a low threshold of CDK activity, beneath which cells forget their position in the cell cycle, causing them to re-enter the null or ground state, G1, without passing through mitosis. They then re-replicate as a consequence. There are

other less well characterised observations that imply that the "pseudo-G1" state, preceding a re-replicative S-phase, may include other aspects of the normal G1 state. It has been noted (Jacky Hayles personal communication) that in a *cdc2*^{ts} induced re-replication, *cdc13* levels may dip following the re-addition of nitrogen allowing the recovery from the heat shock. This suggests the possibility that G1 cyclin degradation is re-activated during re-replication. A similar effect can be postulated during the re-replication caused by the removal of *cdc13*. In this case, cig2p has been shown to be essential for the endo-reduplicative S-phase (Fisher and Nurse, 1996). However, constitutive expression of *cig2* also blocks this re-replication (Paul Russel and Jacky Hayles personal communication). These observations suggest that the decline and subsequent re-accumulation of *cig2* may be a prerequisite for the re-replication induced in the absence of *cdc13*. This may necessitate periodic *cdc10* activity, and possibly also oscillations in cig2p proteolysis. It will be interesting to determine whether G1 proteolysis and pheromone responsiveness are also induced in this re-replicative pseudo-G1 state. Proving this will require further work, in particular the development of a synchronous re-replication system which does not require nitrogen starvation or heat shock.

The idea that removal of G2 CDK activity causes cells to enter a pseudo-G1 state, may also be applied to *S. cerevisiae*. This suggests an alternative explanation for experiments done in budding yeast, where removal of *CLBs(1-4)* activated *SWI4/SWI6* dependent transcription (Koch, *et al.*, 1996). It was concluded from these experiments, that *CDC28* directly inhibits the Swi4p/Swi6p complex in G2 cells. An alternative hypothesis is that inactivation of Cdc28p/Clb(1-4)p has an indirect effect on S-phase transcription by resetting the nuclear cell cycle to a G1 like state. However, it should be noted that this procedure does not induce re-replication in *S. cerevisiae* (probably because of the maintenance of CLB5/6 activity), and does not activate MCB driven transcription (Koch, *et al.*, 1996).

The experiments presented in this Chapter alter the way S-phase transcription in *S. pombe* should be thought about. This led me to reassess the timing of *cdc10* dependent transcription. This work is described in the next Chapter.

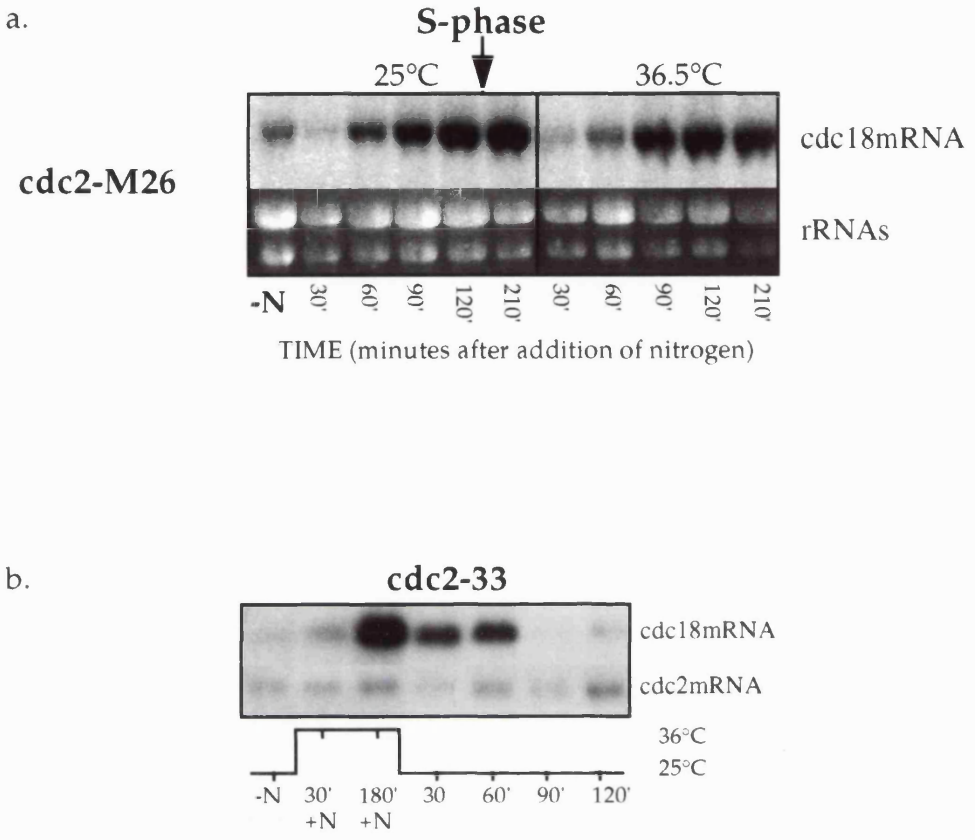


Figure 2.1

***cdc2* function is not required for the activation of *cdc10* dependent transcription following release from nitrogen starvation in G1.**

1a. *cdc2-M26* cells were arrested in G1 by nitrogen starvation and re-fed at 25°C or 36.5°C. A Northern Blot was probed for *cdc18*, rRNA was visualised in the gel using EtBr to control for sample loading. At 25°C cells entered S-phase after 3 hours (arrow), whereas at the restrictive temperature cells remained in G1, (FACS data not shown). In **1b**, *cdc2-33* cells were subjected to a similar procedure. Cells were released from a G1 starvation arrest at 36.5°C into the *cdc2* block. Then, after 3 hours, cells were returned to a permissive temperature (25°C) allowing re-entry into S-phase. RNA was isolated and a Northern blot probed for *cdc18* and *cdc2* transcripts.

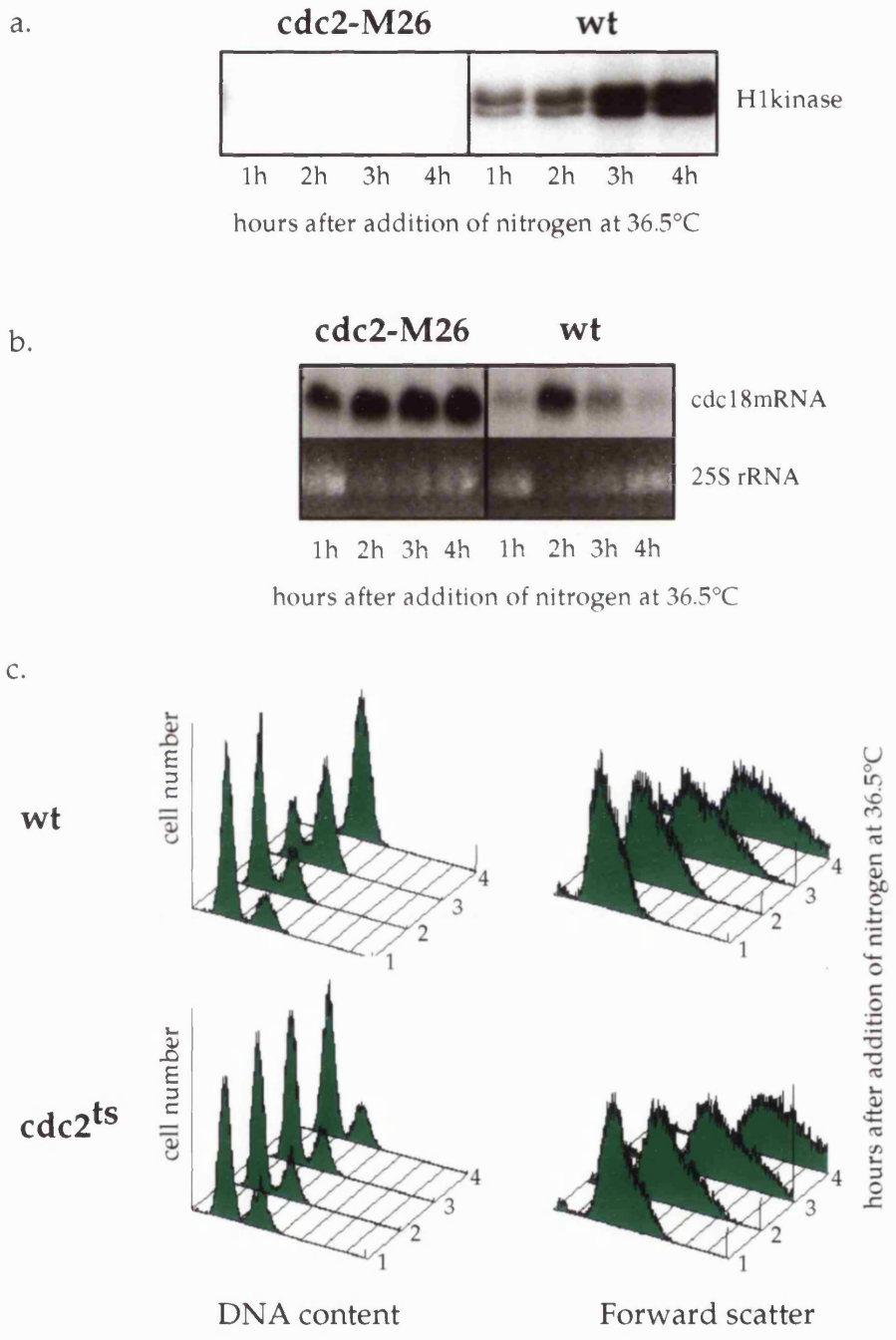


Figure 2.2

***cdc18* expression precedes the peak of H1 kinase activity, and *cdc18* mRNA accumulates in the absence of 99% of the wildtype cdc2p protein kinase activity .**

2a. Wildtype and *cdc2-M26* cells in G1 were re-fed with nitrogen at 36.5°C. H1 kinase assays were carried out at 36.5°C using cdc2p immunoprecipitates from wildtype and *cdc2-M26* cells. **2b.** A Northern Blot is shown of samples taken from the experiment in 2b, the blot was probed for *cdc18*, and EtBr used to visualise rRNA. **2c.** Samples from 2a/2b were taken for FACS analysis. Forward scatter (which represents cell mass) and DNA content are shown on separate histograms.

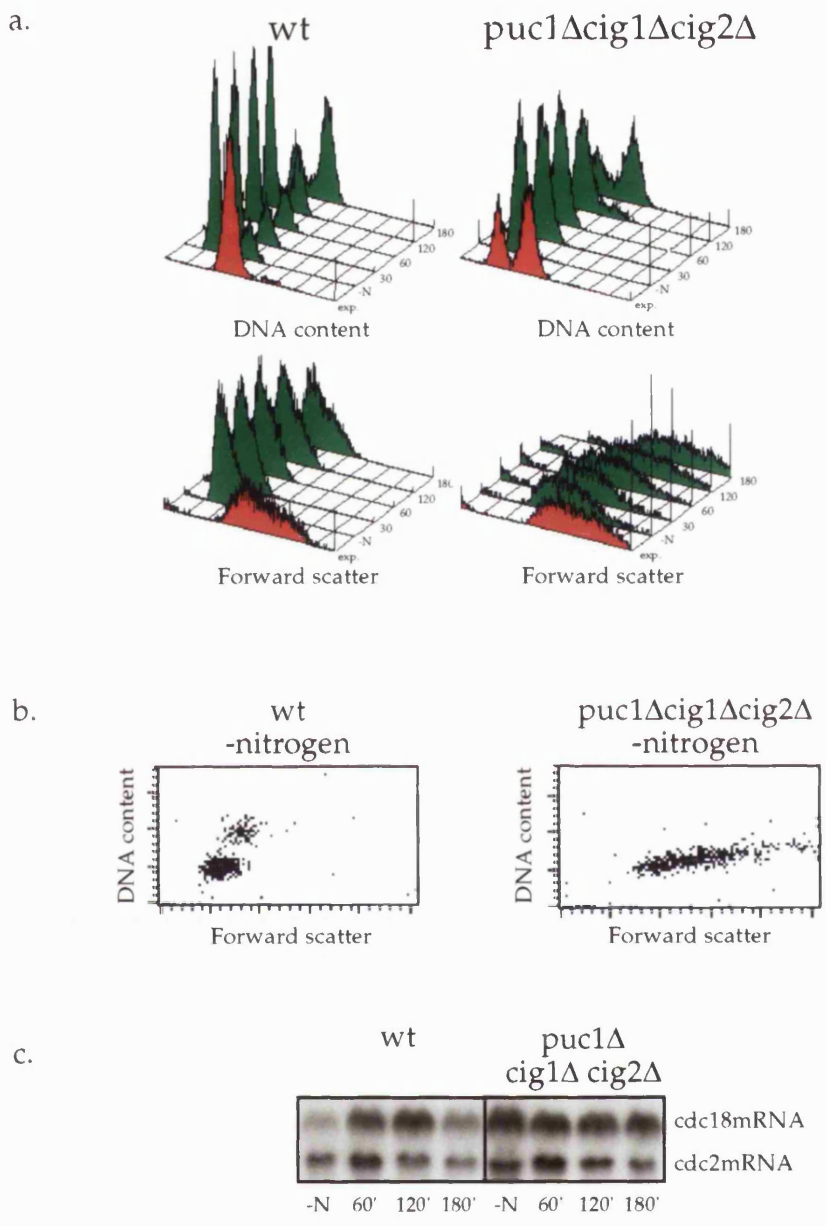


Figure 2.3

***S. pombe* cyclins, *cig1, cig2* and *puc1* are not required for the activation of *cdc10* dependent transcription in G1. The triple cyclin deletion cells arrest in G1 in the first cycle following nitrogen starvation.**

Nitrogen starvation was used to arrest wildtype cells and cells deleted for *cig1, cig2* and *puc1* cyclins in G1. Nitrogen was then added enabling cells to grow and re-enter the cycle at 32°C. **3a.** Samples were taken for FACS analysis. Histograms show both the forward scatter and DNA content (propidium iodide staining). Cells from the two strains differ greatly in size but enter S-phase with similar kinetics. **3b.** A 2D plot of the starved cells is shown to emphasize the difference in cell size. **3c.** After re-feeding, RNA samples from both strains were Northern blotted and probed for *cdc18* and *cdc2* messages.

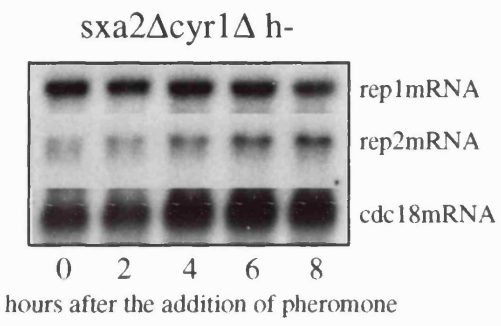


Figure 2.4

In *S. pombe*, S-phase transcription is active in a pheromone induced cell cycle arrest. P-factor was added to *cyr1Δ sxa2Δ h-* cells. After 6h, all the cells are arrested in G1 (FACS data not shown). A Northern Blot was probed for *cdc18, rep1* and *rep2* transcripts. (*his3* expression was used to confirm equal loading, data not shown).

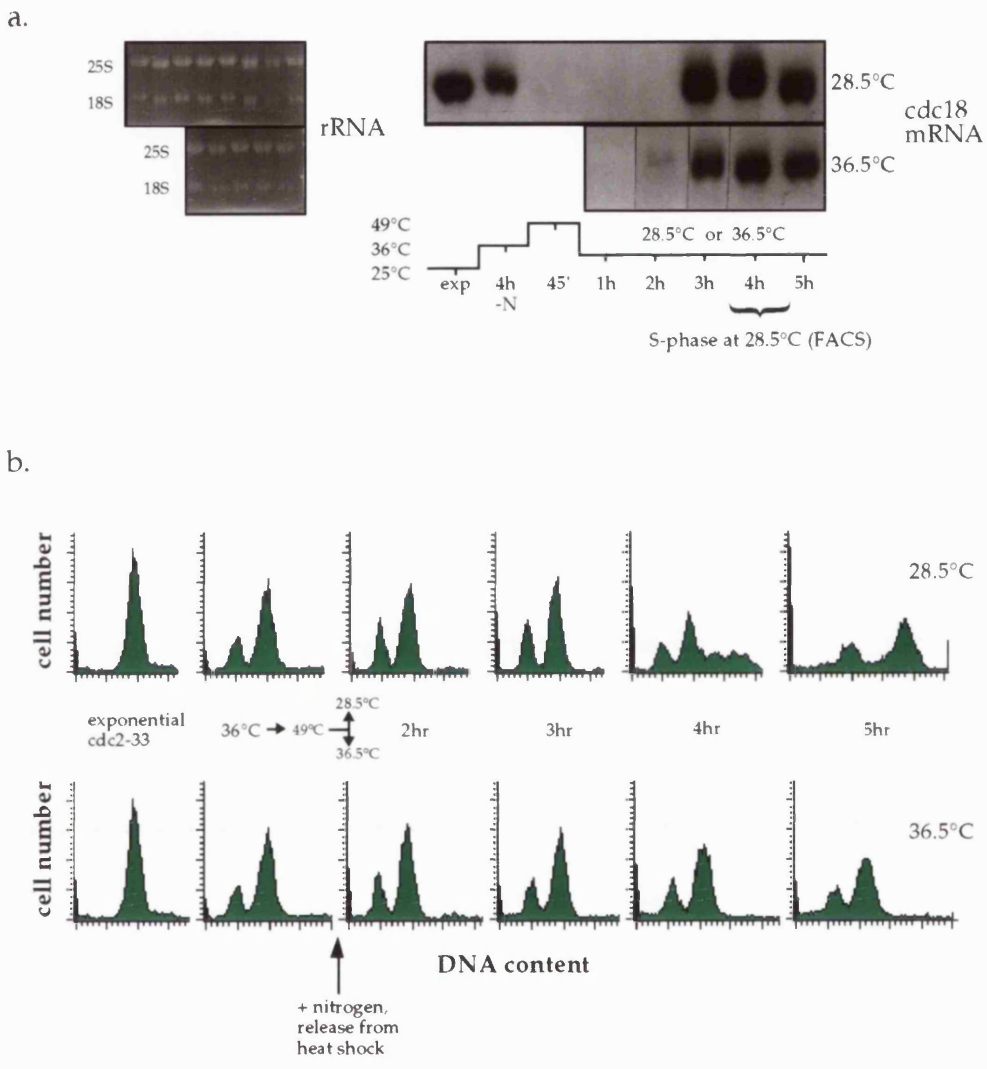
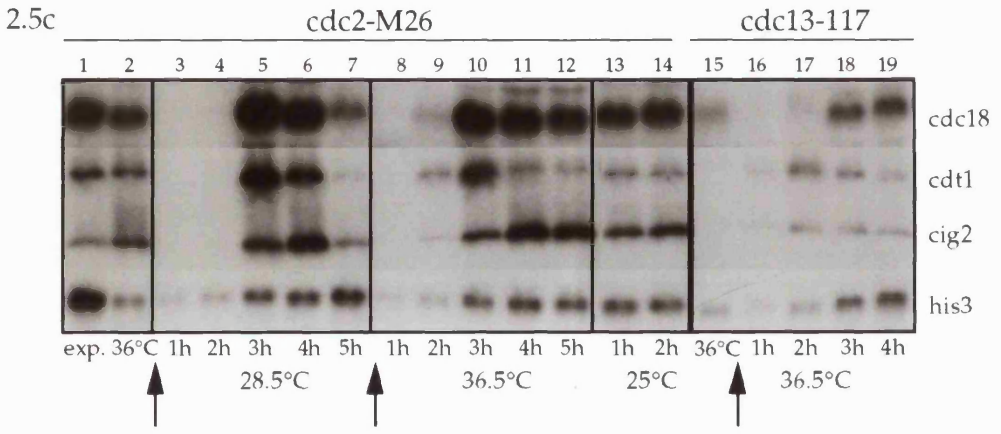


Figure 2.5

cdc2 activity is not required to re-activate the expression of _cdc18_ in re-replicating cells.

cdc2-33 cells were arrested at G2/M, starved of nitrogen and subjected to a heat shock to induce them to re-enter S-phase. Nitrogen was then re-added at the permissive or restrictive temperature for *cdc2-33* (28.5°C or 36.5°C). **5a.** A Northern Blot was probed for *cdc18* mRNA; rRNA is shown as a loading control. 5**b.** Fixed samples were analysed by FACS analysis to determine the timing of S-phase as cells with a 2C DNA content re-replicate their DNA, resulting in a 4C peak.



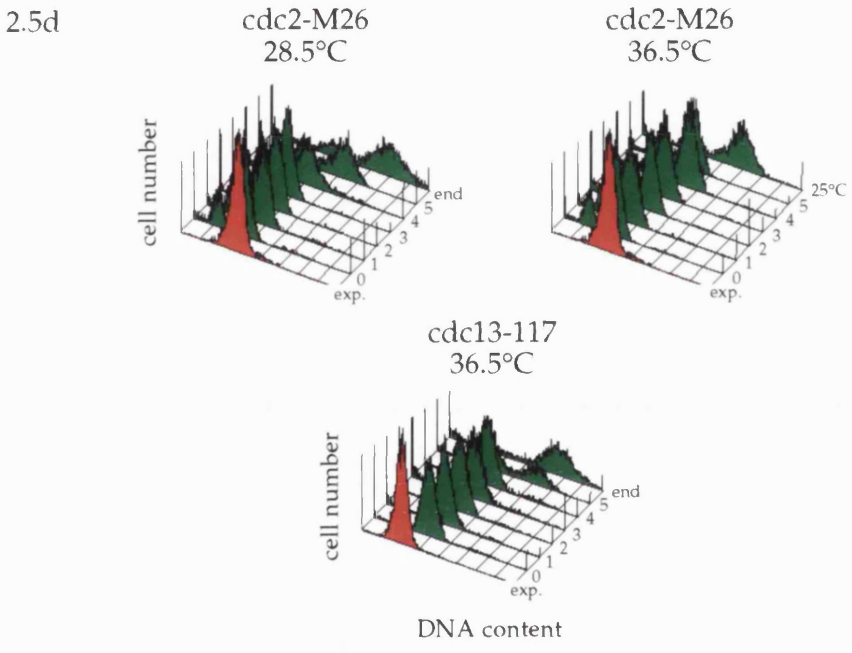


Figure 2.5c and d

***cdc10* activity in re-replicating cells.**

5c. *cdc2-M26* and *cdc13-117* cells were subjected to heat shock and nitrogen starvation to induce them to re-enter S-phase from G2 (as above, Figure 2.5a/d). Cells were sampled each hour for 5 hours after the re-addition of nitrogen (indicated by the arrow), and RNA samples were run on a Northern blot which was probed for the presence of *ura4, cdc18, cdt1* and *cig2* transcripts. In lanes 1-14, *cdc2-M26* cells were re-fed at 28.5°C or 36.5°C. After 5 hours, the cells kept at the restrictive temperature were shifted to 25°C to enable them to enter S-phase (lanes 13 and 14). Nitrogen was re-administered to *cdc13-117* cells at 36.5°C at which temperature they are able to re-replicate with high efficiency (lanes 15-19). **5d.** FACS data shows the DNA content for both strains. In each case, a final sample was taken after 8 hours. *cdc2-M26* cells recovering at 36.5°C were shifted to 25°C after 5 hours at the restrictive temperature and were sampled after an additional 3 hours, to confirm their ability to endoreduplicate (5d right hand panel, final sample).

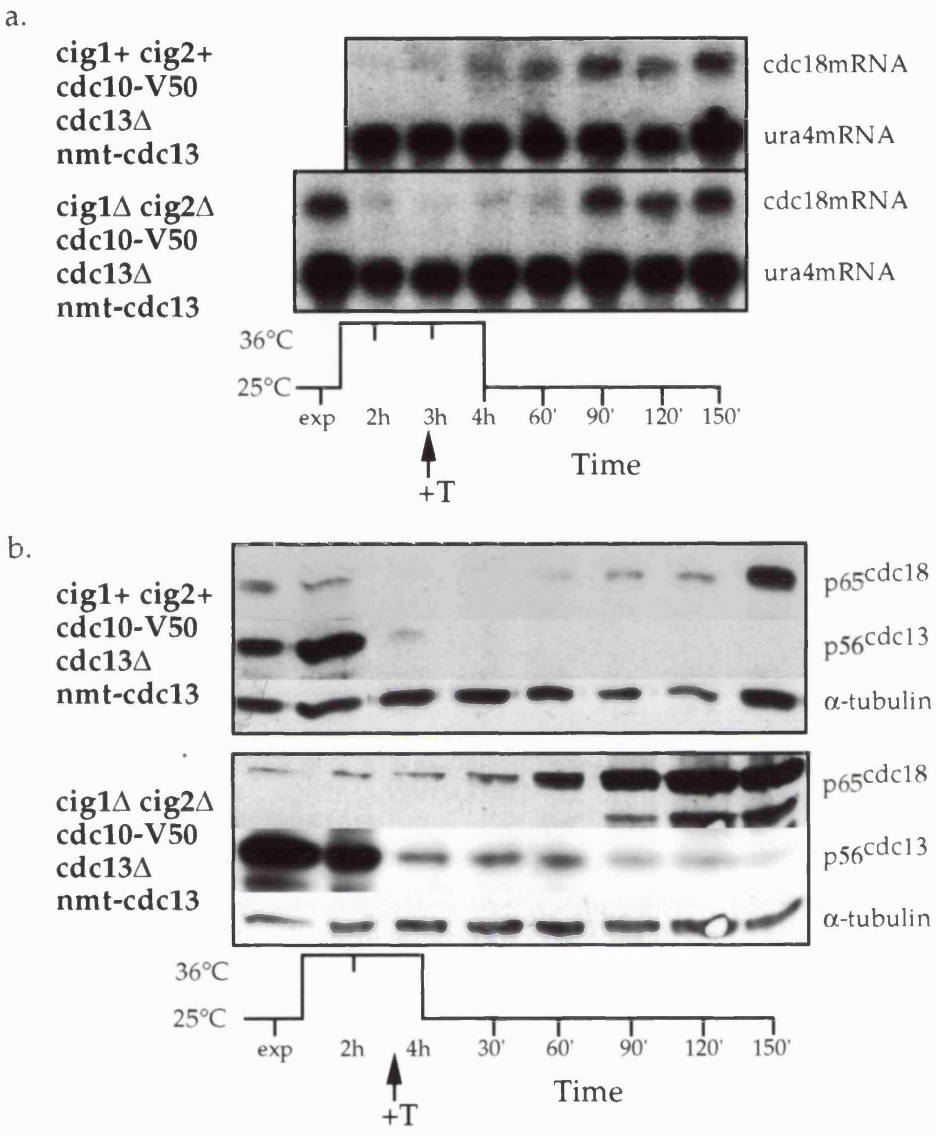


Figure 2.6

***cig1, cig2 and cdc13* functions are not required for the expression of *cdc18* or the accumulation of cdc18p after release from a *cdc10^{ts}* arrest.**

In order to manipulate *cdc10* and CDK activity independently in G1 cells, *nmt-cdc13 cdc10-V50 cig1Δ cig2Δ* and *nmt-cdc13 cdc10-V50 cig1+ cig2+* strains were used. Cells were arrested in G1 by incubation at 36°C, the non-permissive temperature for *cdc10-V50*. After 3h in the arrest, *cdc13* expression was switched off in half the cells by the addition of thiamine. After an additional hour at 36°C, cells were shifted back to 25°C, reactivating *cdc10*. Cells subjected to the *cdc10* block and release were collected for analysis of RNA, protein, and H1 kinase levels. **6a.** A Northern analysis was used to assess levels of *cdc18* and *ura4* mRNA in both cultures, upon release from the *cdc10* arrest, in the presence of thiamine. **6b.** Identical samples were taken to determine the levels of cdc18p, cdc13p and α-tubulin by Western blotting.

2.6c

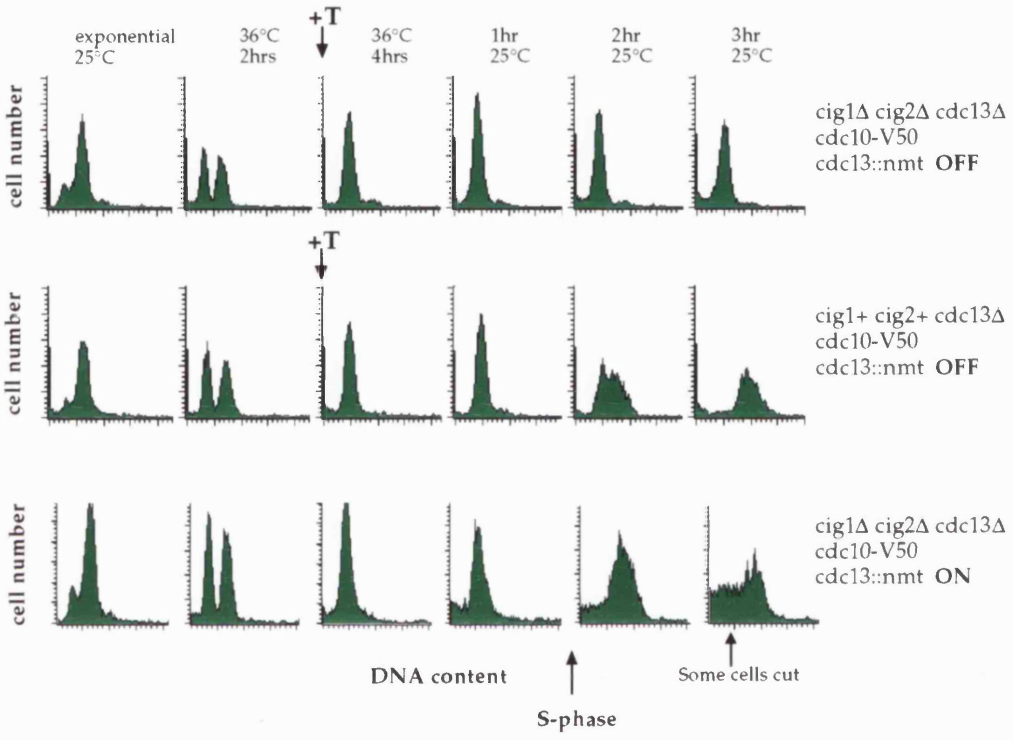


Figure 2.6c

***cig1, cig2* and *cdc13* functions are required after *cdc10* function in G1 to bring about S-phase.**

6c. Samples from the experiment shown in Figure 2.6a/b were taken for FACS analysis. Cells lacking all three cyclins were unable to enter S-phase after release from the *cdc10* block. Arrows indicate the timing of the onset of S-phase in cells expressing B-type cyclins, and the accumulation of cut cells in the *cig1Δ cig2Δ* strain grown in the absence of thiamine. Thiamine addition, after 3 hours at 36°C, is indicated by +T.

2.6d

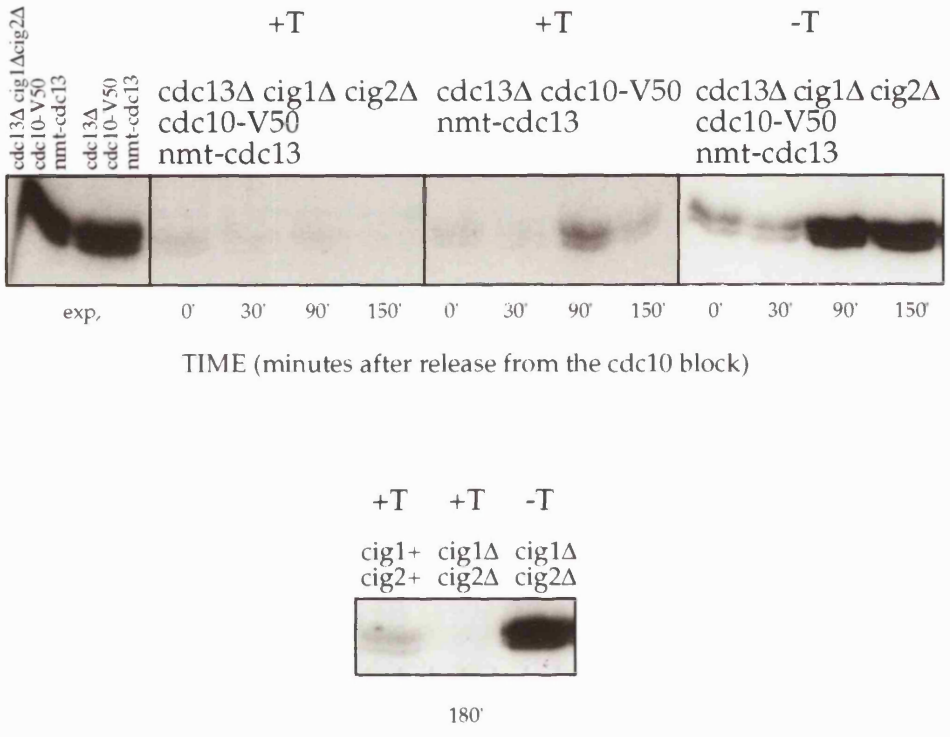


Figure 2.6d

cig1p, cig2p and cdc13p make up 98.5% of the late G1 cdc2p associated H1kinase activity.

6d. Extracts from *nmt-cdc13 cdc10-V50 cig1Δ cig2Δ* cells, in the presence and absence of thiamine, and *nmt-cdc13 cdc10-V50 cig1*$^{+}$*cig2*$^{+}$ cells, in the presence of thiamine, were used in an H1 kinase assay. Cdc2p was immuno-precipitated using polyclonal C2 antibodies and the precipitate added to H1 in the presence of radio-labelled γATP. The levels of phosphorylated H1 were quantified by phosphor-imager analysis. The first 10 samples were run on a single gel. The H1 kinase assays for the *nmt-cdc13 cdc10-V50 cig1Δ cig2Δ* strain expressing cdc13p were run on a separate gel, but contained an exponential control for quantification. The final three samples (corresponding to the 180 minute time-point after release from the *cdc10* arrest) were run on the same gel.

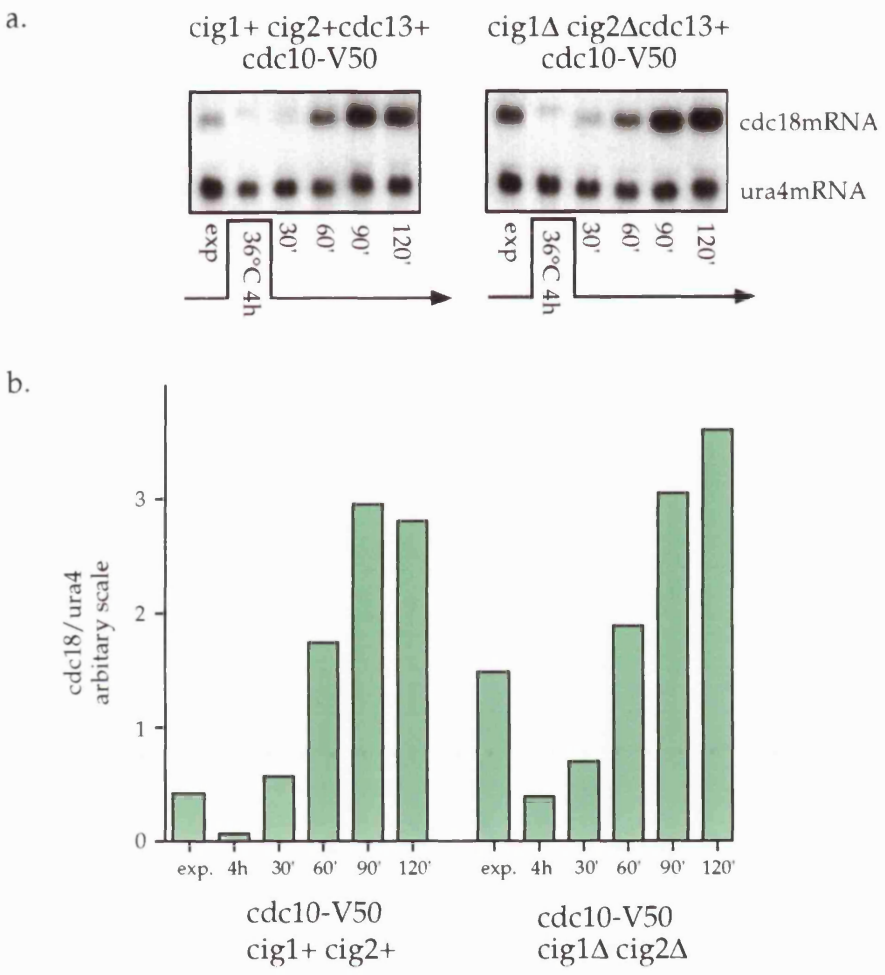
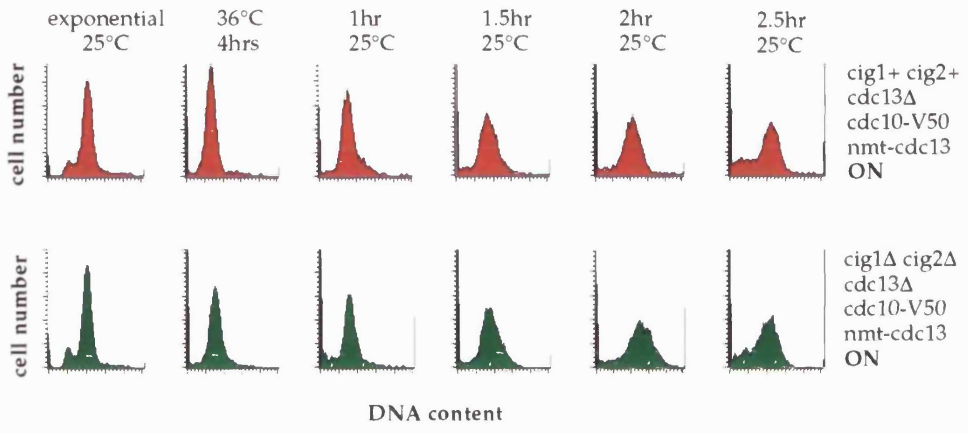


Figure 2.7

Deletion of both *cig1* and *cig2* genes does not effect *cdc10* dependent transcription.

nmt-cdc13 cdc10-V50 cig1Δ cig2Δ and *nmt-cdc13 cdc10-V50 cig1*$^{+}$ *cig2*$^{+}$ cells were subjected to a *cdc10* block and release experiment, in the absence of thiamine, as in experiment in 6a. **7a.** *cdc10* dependent transcription was assayed by Northern blotting, probing for *cdc18* and *ura4*. In **7b,** the levels of *cdc18* and *ura4* mRNAs were quantified and the ratio plotted using an arbitary scale. The FACS analysis is shown in Figure 2.7c.

2.7c.



2.7d.

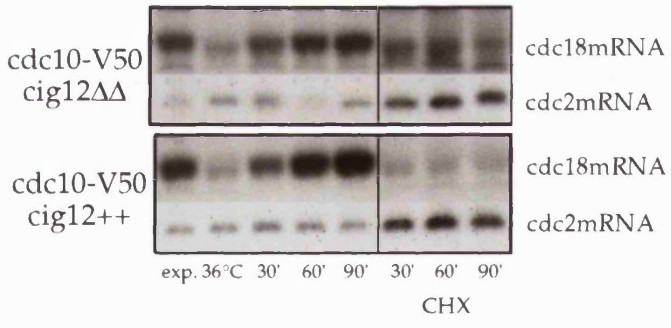
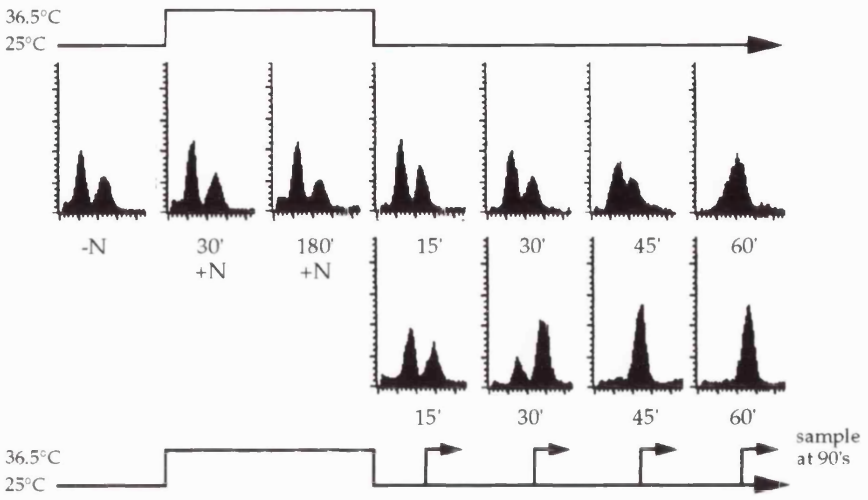


Figure 2.7c/d

Cdc13p alone can drive cells into S-phase upon release from a *cdc10* block. The re-activation of *cdc10* probably requires *de novo* synthesis of cdc10p.

7c. *nmt-cdc13 cdc10-V50 cig1Δ cig2Δ* and *nmt-cdc13 cdc10-V50 cig1*$^{+}$*cig2*$^{+}$ cells were subjected to a *cdc10* block and release experiment in the absence of thiamine, as in experiment in 6a. A FACS analysis is shown. **7d.** The experiment in Figure 2.7 was repeated, with the modification that cyclohexamide (CHX) was added to half of both cultures, as cells were shifted to the permissive temperature. *cdc18* and *cdc2* transcript levels were determined in the presence and absence of cyclohexamide.

a.



b.

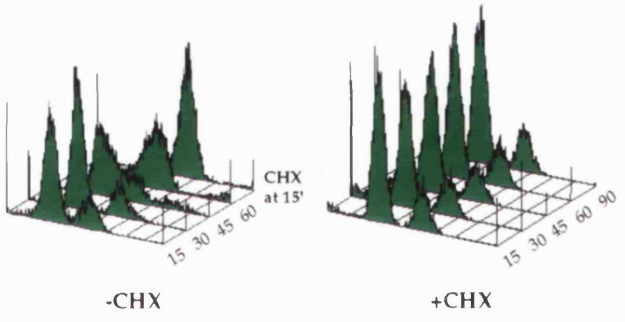
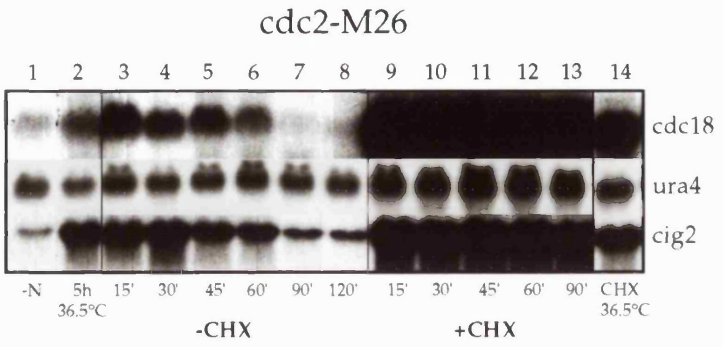


Figure 2.8

The S-phase *cdc2* function is completed between 30 and 45 minutes after the release from a *cdc2* G1 block. This correlates with the timing of the onset of S-phase.

8a. *cdc2-M26* cells were arrested in G1 by nitrogen starvation and then released into the cell cycle at 36.5°C, the restrictive temperature for *cdc2*. After 3 hours, cells were shifted to 25°C to allow cells to enter S-phase. At various times, FACS samples were taken to assess DNA content and an aliquot of cells was shifted back to 36.5°C. To determine when the *cdc2* function was completed, cells returned to the restrictive temperature were analysed by FACS, 90 minutes after the original shift to 25°C. The top panel shows the time-course of release, and the bottom panel the results of the transition point assay. **8b.** In a second experiment cells were again arrested at the *cdc2* block point in G1. In this case, cyclohexamide was added to half the culture upon release from the arrest (right panel). Cyclohexamide was also added to a small sample of cells 15 minutes after the initial shift to 25°C, and the DNA content of these cells was assayed after 90 minutes at the permissive temperature (left panel, final sample).

a.



b.

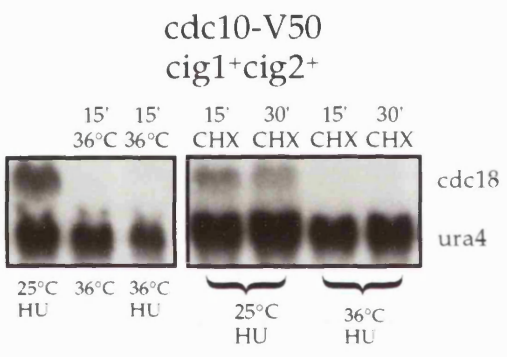


Figure 2.9

Cyclohexamide causes the accumulation of very high levels of *cdc10* target transcripts upon release from a G1 *cdc2* arrest.

9a. RNA samples from cells in the experiment shown in Figure 2.8b was analysed. (*cdc2-M26* cells were arrested in G1 by nitrogen starvation, released into the cell cycle at 36.5°C, and after 3 hours at this temperature, cells were shifted to 25°C in the presence or absence of cyclohexamide). RNA was analysed from cells arrested in the starved state and at 36.5°C,5 hours after the re-addition of nitrogen (lanes 1 and 2), after release from the *cdc10* block in the presence or absence of cyclohexamide (lanes 3-13) and from cells held in the *cdc2* block for 3 hours, 15 minutes after the addition of cyclohexamide (lane 14). The Northern blot was probed for *cdc18, cig2* and *ura4*. **9b.** As a control, *cdc10-V50* cells, either exponentially growing or arrested in HU (lane 1) were shifted to 36°C for 15 minutes (lanes 2-3), and *cdc18* transcript levels monitored. In addition, cyclohexamide was added to cells in HU and half the culture was then shifted to 36°C. *cdc18* and *ura4* mRNA levels were assessed by Northern analysis, 15 and 30 minutes after the addition of cycohexamide (right hand panel) .

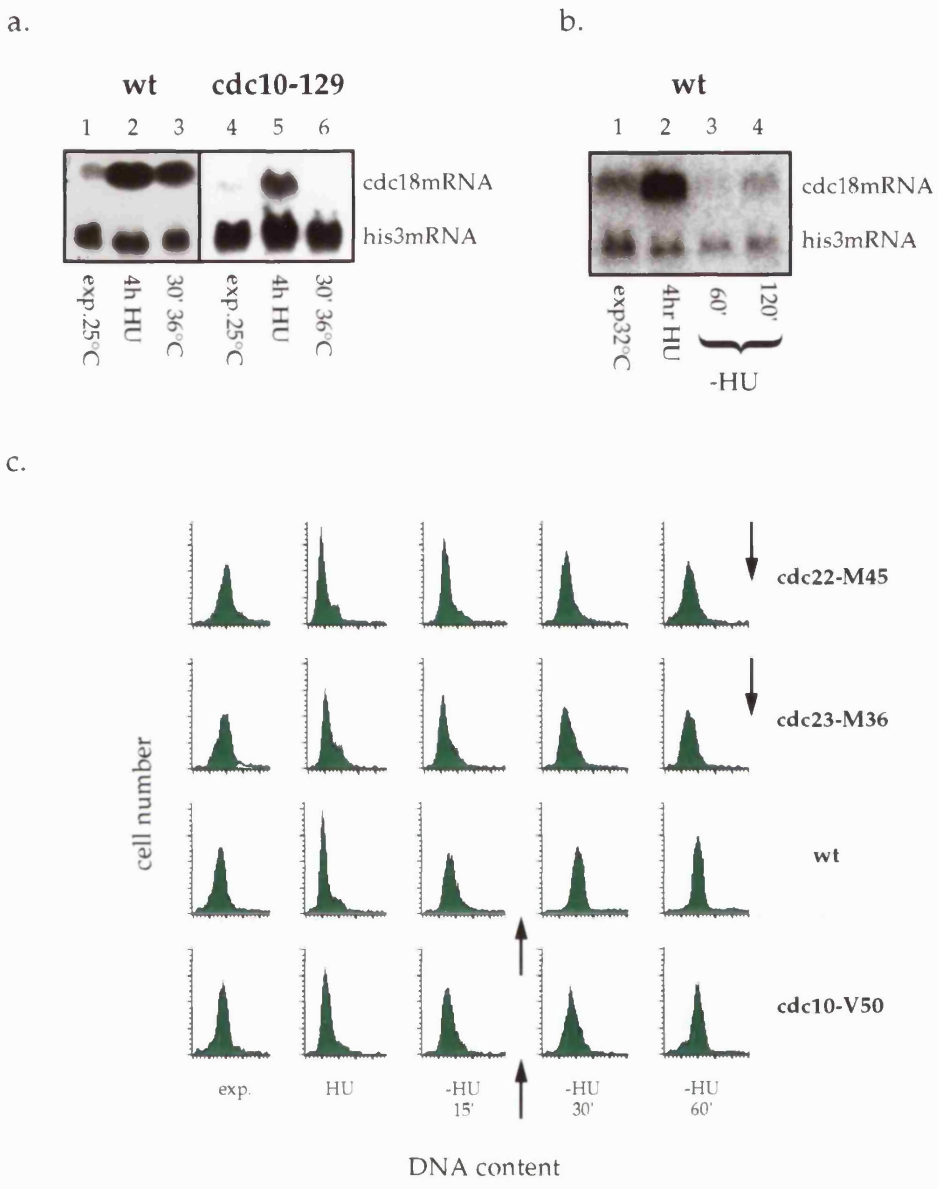
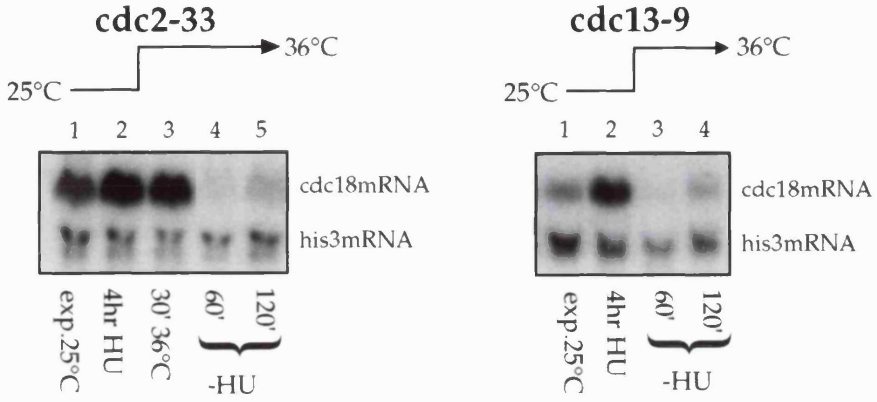


Figure 2.10

Although *cdc10* dependent transcription is active during S-phase, its function is complete in a HU block.

10a. Wildtype and *cdc10-129* cells were arrested at the onset of S-phase by the addition of 11mM hydroxyurea (HU). Cells were then shifted to 36°C for 30 minutes. **10b**. Wildtype cells were arrested in HU for 4 hours and then washed, synchronously releasing cells into S-phase and G2. In both cases, a Northern blot was used to assess levels of *cdc18* and *his3* mRNA. **10c.** In a separate experiment wild type, *cdc22-M45*, *cdc23-M36* and *cdc10-V50* cells were arrested in HU for 3.5 hours, shifted to 36°C for 30 minutes and then washed free of HU. Progression of cells into S-phase and G2 was monitired by FACS analysis. Arrows indicate the completion of S-phase by the majority of cells.

a.



b.

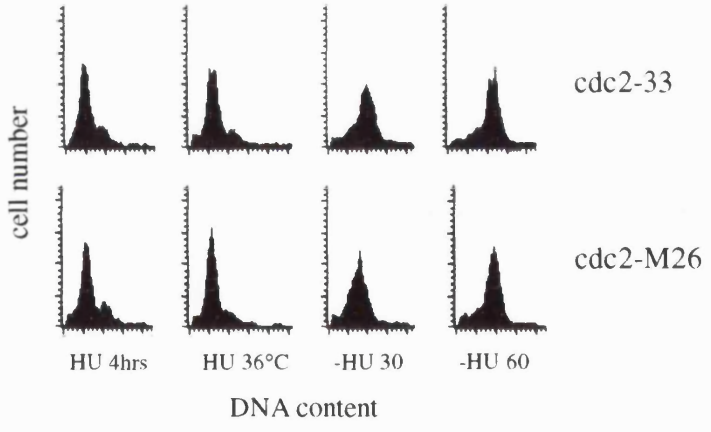
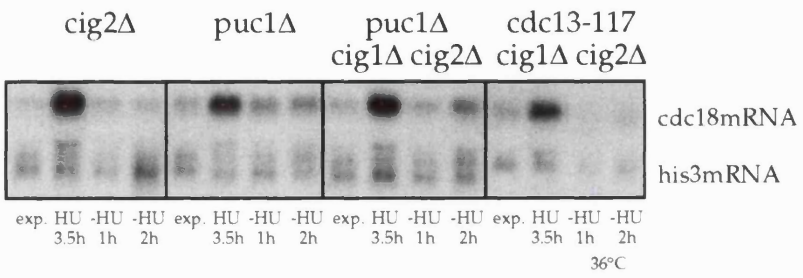


Figure 2.11

***cdc2* function is not required for the maintenance of high levels of *cdc10* dependent transcription in S-phase or for repression of this transcription in G2 cells.**

11a. *cdc2-33* and *cdc13-9* cells were arrested in HU for 4h (lanes 1 and 2) and shifted to 36.5°C for 30 minutes to inactivate the ts-functions (*cdc2-33* , lane 3; data not shown for *cdc13-9*). HU was then washed out at 36.5°C enabling cells to pass through S-phase and into G2 (confirmed by FACS analysis). Samples were taken for Northern analysis and probed for *cdc18* and *his3*. **11b.** *cdc2-33* and *cdc2-M26* strains were subjected to a HU block and release like that carried out in Figure 11a, and samples taken for FACS analysis. Most cells complete S-phase within 30 minutes of the release.

a.



b.

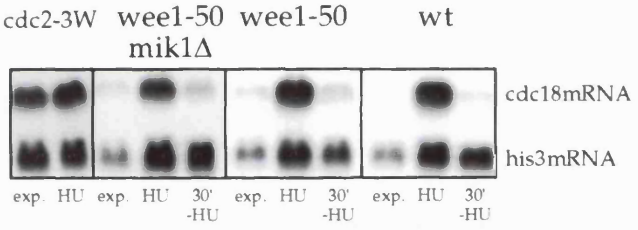


Figure 2.12

***puc1*, *cig1*, *cig2* and *cdc13* cyclins and tyrosine phosphorylation of cdc2p are not required for the G2 repression of *cdc10* dependent transcription.**

12a. Four strains: *cig2Δ*, *puc1Δ*, *puc1Δ cig1Δ cig2Δ* and *cdc13-117 cig1Δ cig2Δ*, were subjected to an HU block and release, as in previous experiments. Cells of the first 3 strains were arrested in HU for 3.5 hours, at 32°C, and HU washed out at the same temperature. *cdc13-117 cig1Δ cig2Δ* cells were arrested at 25°C, shifted to 36°C for 30 minutes, and then released into G2 at the restrictive temperature. In all cases, *cdc18* and *his3* message levels were followed by Northern blotting. **12b.** *wee1-50 mik1Δ*, *wee1-50* and a wildtype strain, were subjected to a HU block and release protocol like that used for *cdc13-117 cig1Δ cig2Δ* cells above. Also, *cdc2-3W* cells were arrested for 3.5 hours in HU at 32°C. In each case, *cdc10* dependent transcription was monitored using *cdc18* and *his3* message levels.

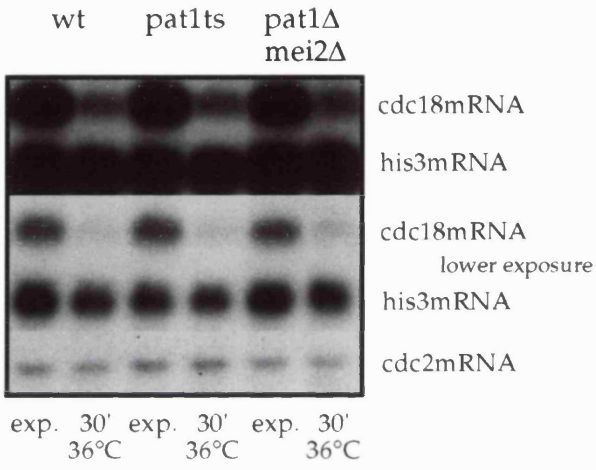


Figure 2.13

***pat1* may not have a direct effect on *cdc10* dependent transcription in the mitotic cycle.**

Wildtype, *pat1-114* and *pat1Δ mei2Δ* cells were grow at 25°C and then shifted to 36°C for 30 minutes. RNA was isolated from cells at both temperatures and a Northern blot used to assess levels of *cdc18, his3* and *cdc2* messages. Two exposures of the blot are shown to clearly show the levels of *cdc18* mRNA before and after the temperature shift.

CHAPTER 3

The timing of S-phase transcription

Introduction

The experiments presented in Chapter 2 bring into doubt the widely accepted notion that the mechanisms governing the periodicity of *cdc10* dependent transcription are similar to those controlling S-phase transcription in *S. cerevisiae*. In particular, and unexpectedly, they show that *cdc10* is active in G1 independently of *cdc2* activity and is active in small cells that are pre-START. One of the problems in addressing the timing of *cdc10* dependent transcription in fission yeast is the short G1 phase. This precludes a simple analysis of the timing of *cdc10* dependent transcription, so previous conclusions about timing of expression of *cdc10* targets with respect to other cell cycle events (which were made on the basis of elutriated wildtype cultures), were necessarily imprecise. In addition, the unusual G1 phase in *S. pombe*, may necessitate the use of mechanisms to control progression through G1/S, that differ from those implicated in other eukaryotes. With these points in mind, I attempted a more careful analysis of the timing of S-phase transcription in the *S. pombe* mitotic cycle. The unexpected results of this investigation may help to explain some of the more suprising observations in Chapter 2.

cdc10 dependent transcription becomes activated early in mitosis

In order to investigate the timing of activation of *cdc10* dependent transcription I followed transcription through a synchronous cell cycle. To do this, a culture of *cdc25-22* cells was incubated at the restrictive temperature for four hours, to arrest cells at the G2/M boundary, and then cells were shifted to 25°C, releasing them into mitosis and a subsequent cell cycle (Figure 3.1). This experiment yields very high levels of synchrony (see Figure 3.1d). As an estimate of the timing of activation of *cdc10* dependent transcription, the accumulation of *cdc18* mRNA was correlated with markers of mitosis (shown in Figure 1c/1d): cdc13p associated H1-kinase levels (in immuno-precipitates using cdc13p), the state of the microtubular cytoskeleton and nuclear division, using criteria shown in Figure 3.1f. The Northern blot shown in Figure 3.1a is quantified and shown in Figure 3.1c/d/e. *cdc18* message levels rose within twenty minutes of the shift to the permissive temperature, as the levels of *cdc13p* associated mitotic H1 kinase and the percentage of cells with a short spindle were still increasing, coinciding with entry into mitosis (Figure 3.1c/d). Another *cdc10* target, *cig2*, showed a similar profile of expression but exhibited less periodicity (Figure 3.1a: bottom panel, quantified in Figure 3.1e). *cdc18* expression persisted after the kinase levels dropped as cells exited mitosis and then fell as cells passed from G1 into S-phase and G2, suggesting that *cdc10*-dependent transcription is switched off at this time (Figure 3.1c). These data imply that, in *S. pombe*, "S-phase" transcription is active in the window from early mitosis until late G1/S-phase. The cdc18p protein level also showed a periodic change that closely mirrored that of the transcript (Figure 3.1b). The profile of cdc18p accumulation appears to be the inverse of the changes in cdc13p levels, (although the alterations in cdc18p levels appear more dramatic than those of cdc13p).

The *cdc25* block and release protocol was then repeated, in order to compare the timing of *cdc18* mRNA accumulation with the behaviour of the cdc10p bandshift complex, DSC1 (Figure 3.2). Passage through the cell cycle was monitored by following the septation index and expression of histone message, both of which peak during S-phase (Figure 3.2a). As before, *cdc18* mRNA began to accumulate very soon after the release from the *cdc25* block. *cdc22* transcripts were also monitored in this experiment. *cdc22* mRNA, like *cig2* mRNA, accumulated in a broad peak relative to

cdc18 mRNA, and reached a maximum level approximately fifteen minutes later than *cdc18* transcripts. The difference between the behaviour of *cdc22* and *cdc18* transcripts may result simply from differences in their rates of transcription and mRNA turnover. The accumulation of *cdc18* mRNA preceded the peak of histone transcription by 40 minutes, and the peak of septation (which is coincident with S-phase) by more than an hour. This confirms that *cdc18* mRNA is not transcribed in late G1, but in mitosis or early G1. DSC1 bandshift activity was then investigated after release from the *cdc25* block (Figure 3.2b, quantified in Figure 3.2c). A radio-labelled fragment of the *cdc18* promoter (Zhu, *et al.*, 1994) (containing the putative MCB elements and shown in Figure 5.9a), was added to cell extracts to detect a mobility shift corresponding to the formation of a cdc10p containing protein/DNA complex. (This is characterised and described in more detail in chapter 4). A limited oscillation in the level of DSC1 activity was seen through the cycle. DSC1 was present in G2 cells, transiently disappeared on release into mitosis and then reformed. The peak of DSC1 formation occurred after the peak of *cdc10* transcription, 60 minutes after the release from the G2/M arrest, at which time *cdc18* transcripts were barely detectable. This is in agreement with published data showing the temporal change in DSC1 in extracts from cells undergoing a *cdc25* block and release (Reymond, *et al.*, 1993). DSC1 was previously thought to correspond to the active complex. However, a comparison between the level of DSC1 complex formation and the profile of accumulation of *cdc10* target transcripts clearly shows that DSC1 only appears after S-phase transcription is downregulated. This interpretation is further supported by data presented in Chapter 5. These data bring the nature of DSC1 into doubt and are discussed further in Chapter 5.

It is possible, that the timing of *cdc18* transcription observed in the previous experiment, was simply due to the abnormally large size of cells following release from the *cdc25-22* block, (after 4 hours at 36°C, cells accumulate approximately three times the wildtype mass). To confirm that this is not the case, a second set of experiments were conducted, in which *cdc25-22* cells were simultaneously arrested at the restrictive temperature and starved for nitrogen (Figures 3.3a and 3.3b). Under these conditions cells do not elongate significantly (Figures 3.3c). *cdc25-22* cells were arrested at 36°C and nitrogen was removed, either after 2 hours at 36°C, or simultaneously as cells were shifted to the restrictive temperature.

Cultures were then returned to 25°C (still in the absence of a nitrogen source) to release cells synchronously into the subsequent cell cycle. In both cases, upon release to the permissive temperature, cells passed rapidly from mitosis into S-phase without a visible G1 phase (measured by FACS analysis, data not shown). This indicates that S-phase and septation occurred concurrently. However, because there is little further growth in the absence of nitrogen, the timing of cell division is clearly observed using forward scatter data from the FACS analysis (Figure 3.3c). Levels of *cdc18* mRNA (and *cdc22* mRNA in cells starved for 2 hours) were monitored. In this first experiment, after the shift to 25°C, cells starved for 2 hours entered the next cell cycle with similar kinetics to that seen in complete medium. *cdc18* and *cdc22* were expressed in a similar profile (Figure 3.2). In this culture, a similar temporal pattern of expression of *cdc10* targets was also observed in the second cycle after the release; although these cells had been starved of nitrogen for a further 3 hours. Interestingly, in this second cycle, the peak of *cdc18* mRNA only seemed to reach an intermediate level. In the second nitrogen starvation experiment, *cdc25-22* cells were grown for 4 hours at 36°C in the absence of nitrogen (Figure 3.3b). In this case, cells in the G2/M block at 36°C accumulated only half the mass of those starved after 2 hours at the restrictive temperature (Figure 3.3c). Upon release, at the permissive temperature, cell synchrony was still good but the period prior to nuclear division and septation was dramatically extended. (No septation occurred during the 90 minute time-course of RNA analysis). One possible explanation for the delay over mitosis is that the nitrogen starvation treatment reduces the stability of proteins essential for the onset of mitosis e.g. cdc25p or cdc13p, below a threshold level required for mitosis. Again, in this experiment, *cdc18* mRNA levels peaked well before S-phase, coinciding with the early nuclear events of mitosis (as measured by the percentage of cells in anaphase, shown in Figure 3.3b). The rapid passage through M-phase and G1 into S-phase is delayed somewhat in these cells, emphasising the difference between the timing of the activation of transcription and S-phase. *cdc18* mRNA accumulates approximately 70 minutes after the shift to 25°C, close to the onset of anaphase, and S-phase begins (coinciding with septation; data not shown) after approximately 140 minutes at 25°C (Figure 3.3c). These results confirm that, irrespective of cell mass and nutrient conditions, *cdc18*, *cig2* (and *cdc22* with some slight delay), are expressed shortly after cells are released from a *cdc25* block into the subsequent round of the cell cycle, probably early in mitosis.

A similar experiment was conducted, using *cdc2-M26* cells released from a G2/M arrest at 36°C, into mitosis at 25°C (Figure 3.4). In this case, *cdc18* mRNA accumulated very early even before the major peak of anaphase, and transcript levels even appeared to decline before cells had completed mitosis. The addition of cyclohexamide to the culture upon release from the $cdc2^{ts}$ block, delayed nuclear division, but did not prevent the accumulation of *cdc10* target transcripts early on in mitosis. (In this case, cyclohexamide did not cause the hyperactivation of *cdc10*, as seen in cells released from the G1 *cdc2* block (Figure 2.9)).

cdc10 dependent transcription is activated in cells arrested in mitosis.

In order to verify that *cdc10* dependent transcription is switched on at the onset of mitosis, and to distinguish between transcription during metaphase and anaphase, I next assayed *cdc10* dependent transcription in cells arrested in mitosis. *nuc2-663* cells are defective in the APC complex which controls the proteolysis of B-type cyclins and other specific targets. This is essential for the initiation of anaphase and the exit from mitosis (Kumada *et al.*, 1995). Therefore, at the restrictive temperature, $nuc2^{ts}$ cells accumulate at the metaphase-anaphase transition with high levels of the mitotic kinase and condensed chromatin (Kumada, *et al.*, 1995). Early G2, $nuc2^{ts}$ cells, were isolated by centrifugal elutriation at 25°C. They were then shifted to the restrictive temperature, 36°C, and followed as they entered the mitotic block (Figure 2.5a/b). As cells entered mitosis (which was assessed by visualising chromatin condensation using DAPI), *cdc18* and *cdc22* transcripts accumulated (Figure 3.5a and 3.5b). No increase in histone transcription was observed, suggesting that cells did not leak through the block and into S-phase (Figure 3.5b). This shows that *cdc10* dependent transcription is active in metaphase arrested cells.

The activation of *cdc10* during metaphase was confirmed using a cold sensitive mutation in β-tubulin, *nda3-km311*, which arrests cells at the metaphase anaphase transition, transiently at 20°C and for longer periods at 18°C, as a result of a spindle checkpoint. These cells arrest with high levels of cdc2p/cdc13p kinase activity and condensed chromatin (Umesono *et al.*, 1983; Hiraoka *et al.*, 1984; Moreno, et al., 1989). In two experiments,

nda3 cells were shifted to 20°C (Figure 3.5c/d). In both cases *cdc18* mRNA accumulated 3-4 hours after cells were shifted to the restrictive temperature. In the longer time-course (Figure 3.5c), this was shown to parallel the proportion of cells containing condensed chromatin. *cdc22* and *cdt1* mRNAs behaved similarly to *cdc18*. In contrast, *cig2* mRNA levels remained unaffected by the transient mitotic arrest (an observation which was repeatable (data not shown)). In the first experiment, *cdc18* and *cdt1* expression was downregulated, as cells leaked out of mitosis into S-phase. These experiments indicate that *cdc18, cdc22* and *cdt1* are expressed in mitosis, whilst *cig2* appears to be expressed similarly in both G2 and mitosis.

The mitotic expression of *cdc18* requires *cdc10* function.

To show that the *cdc18* transcript accumulating in mitosis is the result of active *cdc10* dependent transcription, two experimental procedures were used.

First, cells were arrested in mitosis, and *cdc18* transcript levels were followed after inactivating the *cdc10* function, using a temperature sensitive allelle (Figures 3.6a and 3.6b). A strain was constructed carrying the *nda3* mutation together with *cdc10-129*. (Figure 3.6a) These cells were arrested at the *nda3* block for 6 hours at 18°C and then shifted to 36°C, to inactivate $cdc10^{ts}$. The addition of thiobendazole temporarily delays their exit from mitosis. In cells containing a $cdc10^{ts}$ allele, *cdc18* mRNA levels fell to background within 5 minutes of the shift to 36°C. No effect was seen of the temperature shift in control cells, and *cdc18* mRNA levels remained high. This demonstrates that the high levels of *cdc18* mRNA in mitotic cells are the result of active *cdc10* dependent transcription, and that in these cells, *cdc18* mRNA is still rapidly turned over.

Although the activity of *cdc10* is independent of *cdc2* in G1, it is still possible that cdc2p/cdc13p activates *cdc10* at the onset of mitosis. If this was the case, continued *cdc10* activity in mitosis may require maintenance of the mitotic kinase, (if the activated state of *cdc10* is labile until cells pass into G1). To test this hypothesis, a *cdc2-L7 nda3* double mutant was subjected to a similar procedure to that used above (Figure 3.6a middle panel). Cells were arrested in mitosis and then shifted to the restrictive

temperature for *cdc2*, 36.5°C. No difference was observed between the level of *cdc18* mRNA in the control and the *cdc2-L7 nda3* strain. It should not be concluded that this rules out the activation of *cdc10* by *cdc2* because: **i)** it is not certain how quickly *cdc2* function is inactivated and **ii)** if *cdc10* is activated by *cdc2*, once activated, the cdc10p complex may preserve a molecular memory of the activation signal to ensure that target genes are still transcribed after the kinase level falls, into the subsequent G1 phase of the cycle. For these reasons, it is hard to assess whether *cdc10* activity at metaphase is dependent on events of mitosis or more directly on the mitotic cdc2p/cdc13p kinase.

The dependence of *cdc18* levels upon *cdc10* function in mitosis was also shown in cells over-expressing the non-degradable B-type cyclin, *cdc13Δ90* (Figure 3.6b) (Murray, *et al.*, 1989). In this case, cells are able to undergo nuclear division but cannot leave mitosis or decondense their chromosomes, because of the continued presence of the cdc2p/cdc13p mitotic kinase. *cdc18* mRNA levels were elevated as cells entered the mitotic arrest, induced by *cdc13Δ90* expression, equivalent to that observed in HU arrested cells (Figure 3.6b lanes 1-4). To confirm that this is the result of ongoing *cdc10* dependent transcription, an integrated copy of *cdc13Δ90* was expressed in a $cdc10^{ts}$ background (Figure 3.6b lanes 5 and 6). Upon induction of *cdc13Δ90*, the majority of cells were arrested in mitosis (as measured by chromatin condensation, data not shown) with elevated *cdc18* mRNA levels. After shifting to the restrictive temperature for 30 minutes, *cdc18* transcript levels fell to very low levels. In conclusion, *cdc18* mRNA levels are maintained at a high level during mitosis as a consequence of continuous *cdc10* activity. This conclusion is also supported by experiments in which *cdc13Δ90* was ectopically expressed in *res1Δ* and *res2Δ* strains (shown in Figure 5.4).

cdc18 is actively transcribed in cells arrested in mitosis.

Finally, in order to show directly that *cdc18* is actively transcribed during mitosis, a nuclear run-on experiment was carried out (Figure 3.7) (Humphrey *et al.*, 1994). This method can be used to differentiate between changes in transcription and in mRNA stability, both of which can alter the levels of mRNA observed by Northern analysis. Cells were isolated, placed on ice and treated with sarcosine to permeabilise the cell nuclei. Actively

transcribing RNA polII molecules remain bound to the DNA, and transcriptional elongation can be assayed by the incorporation of exogenous radiolabelled UTP into nascent mRNA strands. Levels of labelled RNA were measured by hybridisation to single stranded DNA on a nylon membrane. I compared the level of ongoing *cdc18* mRNA synthesis in exponentially growing cells, to those arrested in mitosis. Background was controlled by measuring hybridisation to ssDNA from the sense strand of *cdc18*, i.e. non-complementary to *cdc18* mRNA, (data not shown), and to antisense *ura4* and *cdc2* ssDNA. The relative levels of ongoing transcription were quantified (Figure 3.7b,) by first subtracting the background, and then comparing the *cdc18/cdc2* signals. In cells arrested at the *nda3* block, or in S-phase using HU, the rate of ongoing transcription was higher than that seen in control wildtype cells at 18°C (or 32°C, data not shown). *cdc18* transcription was low, as expected, in *cdc10-129* cells at the restrictive temperature. This experiment supports the conclusion that *cdc18* is actively transcribed in mitotic cells.

Synthetic MCB elements can drive the expression of a reporter gene in mitosis.

In order to verify whether the cdc10p transcriptional machinery alone is capable of driving mitotic transcription of target genes, a reporter construct was introduced into cells which expresses *LacZ* from behind a synthetic triple MluI promoter element. (Three tandem copies of the MluI site, ACGCGT, equivalent to an MCB element, are present in the vector pSPΔ178.3M (Lowndes, *et al.*, 1991)). *nuc2* and *nda3* cells containing this plasmid were arrested in mitosis and the level of *lacZ* transcript measured (Figure 3.8). *cdc10-129* cells carrying the plasmid were used as a control. Before running the RNA on a gel, polyA$^+$ RNA was isolated to eliminate incompletely processed RNAs (multiple transcripts are seen when using the β-gal gene as a reporter in *S. pombe*) and plasmid DNA from the preparation. Both mitotic arrests resulted in an elevated level of *lacZ* transcript. This result suggests that MCB elements are sufficient to confer mitotic expression on a reporter gene. This implies that *cdc10* itself is responsible for the mitotic expression of target genes.

Chromatin condensation *per se* does not activate *cdc10* dependent transcription.

The experiments described so far in this chapter demonstrate that *cdc10* dependent transcription is activated during mitosis in cycling cells, and in cells arrested in metaphase or anaphase with condensed chromatin. It is not clear though, whether *cdc10* becomes activated as a direct consequence of the mitotic kinase, or whether its activity is controlled by some downstream mitotic event. One possibility, is that the physical changes accompanying mitosis, e.g. chromatin condensation could in some way activate *cdc10*-dependent transcription. This is an appealing hypothesis because *cdc10* activity would then be able to respond to the physical events accompanying progression through the cycle. In order to test this possibility, chromatin condensation was forced in G2 cells by the expression of *nimA*. *nimA* is a gene from *Aspergillus nidulans* which acts genetically downstream of *cdc2* to cause chromatin condensation (Osmani *et al.*, 1988; Osmani *et al.*, 1994) (Figure 3.9a). Expression of *nimA* can drive chromatin condensation in yeast and mammalian cells from all phases of the cell cycle (O'Connell *et al.*, 1994). *nimA* was ectopically expressed in a *cdc25-22* strain at the restrictive temperature to prevent other aspects of mitosis from occuring (Figure 3.9). In G2 arrested cells, the ectopic expression of *nimA* induced chromatin condensation, but not *cdc18* transcription. However, because *nimA* causes extreme chromatin condensation, this may not accurately reflect the physiological state of mitotic DNA.

Expression of *cdc10* targets persists from mitosis until S-phase in a single smooth oscillation in cells with an extended G1 phase.

Having established that *cdc10* dependent transcription is activated during mitosis, I next investigated at what cell cycle stage transcription is switched off (Figure 3.10). Earlier experiments already indicate that *cdc10* is inactivated at some time around S-phase, e.g. on release from a *cdc2* block in G1 and in *cdc25* block and release experiments. However the apparent timing between experiments is a little variable. To separate mitosis from S-phase, enabling a more accurate determination of the timing of transcription, I prepared an elutriated synchronous culture of *wee1-50* cells. (Elutriation is the least invasive method of synchronising populations of cells). In *wee1-50* cells, G1 is expanded at 36°C, as compared to the wildtype. At this temperature, the wee1p kinase is inactive, and cells are

unable to restrain the cdc2p/cdc13p G2 complex by tyrosine phosphorylation. As a consequence, cells enter mitosis prematurely, at a smaller size. In the following G1, *wee1-50* cells therefore require an extended period of growth to reach the minimum size required for S-phase, revealing the normally cryptic G1 size control (Nurse and Thuriaux, 1977). *wee1-50* cells were grown at 25°C, synchronised by centrifugal elutriation and then shifted to 36°C to inactivate the $wee1^{ts}$ gene. The long G1 is accentuated in the second cycle following inactivation of the wee1p kinase. *cdc18* transcripts were followed with respect to septation, cell number (Figure 3.10c), DNA content (Figure 3.10b) and histone transcript levels (Figure 3.10d). *cdc18* mRNA levels rose early, as expected, preceding septation (which shortly follows mitosis) in both cycles and remained elevated for a large portion of the cell cycle compared to the wildtype (Figure 5.3a). This elevation of *cdc18* mRNA is also evident in exponential cultures of "wee" cells (Figure 2.12b). In the first cycle, the fall in *cdc18* transcript levels was coincident with the fall in the percentage of septated cells, and in the second cycle the decline in *cdc18* mRNA levels is delayed as is entry into S-phase. Therefore *cdc10* is probably active from mitosis throughout an extended G1 until some time close to the onset of S-phase, apparently in a single smooth oscillation. This suggests that, even in cells with an extended G1, there is only one phase of *cdc10* activity and inactivity per cycle.

cdc10 remains active in cells unable to complete DNA replication.

It was already been shown, in the nuclear run on analysis and in Chapter 2, that *cdc10* dependent transcription is active in cells arrested at the onset of S-phase in HU. In order to substantiate the timing of the downregulation of *cdc10* dependent transcription, wildtype cells, *cdc23-M36* and *cdc1-7* cells were arrested in HU, shifted to 36°C for 30 minutes and released into S-phase and G2. The *cdc23* mutation impedes early S-phase (Figure 2.10c) and *cdc1* encodes a polymerase ∂ subunit required for the completion of DNA synthesis (MacNeill *et al.*, 1996). In the wildtype, *cdc10* was rapidly inactivated as cells proceeded through S-phase into G2, however, in the two mutants, *cdc18* levels remained somewhat elevated suggesting that *cdc10* dependent transcription may be inactivated, during, or at the end of S-phase. This agrees with much of the previous data and suggests that *cdc10* remains active until some aspect of DNA replication, is complete. It is

nevertheless conceivable that *cdc10* remains active in these mutants because it responds in some way to a defective DNA replication checkpoint.

Discussion

The work presented in this Chapter shows that so called "START" (Sturm and Okayama, 1996: Zhu, *et al.*, 1997) or "S-phase" *cdc10* dependent transcription is, in fact, activated in mitosis; in cells with condensed chromatin and high levels of the mitotic kinase. This was shown using both synchronous cultures and mitotic arrests, in metaphase and telophase. *cdc10*^{ts} mutations were used to show that this mitotic transcription of *cdc18* requires the continuous presence of *cdc10*. Also, a nuclear run on assay confirmed that the accumulation of *cdc18* message in mitosis is the result of on-going transcription. Therefore, the elevated levels of *cdc18* mRNA in mitosis, reflect active *cdc10* dependent transcription and not, for instance, changes in *cdc18* transcript stability. It was also shown that the synthetic, triple MluI promoter could mediate the mitotic expression of a reporter gene, *lacZ*. This suggests that the MCB element, present in the promoters of *cdc10* targets, is itself, likely to be responsible for the active S-phase transcription seen in mitosis. Proving that this element is responsible for the mitotic expression of *cdc10* target genes, in its native context, will require a detailed dissection of their promoters.

In several experiments *cdc18* and *cdt1* mRNAs were shown to be expressed in mitosis with a similar profile. Although *cdc22* message accumulates in a mitotic arrest, it is expressed a little later in synchronous cell cycles and persists for some time after the disappearance of *cdc18* transcripts. This suggests that the differences in the profiles of *cdc18*, *cdt1* and *cdc22* mRNAs are largely the result of differences in mRNA stability. In contrast, *cig2* expression was not induced in a mitotic block, while in a synchronous culture, *cig2* mRNA accumulated with comparable kinetics to *cdc18* mRNA. This latter observation could reflect the difficulties in pinpointing an event in cell cycle time in a synchronous culture, because of the rapid succession of M, G1 and S-phases in *S. pombe*. Alternatively, it might be a manifestation of a physiological difference between the mitosis in the two different experimental approaches. The fact that chromatin condensation is visibly more pronounced in a mitotic block than in the transient mitosis seen in cycling cells, is one indication that this might be the case. Arresting cells in mitosis, could induce a checkpoint, which then alters gene expression. There are other indications that *cig2* expression may be

controlled differently from that of other *cdc10* targets. Firstly, the published sequence of *cig2* does not contain an MCB element, although one may lie several hundred base pairs 5′ to the coding sequence (J. Ayte personal communication); other *cdc10* targets have MCB elements very close to the transcription start site. In addition, in the presence of pheromone and in cells entering meiosis, *cig2* message accumulates in a form that lacks polyadenylation (Obara-Ishihara and Okayama, 1994). Furthermore, this form persists in the absence of *cdc10* function (Bodo Stern unpublished data). This indicates that, either this represents a very stable form of the message or that, under these conditions, another transcriptional complex drives *cig2* expression. (*cdc18, cdc22* and *cdt1* mRNAs are not subject to this phenomenon). Also, in the mitotic cycle, *cig2* expression is not so profoundly affected by $cdc10^{ts}$ mutations as other targets (data not shown). Thus, it is likely that the expression of *cig2* may be influenced by factors other than *cdc10.*

Interestingly, *cut5* expression is also activated in cells arrested in mitosis (Saka, *et al.*, 1994). Although the *cut5* promoter does contain a single MCB element, it is not clear if this mitotic expression is *cdc10* dependent. Surprisingly, in spite of the induction of *cut5* expression in a mitotic block, *cut5* mRNA is expressed aperiodically in cycling cells, and the level of expression is independent of *cdc10* activity in a ts-mutant (Saka, *et al.*, 1994). As in the analysis of *cig2* expression, this suggests that there may be differences between the state of a cell in a mitotic arrest and when passing through mitosis during the normal cell cycle. The fact that the synthetic, triple MluI promoter can drive mitotic gene expression, indicates that this element alone may be responsible for the transcription of *cdc18, cdc22* and *cdt1* in mitosis. This implies that other unidentified elements present in the promoters of *cut5* and *cig2* genes may alter this MCB expression profile. A dissection of *cdc18, cig2* and *cut5* promoters should be made an important priority in future studies.

Despite the potential complexities influencing S-phase transcription suggested in this discussion, it seems likely, nevertheless, that *cdc10* is able to drive the transcription of several "S-phase genes" in mitosis. This mitotic expression is surprising, firstly, because it was always assumed that, like *SWI4/SWI6/MBP1* in *S. cerevisiae, cdc10* would be activated in late G1 (Johnston, 1992; Breeden, 1996), although the exact timing was hard to

discern. Secondly, it has been known for over 30 years that general transcription in eukaryotes is inhibited in mitosis, seemingly because condensed chromatin is unlikely to be accessible to RNA polymerase complexes (Johnston and Holland, 1965). While general mitotic repression of transcription has been best studied in mammalian cells, it is also thought to occur in *S. pombe.* However, the data in the literature concerning mitotic transcription in *S. pombe,* contains several instances where a dip in general transcription during mitosis was not observed, in synchronous cultures (Creanor and Mitchison, 1982 earlier work discussed therein; Elliott, 1983) or even in a mitotic arrest (Novak and Mitchison, 1990). In this latter study, RNA synthesis was shown to continue in *cdc13-117* cells arrested in mitosis with condensed chromatin at 35°C. For these reasons, the apparently heretical observation, that *cdc10* target genes are expressed in mitosis, appears less surprising, and this will be an interesting area for future work. In particular, it will be important to take a fresh look at the question of whether general polII transcription is maintained in mitotic chromatin.

In "wee" cells with an elongated G1 period, *cdc10* dependent transcription remains active for much of the cycle, probably from the onset of mitosis through G1 until S-phase. *cdc18* mRNA levels also remain high in *cdc2* arrested cells in G1, in a pheromone induced cell cycle arrest, in HU, and in several mutant cell cycle blocks within S-phase. These data suggest that *cdc10* is active in a single broad peak from mitosis until S-phase. The ability of cells to activate "S-phase" transcription in mitosis, and to remember this activation signal into the subsequent G1 and S-phase, may explain why *cdc10* dependent transcription is active early in G1, irrespective of the level of G1 CDK activity. (The low levels of *cdc10* target transcripts in G1 cells starved for nitrogen, may result from general mechanisms governing mRNA transcription and turnover). This reassessment of the timing of *cdc10* dependent transcription, also emphasizes the observation, described in Chapter 2, that *cdc10* is not activated as a result of the attainment of a critical cell mass or by passage through START. This is the case even though the decision to pass START requires *cdc10* activity. Therefore, cells probably become committed to the cycle as the result of the activation of some other gene function. One likely possibility, is that the accumulation of B-type cyclins, in late G1, is responsible for this transition. CDK activity may then inhibit pheromone signalling and the ability of cells to activate the

meiotic programme (Obara-Ishihara and Okayama, 1994; Oehlen and Cross, 1994).

So far, the mechanisms controlling the periodicity of *cdc10* dependent transcription, both its activation and inactivation, remain unresolved. Chromatin condensation *per se* is probably not responsible for the activation signal because the ectopic expression of *nimA*, which induces condensation without mitosis (O'Connell, *et al.*, 1994), does not lead to an increase in the *cdc18* transcript levels. Although I have been unable to identify a relationship between CDK function and *cdc10* activity, it is still possible that the mitotic activation of transcription, is the direct result of the phosphorylation of a component(s) of the cdc10p complex by the mitotic kinase. In fact, a consensus *cdc2* phosphorylation site within *cdc10,* has been shown to be critical for its activity (Connolly, *et al.*, 1997). The cdc10p complex would then retain a memory of this signal through G1 until S-phase. However, the data presented in Chapter 2 suggest that *cdc2* protein kinase activity is unlikely to mediate the inactivation of *cdc10* dependent transcription in G2. An alternative explanation is that, since *cdc10* is inactivated during S-phase, passage of the replication fork itself could disrupt the complex and constitute an off-switch. Such a mechanism is appealing, because it would enable *cdc10* to directly measure the achievement of its goal, DNA replication. A model could be constructed in which *cdc10* would monitor both mitosis and S-phase. Entry into mitosis would signal that S-phase functions are imminently required, and DNA replication would serve as the cue to inactivate *cdc10,* as its function is completed. Unfortunately, such schemes are difficult to test, although *cdc10* target genes could be placed adjacent to early and late origins of replication, if they were identified in *S. pombe.*

Finally, I will briefly discuss two possible implications of the unexpected mitotic expression of S-phase functions. The initiation of S-phase events in mitosis could simply prepare cells for DNA replication, since fission yeast has an unusually short G1 period. However, if this was the case, it is not clear why *cdc10* dependent transcription should be activated during mitosis in *wee1* mutant cells, in which the G1 phase is considerably extended, or in cells arrested in G1 in the presence of pheromone (Stern and Nurse, 1997). Interestingly, in *S. cerevisiae,* the *cdc18* homologue, *CDC6,* is expressed during late mitosis (Zwerschke, *et al.*, 1994; Piatti, *et al.*,

1995; Detweiler and Li, 1997). The conservation of the mitotic expression of *cdc18/CDC6* suggests that it may be a conserved feature of the eukaryotic cell cycle and therefore of functional relevance. In *S. cerevisiae,* expression of *CDC6* early in the cycle has been shown to be critical for entry into the subsequent S-phase (Piatti, *et al.*, 1996; Piatti, *et al.*, 1995; Detweiler and Li, 1997). Perhaps, *cdc10* dependent transcription of *cdc18,* at the boundary of mitosis and G1, is similarly critical for *cdc18* function. This latter explanation for the mitotic activation of *cdc10* is potentially interesting and is explored in the next Chapter.

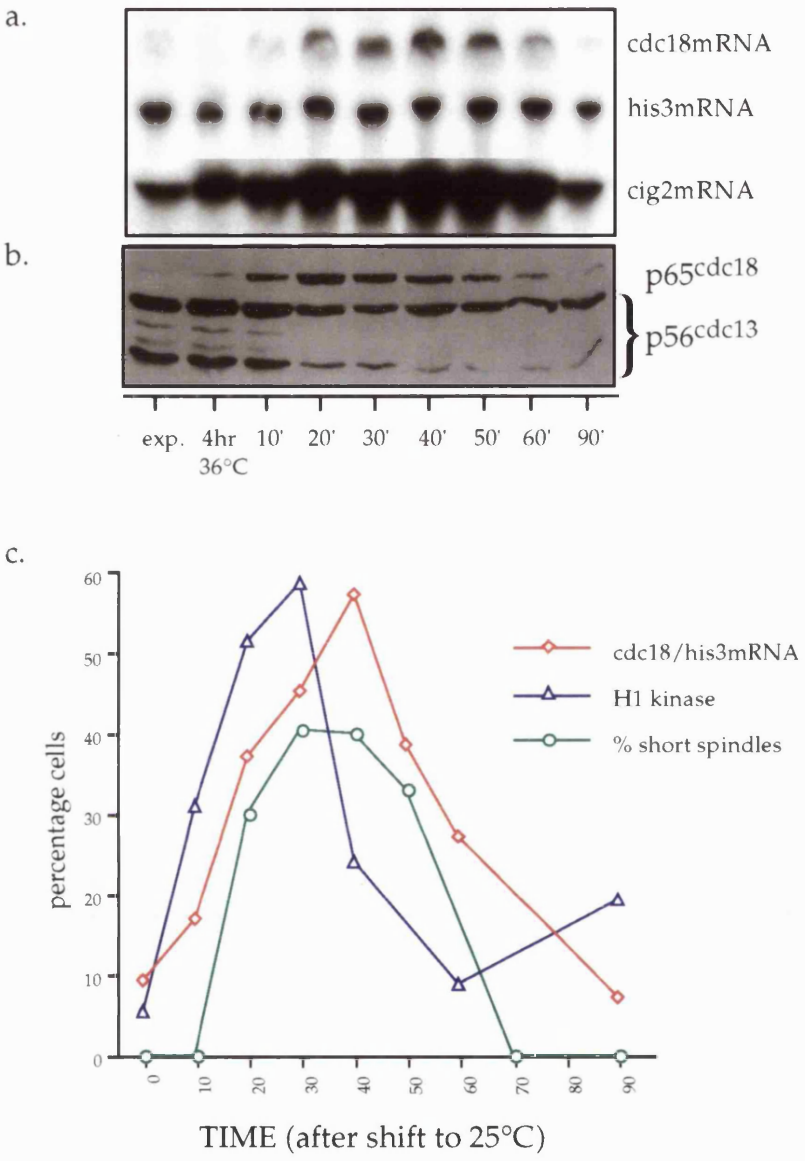
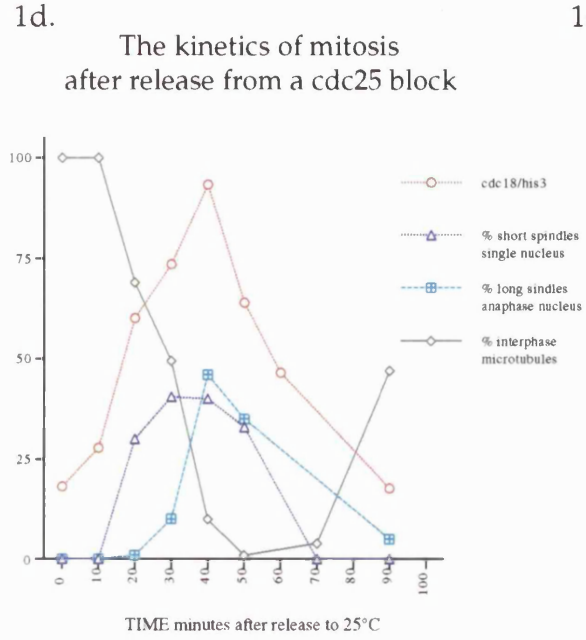


Figure 3.1

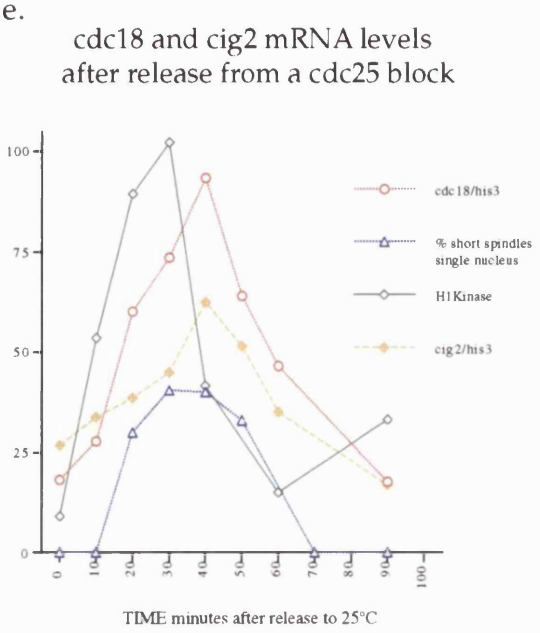
***cdc18* is expressed in mitosis in cells released from a *cdc25* block.**

1a. *cdc25-22* cells were blocked for 4 hours at 36°C to arrest them in G2 and then synchronously released into mitosis and G1 by shifting the culture to 25°C. Samples were taken every 10 minutes and *cdc18* and *cig2* transcript levels were followed by Northern analysis, with *his3* mRNA serving as a control. **1b.** cdc18p and cdc13p levels were determined by Western blotting. (Full length cdc13p runs close to cdc18p, the lower band is partially degraded cdc13p). **1c.** The *cdc18* message level was quatified by phosphor-imager analysis and standardised with respect to *his3*. Mitotic progression was monitored by measuring cdc13p associated H1 kinase activity and the percentage of cells with short spindles, (determined by immuno-labelling with anti α-tubulin antibodies). The criteria for assessing mitotic states are marked on the diagram in 2.**1f**.

1d.



1e.



1f.

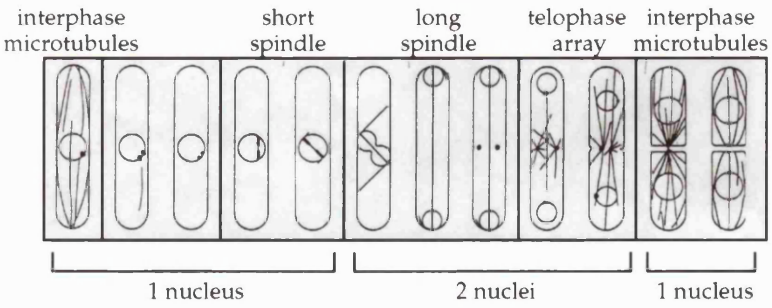


Figure 3.1 d, e & f.

A comparison of the timing of *cdc18* mRNA accumulation, progression through mitosis and cig2 expression.

Figures **1d** and **1e** show graphs quantifying events within the first 90 minutes following release from the *cdc25* block. Both include levels of *cdc18* mRNA, standardised against *his3*. In **1d**, *cdc18* mRNA levels are compared to the state of the microtubule cytoskeleton and in **1e**, to standardised levels of *cig2* transcript. The criteria for assessing the mitotic state of the cell are indicated in the diagram in **1f**.

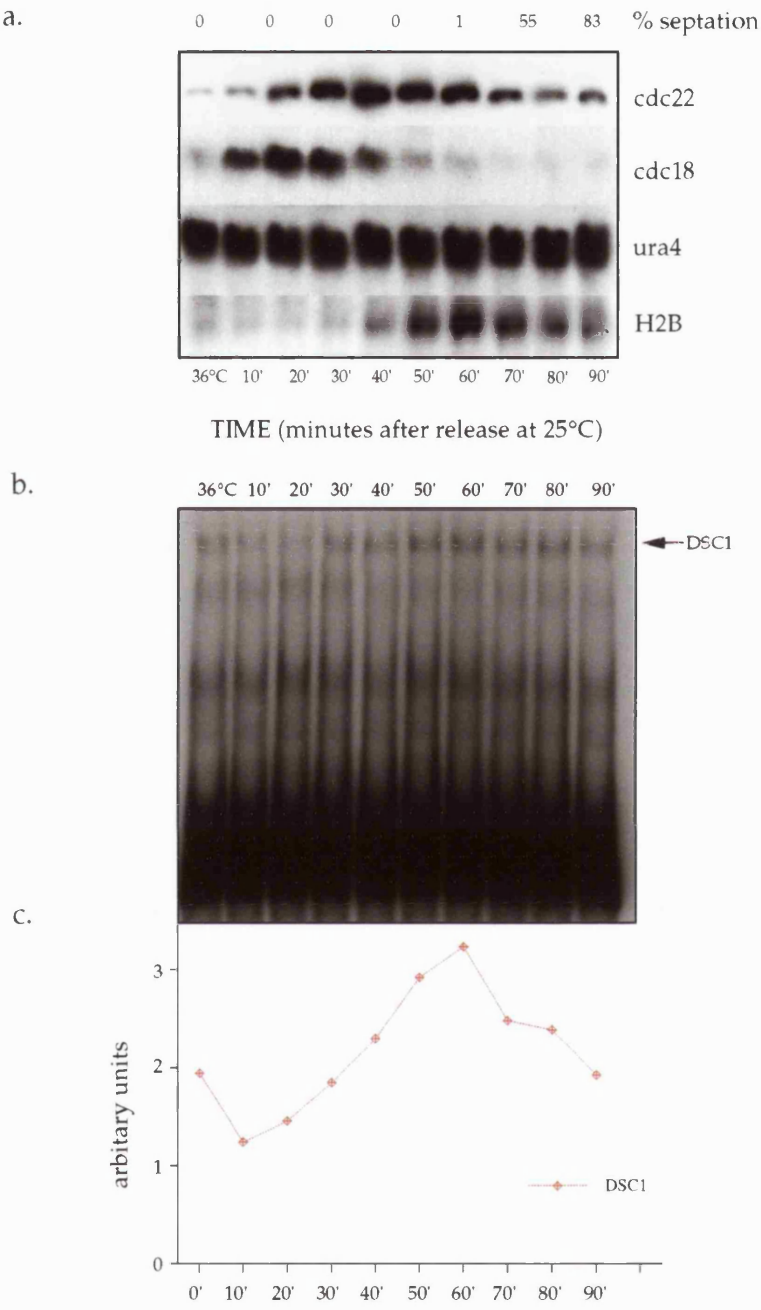


Figure 3.2

The presence of DSC1 through the cycle does not correlate with active transcription. The *cdc25* block and release experiment was repeated and samples taken for both a Northern (**2a**) and bandshift analysis (**2b**/**2c**). **2a.** *cdc18, cdc22, ura4* and histone transcript levels were monitored by Northern blotting. **2b.** A radio-labelled ds-DNA fragment of the *cdc18* promoter was added to cell extracts. DSC1 contains cdc10p (see Chapter 5) and is marked. **2c.** DSC1 was quantified by phosphor-imager analysis using the probe signal as a control.

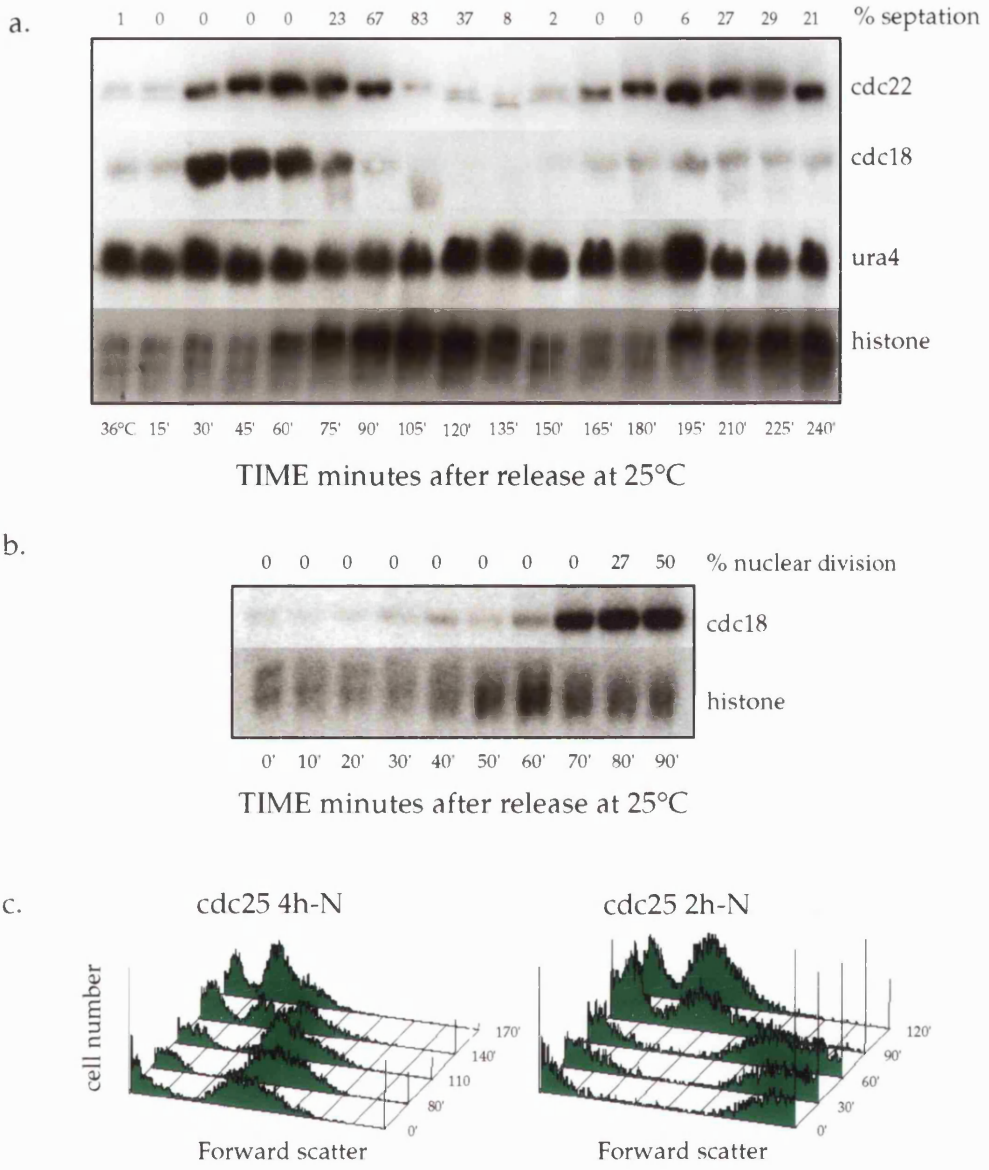


Figure 3.3

***cdc10* dependent transcription is activated in mitosis irrespective of cell size and nutrient conditions.** The *cdc25* block and release experiment was repeated with 2 variations; **3a**. nitrogen was removed from the media either after 2 hours at the restrictive temperature or **3b**, simultaneously with the shift to 36°C. In both cases, 4 hours after the shift to 36°C, cells were returned to 25°C in the same media, enabling entry into the cell cycle. *cdc18, cdc22, ura4* and histone transcripts were monitored by Northern blotting. As an indication of cell cycle time, the septation index was monitored in 3a, and the percentage of cells undergoing nuclear divisionin 3b (becauses in 3b, cells were unable to undergo septation in the 90 minutes sampled). Finally in **3c**, forward scatter data from a FACS analysis is presented as a measure of cell division.

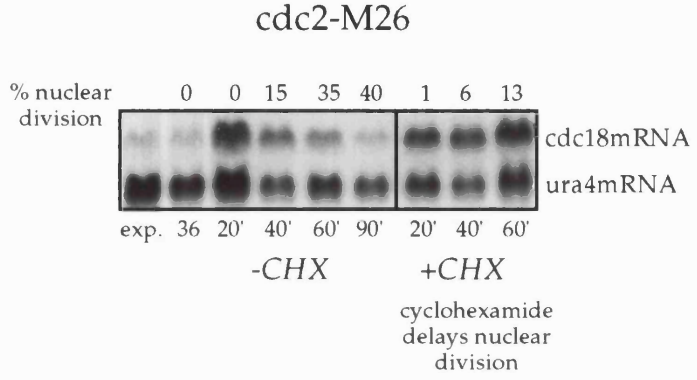


Figure 3.4

***cdc18* mRNA levels peak before the onset of anaphase upon release from a G2 *cdc2* block.**

cdc2-M26 cells were arrested at 36°C for 4 hours at the G2/M transition and then released into mitosis at 25°C, in the presence (lanes 3-6) or absence of cyclohexamide (CHX), (lanes 7-9). Cyclohexamide delays nuclear division. The percentage of cells with anaphase nuclei was determined by DAP I staining cells.

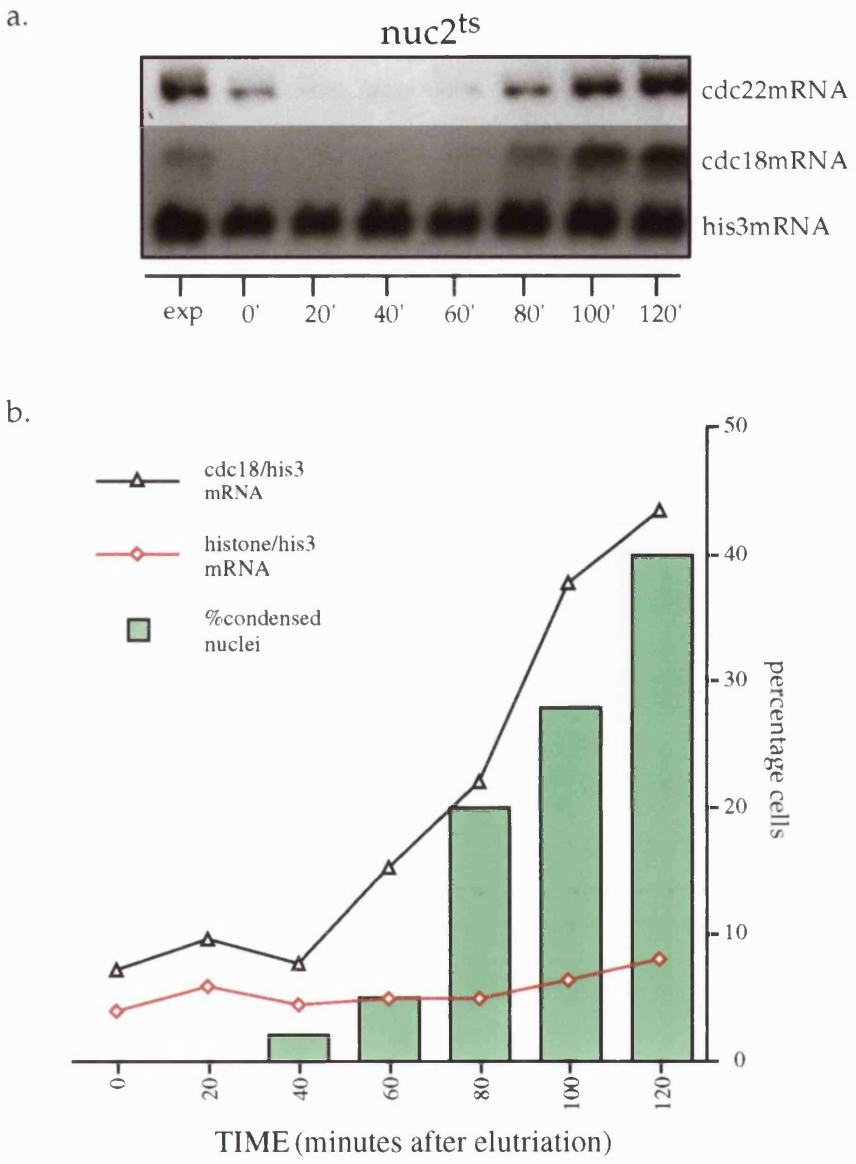
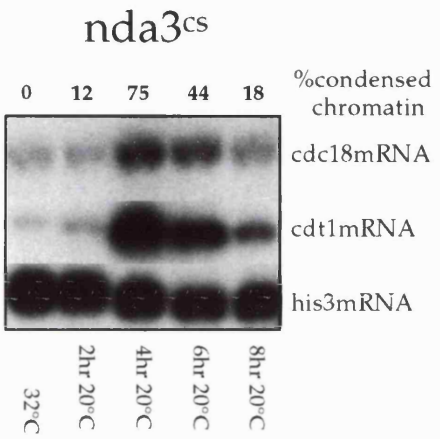


Figure 3.5

***cdc10* target transcripts accumulate in a mitotic block.**

A synchronous culture of*nuc2-663* cells in early G2 was obtained by elutriation at 25°C and then shifted to 36°C. Cells were followed as they entered the*nuc2* block. **5a.** *cdc18, cdc22* and *his3* mRNA levels were monitored by Northern analysis. **5b.** *cdc18* mRNA levels were quantified with respect to *his3* mRNA by phosphor-imager analysis, and histone transcript levels were monitored as a measure of leak through into S-phase. Entry into the metaphase arrest was visualised by determining the percentage of cells with condensed nuclei by DAPI staining cells.

5c.



5d.

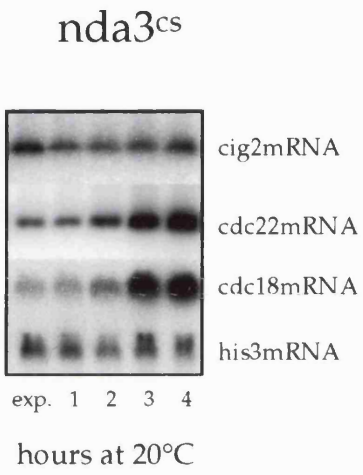


Figure 3.5c/d

***cdc10* target transcripts accumulate in a mitotic *nda3* block.**

5c. An asynchronous culture of *nda-km311* cells growing at 32°C, was transiently arrested in mitosis by a shift to the restrictive temperature of 20°C. After 6 hours at 20°C, *nda3* cells leak through the block, into the following cell cycle. *cdc18* and *cdt1* trancsript levels were followed, *his3* mRNA serving as a control. The percentage of cells with condensed nuclei was determined by microscopic analysis of DAPI stained cells, (see Figure 3.5e). In **5d,** the *nda3* block was repeated and a Northern probed for *cdc18, cig2, cdc22* and *his3* transcripts.

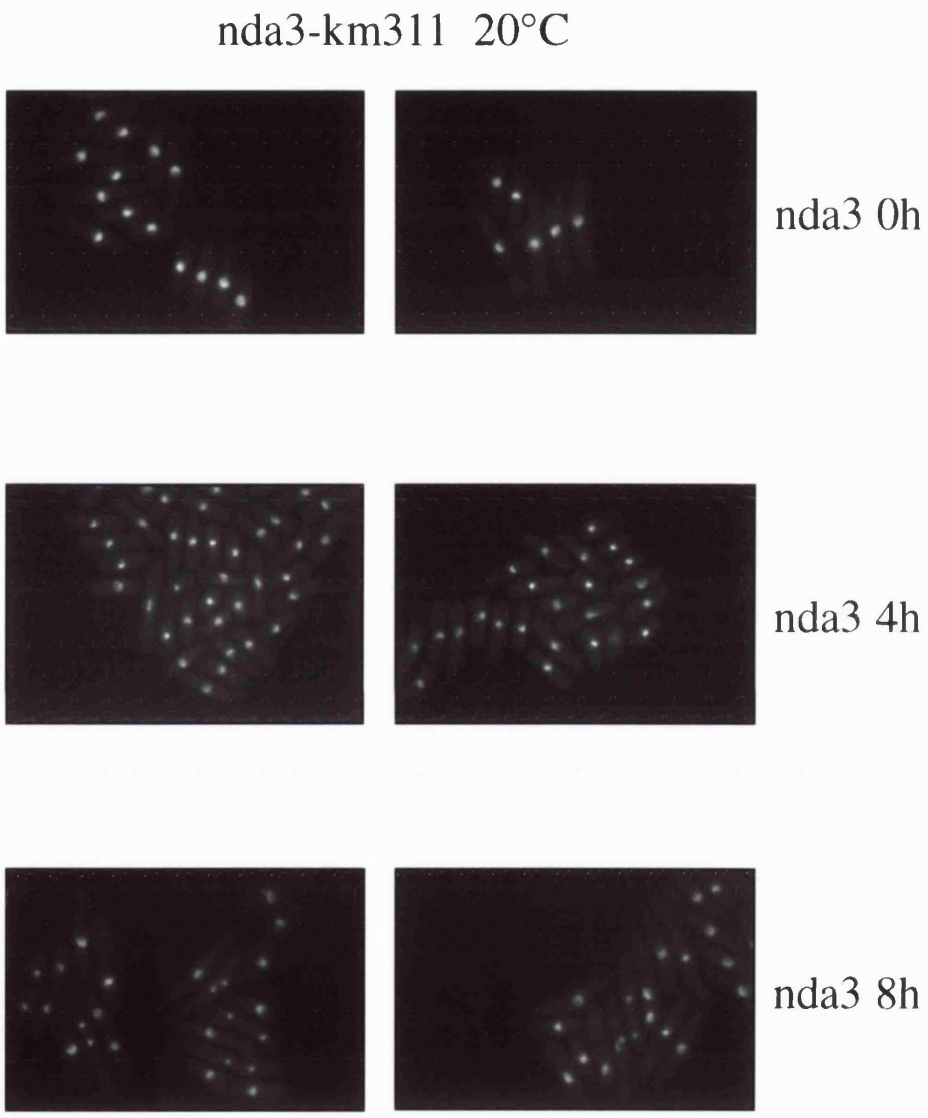


Figure 3.5e

DAPI stained cells in a transient *nda3* induced arrest.

Cells from 3.5c were DAPI stained at 32°C and after 4 and 8 hours at 20°C in minimal media.

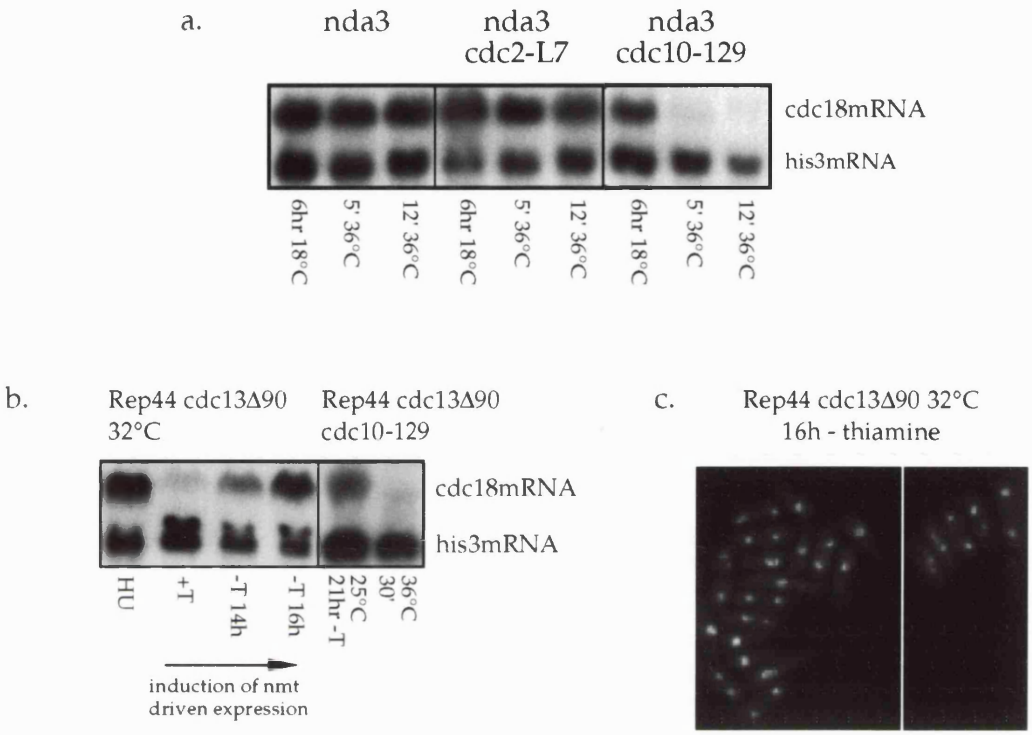
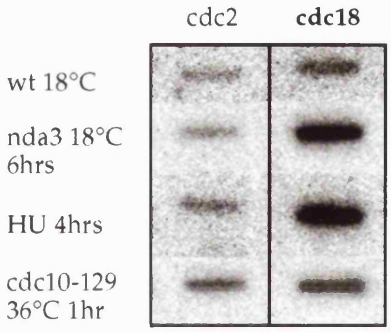


Figure 3.6

The mitotic expression of *cdc18* is dependent on *cdc10* function.

6a. *nda3, nda3 cdc10-129* and *nda3 cdc2-L7* cells were arrested at metaphase for 6 hours at 18°C. Cells were filtered and resuspended in minimal media at 36°C, containing 150µg/ml of thiabendazol to delay the onset of anaphase. Samples were taken for Northern analysis 0, 5 and 12 minutes after the temperature shift. *cdc18* and *his3* transcript levels were followed. (Cells were fixed and stained with DAPI to assess the state of the nuclei in the block and after the shift). In **6b**, cells were arrested in anaphase by the ectopic expression of *cdc13Δ90*. Two strains containing an integrated copy of *cdc13Δ90* behind the weak *nmt* promoter were used, one also carried the *cdc10-129* mutation. Exponentially growing cells were washed free of thiamine (4 washes) to induce *nmt* driven expression (after approximately 12 hours at 32°C and 20 hours at 25°C). At 32°C, *cdc18* mRNA levels were analysed after 14 and 16 hours (6b left hand panel). (An RNA sample from wildtype cells arrested in HU for 4 hours was included as a control). 21 hours after the removal of thiamine *cdc10–129* cells overexpressing *cdc13Δ90* were shifted to 36°C for 30 minutes to inactivate *cdc10* function (6b right hand panel). A Northern blot was again used to assess levels of *cdc18* and *his3* transcripts. DAPI was used to confirm the mitotic arrest. In **6c,** cells ectopically expressing *cdc13Δ90* for 16h at 32°C (6b, lane 4) were fixed and DAPI stained to visualize nuclei.

a.



b.

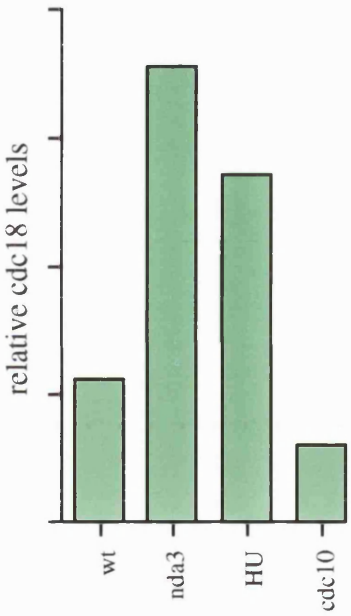


Figure 3.7

***cdc18* is actively transcribed in a mitotic block.**

To determine whether *cdc18* is actively transcribed in mitosis, and to rule out an effect of RNA stability, I carried out a nuclear run on experiment. Nascent transcription was analysed in wildtype cells grown at 32°C and 18°C, in cells blocked in hydroxyurea for 4 hours, in *nda3-km311* cells blocked in mitosis for 6 hours at 20°C and in *cdc10-129* cells at the restrictive temperature (36°C). Radiolabelled RNA was isolated and hybridised to single stranded DNA crosslinked to a membrane. Single stranded *cdc18*, *ura4*, and *cdc2* probes, in both orientations, were generated using the M13 origin in the KS vector. Vector alone was used to determine the background signal. The raw data is presented for the experiment in **3a.**, together with a quantification by phosphor-imager analysis, in **3b**. . The ratio of the *cdc18/cdc2* signal was calculated after the background signal was subtracted from each.

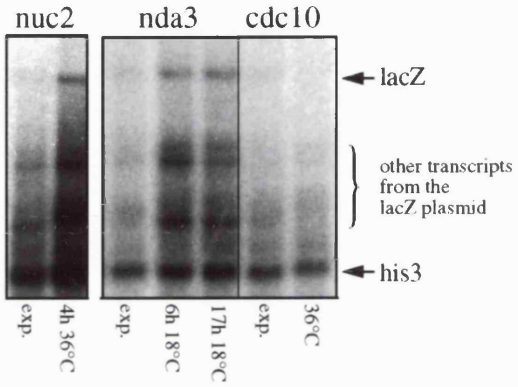


Figure 3.8

In *S. pombe* transcription from the synthetic triple Mlu1 promoter is active in a mitotic arrest. A plasmid containing *LacZ* behind a synthetic triple MCB element (in the vector pSPΔ178.3M) was transformed into *cdc10-129*, *nuc2-663* and *nda3* cells. *nuc2* cells were shifted to 36°C for 4 hours, and *nda3* cells were shifted to the restictive temperature of 18°C, for 6h and 17h, to accumulate cells in mitosis. *cdc10-129* cells were shifted to 36°C for 30 minutes, to inactivate *cdc10*. RNA was isolated and polyA+ RNA purified using a Promega kit. The equivalent of 10μg of RNA was analysed by Northern blotting. PolyA+ RNA was probed to determine levels of *lacZ* and *his3* transcripts.

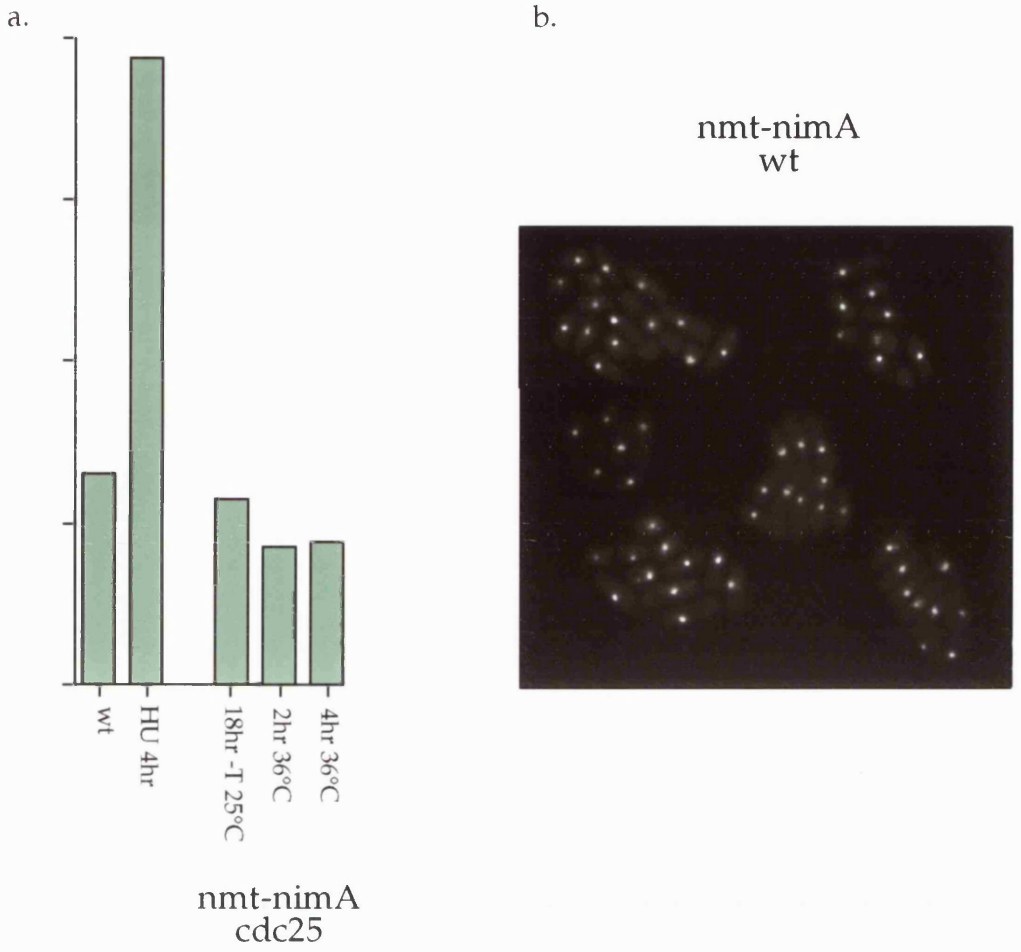


Figure 3.9

Chromatin condensation *per se* cannot induce *cdc10* dependent transcription. **9a.** A *cdc25-22* strain containing an integrated copy of *nimA* behind the weak *nmt* promoter was grown at 25°C, in the absence of thiamine, for 18 hours to induce expression of *nimA* (this was confirmed by DAPI staining cells). Cells were then shifted to 36°C for 4 hours to prevent mitosis. A Northern analysis was used to determine the levels of *cdc18* and *his3 mes*sages, which were then quantified by phosphor-imager analysis. RNA samples, from exponential wildtype cells and cells arrested in 11mM HU for 4 hours, were run on the same gel as controls. **9b.** The terminal phenotype of wildtype cells expressing *nimA* is shown by DAPI staining to demonstrate the dramatic nuclear condensation.

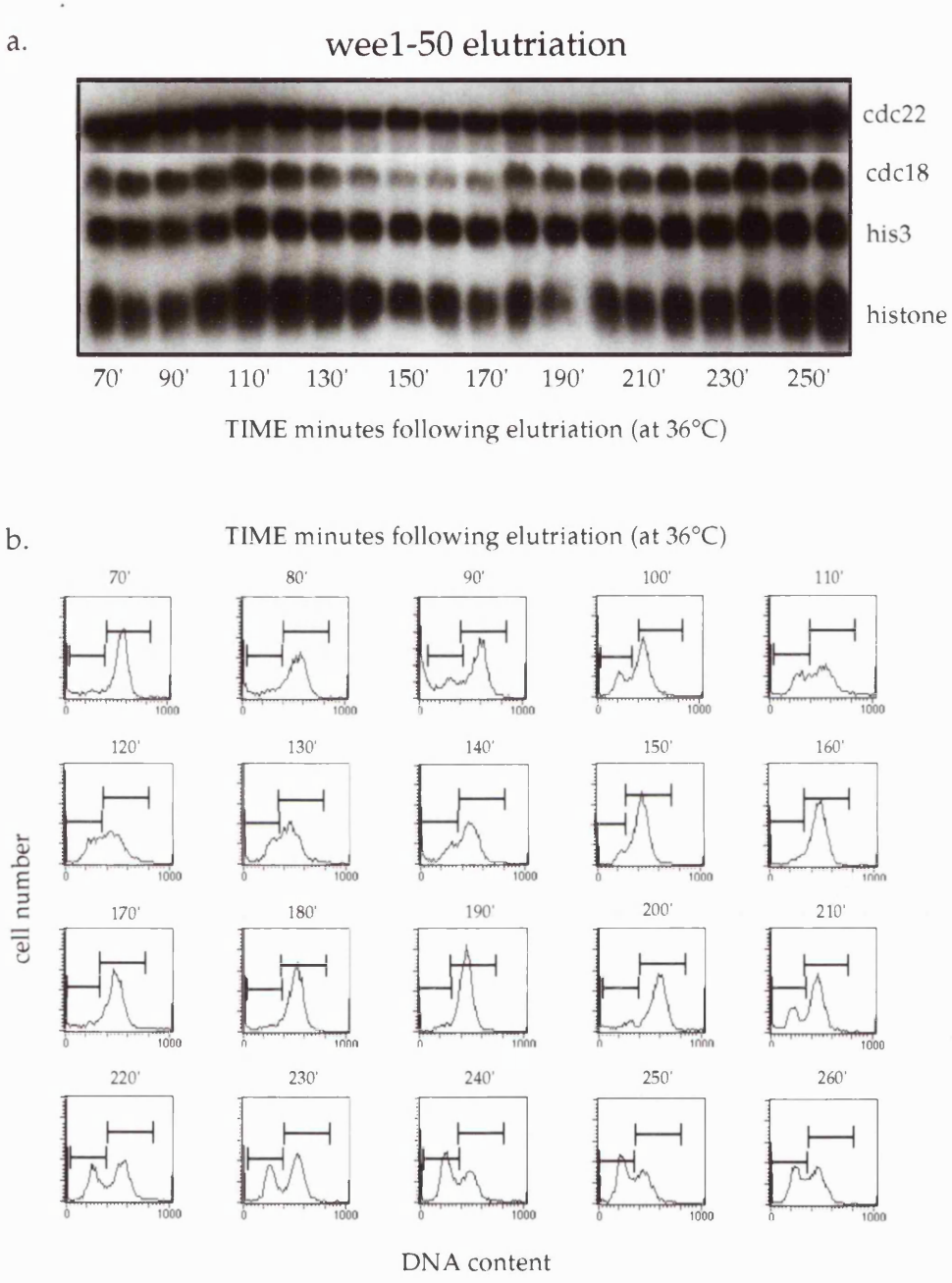
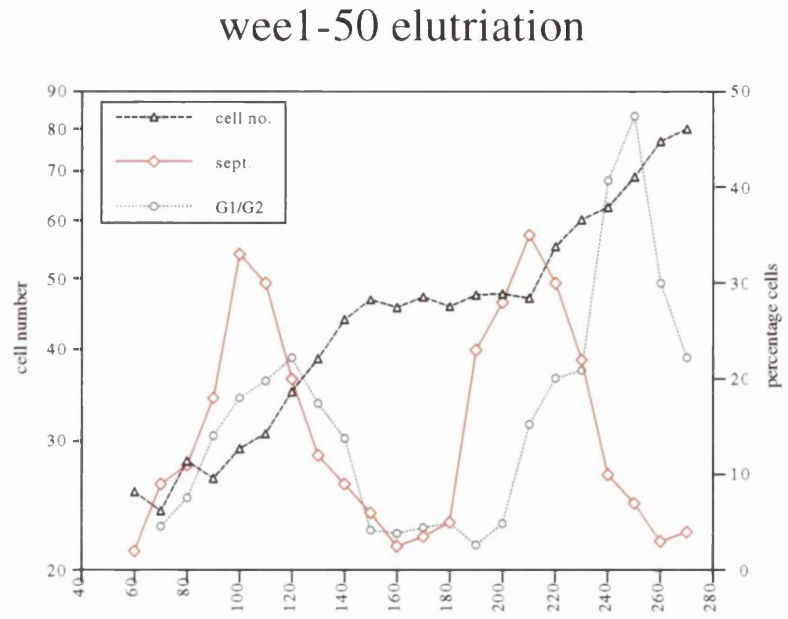


Figure 3.10

A synchronous culture of *wee1-50* cells was used to separate mitosis and S-phase in time, to facillitate the analysis of *cdc10* activity through the cycle.

wee1-50 cells were elutriated at 25°C, shifted to 36°C and followed through two synchronous cell cycles. In **10a**, RNA samples were run on a Northern Blot which was probed for *cdc18, cdc22, his3* and histone transcripts. In **10b**, the raw FACS data is presented showing the DNA content. The regions used to define the percentage of G1 and G2 cells are marked (and quantified in Figure 3.10c).

3.10c.



3.10d.

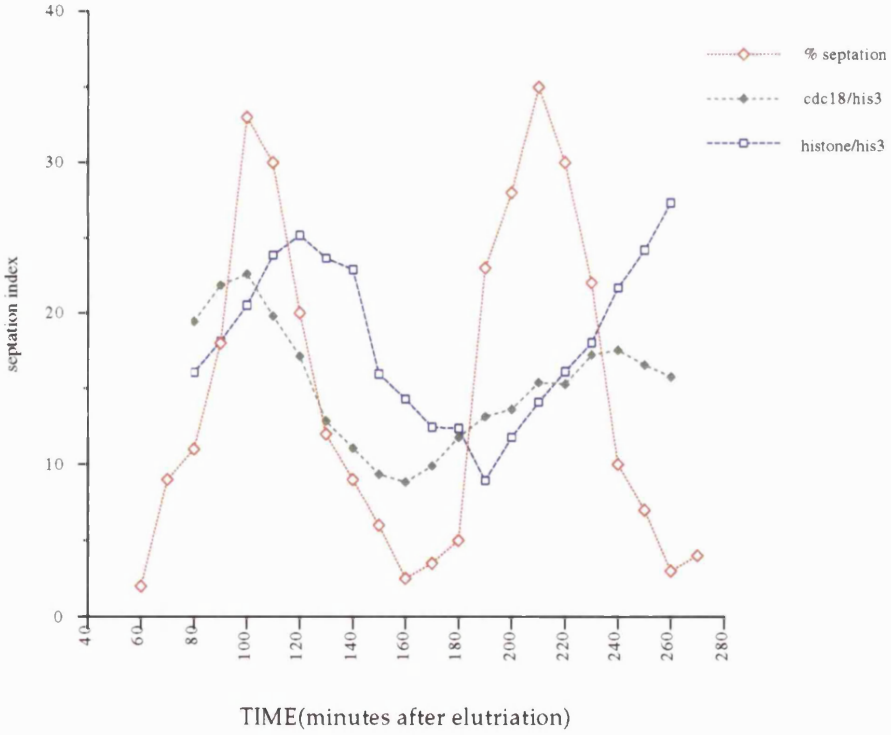


Figure 3.10

The synchrony of the elutriated culture of *wee1-50* cells.

10c. The septation index, cell number data and the ratio of G1/G2 cells, as determined by FACS analysis (in 3.10b), are presented together to show synchrony in the elutriated *wee1-50* culture. In **10d**, *cdc18, histone* and *his3* mRNA levels were quantified by phosphor-imager analysis and are shown graphically for comparison.

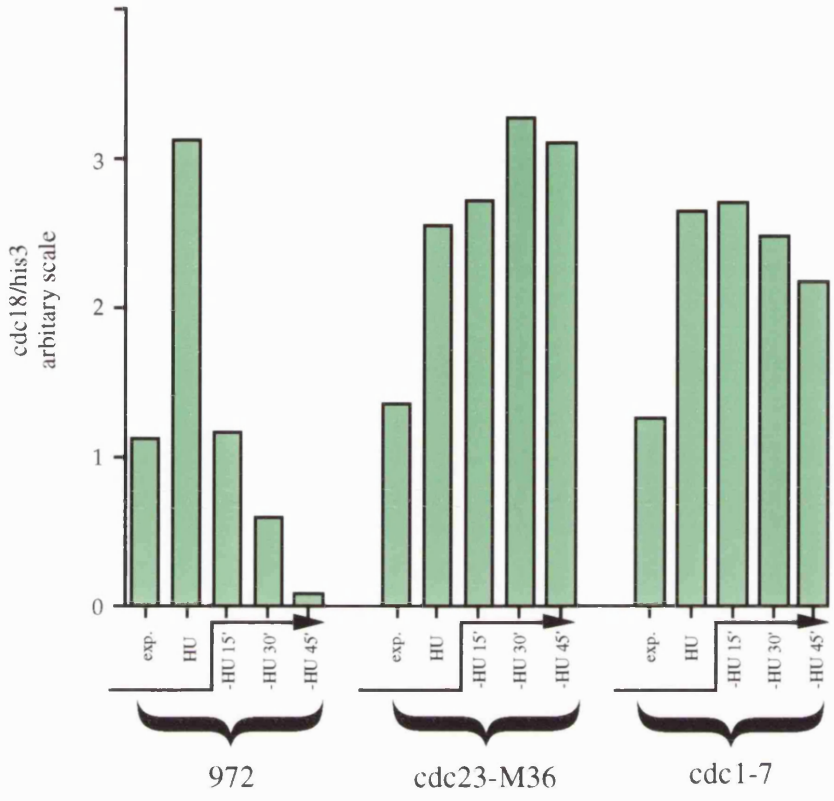


Figure 3.11

***cdc18* expression remains elevated in cells delayed in their progression through S-phase.**

Wildtype, *cdc23-M36* and *cdc1-7* mutant cells were arrested in HU for 3.5 hours, shifted to 36°C and then HU was washed out at the high temperature. Samples were taken at various times after release from the block. *cdc18* and *his3* message levels were analysed by Northern blotting and quantified by phosphor-imager analysis. The *cdc18/his3* ratio is shown graphically. (Similar results were obtained with *cdc27* and *cdc22* mutations, data not shown).

CHAPTER 4

The function of mitotic *cdc18* expression, and the accumulation of cdc18p.

Introduction

In this chapter, I address the functional significance of the timing of *cdc10* dependent transcription described in Chapters 2 and 3. The finding that *cdc10* is active in mitosis was initially surprising because *cdc10* function is required for S-phase, and cells happily proceed through mitosis into G1 in its absence. In this Chapter, I investigate the timing of oscillations in the level of cdc18p, the major target of *cdc10*, and define a mechanism, in addition to periodic transcription, used to control cdc18p accumulation through the cell cycle. This goes some way towards explaining how passage through mitosis is able to re-set a cell's capacity to enter S-phase. The mechanism identified may also be important for coupling the exit from mitosis to entry into S-phase. This analysis highlights the fact that, although the transcriptional controls appear to be different between budding and fission yeast, there is another level at which both organisms use similar, conserved machinery to control the onset of S-phase.

cdc10 is unable to complete its function in a metaphase arrest.

In the previous chapter, I demonstrated that S-phase transcription is active in a metaphase arrest. This lead me to ask whether *cdc10*, which is required for the subsequent round of DNA replication, is able to complete its S-phase function in the previous mitosis (Figure 4.1). This would indicate that the activity of *cdc10* in mitosis is of functional significance. To do this, an *nda3-km311* strain was used carrying a ts-mutation in *cdc10*, *cdc10-129*. *nda3-km311* and *nda3-km311 cdc2-33* cells were used as controls. Cells were arrested in metaphase for 6hrs at 18°C and then shifted to 36.5°C the restrictive temperature for *cdc10* and *cdc2*. This allows cells to rapidly exit from mitosis. Samples were collected for FACS analysis to determine whether these cells were able to enter and complete the subsequent S-phase, in the absence of *cdc2* or *cdc10* function. *nda3* cells left mitosis and entered G1 and S-phase without an observable G1 population (the full time course is not shown), since in rapidly growing *S. pombe*, cell separation and S-phase occur simultaneously. However, in the strain mutated for *cdc10* function, cells were unable to enter S-phase upon release from the *nda3* block and accumulated in G1 of the first cycle after the release (Figure 4.1, panel 3). This shows that, although *cdc10* dependent transcription is fully active in mitosis, it is unable to complete its S-phase function in a metaphase arrest. *nda3-km311 cdc2-33* cells were also able to exit mitosis, and 80% of cells arrested in G1 of the subsequent cycle (Figure 4.1, panel 2). This implies that *cdc2* may not be required after metaphase for the completion of mitosis. *cdc2* function is required for both the G1/S and G2/M transitions, therefore this indicates that before the shift to 36.5°c, 80% of the *nda3 cdc2-33* cells in this experiment were arrested after the G2 *cdc2* requirement, i.e. in mitosis.

Cdc18p does not accumulate in cells arrested in mitosis.

The above experiment suggests that targets of *cdc10* cannot fulfil their S-phase function in mitosis. Therefore, in order to address whether cdc18p, a critical target of *cdc10*, is able to accumulate in metaphase arrested cells, cdc18p levels were monitored in an *nda3* block and release experiment. A mitotic arrest was induced by shifting *nda3* mutant cells to 18°C for 6 hours. Cells were then shifted to 32°C, by the addition of warm media, to release them into the subsequent cycle (Figure 4.2a). (DAPI stained *nda3* cells are shown, 5 and 10 minutes after the release, in Figure 4.4a). Although cdc18p

levels are low in exponentially growing cells, cdc18p levels were further reduced in the block, in spite of the elevated levels of *cdc18* mRNA (see Figure 3.5c). However, within 15 minutes of release from the block at 32°C, 50% of cells were synchronously undergoing anaphase as measured by DAPI staining, (Figure 4.4) and cdc18p had accumulated to high levels. Next, the *nda3* block and release experiment was repeated, this time shifting cells to 36°C (Figure 4.2b). A wildtype control was included to demonstrate that this accumulation of cdc18p is not a side effect of the extreme temperature shift. In the wildtype, there was a slight decrease in cdc18p levels after the temperature shift, (Figure 4.2b, right hand panel). Again in the experimental strain, cdc18p accumulated very rapidly, reaching high levels within 5 minutes of the release. Cdc18p is thought to be the critical target of *cdc10*, so the inability of cdc18p to accumulate in a metaphase arrest offers a possible explanation for the observation that *cdc10* function is not complete in cells arrested in an *nda3* block (Figure 4.1).

In S-phase cells, cdc18p is unable to accumulate to high levels and the protein is smeared towards higher molecular weights.

I next measured cdc18p levels in cells at the other end of the active *cdc10* oscillation, in cells arrested in early S-phase in HU. Although *cdc10* is still maximally active, cdc18p was unable to accumulate to high levels (Figure 4.2c). In these cells, cdc18p is visible in a smear of co-migrating bands when compared to the exponential cells, suggesting some post-translational modification. This retarded mobility is also seen occasionally in a mitotic arrest (data not shown). Like cells arrested in metaphase, cells in HU have elevated levels of B-type cyclin and CDK activity. This result suggests the possibility that high levels of *cdc2* kinase may de-stabilise cdc18p in both mitosis and S-phase. Therefore, although *cdc18* transcripts are present from M-phase through to S-phase, the protein only accumulates in a window of low kinase within this period, from anaphase up until late G1. The inability to degrade all the cdc18p in HU, may be a reflection of the fact that a subset of the protein remains in a complex e.g. at origins. In *S. cerevisiae*, some origins are unable to fire in wildtype cells arrested in HU (personal communication John Diffley), and a similar phenomenon may exist in *S. pombe*.

Oscillations in cdc18p periodicity can be controlled by post-transcriptional controls alone.

I next investigated the mechanism by which this dramatic change in cdc18p levels occurred on exit from a metaphase arrest. The data shown in Chapter 3, imply that the rapid increase in the level of cdc18p is unlikely to be the result of changes in *cdc18* transcript levels (Figure 3.6a). To confirm whether it is the result of post-transcriptional and post-translational controls, I used an *nda3-km311 cdc18Δ* strain, kept alive by a plasmid borne copy of *cdc18* expressed from the weak *nmt* promoter (Figure 4.3b, lanes 1-3). In the absence of thiamine, *cdc18* is constitutively transcribed and translated in this strain, so any oscillation in cdc18p levels probably occurs via alterations in the turnover of the protein. This strain was subjected to an *nda3* block and release protocol. DAPI stained cells from this experiment, 10 minutes after release from the mitotic block, are shown in Figure 4.4b. Cdc18p expressed from the *nmt* promoter, like the endogenous cdc18p in *nda3* cells, was undetectable in the mitotic arrest, but accumulated soon after the release. Therefore, regulated proteolysis is sufficient to explain the accumulation of cdc18p on exit from mitosis, and strains constitutively expressing *cdc18* are perfectly viable. This implies that the periodic expression of *cdc18* is dispensible for cell cycle regulation of cdc18p levels under most conditions. This second layer of control over cdc18p stability may also explain, in part, the viability of strains with aperiodic *cdc10* dependent transcription (see Chapter 5). The proteolysis of cdc18p is known to be mediated by *pop1*, which is part of a complex which directs ubiquitin-proteasome mediated degradation of cdc18p and rum1p (Kominami and Toda, 1997). Therefore, cell cycle dependent changes in the ability of the pop1p complex to target cdc18p for degradation could control the periodic accumulation of cdc18p.

The N-terminus of cdc18p mediates its periodic instability.

How is the mitotic instability of cdc18p mediated? Cdc18p contains 6 cdc2p consensus phosphorylation sites of the sequence (S/T-P-X-K/R); 5 lie in its N-terminus while the sixth lies in the central portion of the protein. Hideo Nishitani has shown that a mutant version of cdc18p lacking these sites is still able to induce over-replication when ectopically expressed. This implies that phosphorylation of cdc18p at these residues may not be critical for the S-phase function of cdc18p. Given that cdc18p only

accumulates when cdc2p kinase levels are low, after the exit from mitosis, it is possible that these sites play a role in generating the mitotic instability of cdc18p. In order to test the hypothesis that these sites mediate the periodic accumulation of cdc18p, I used, 2 mutant forms of cdc18p, constructed by Hideo Nishitani, lacking the N-terminus and all 5 putative cdc2p phosphorylation sites. A schematic of both wildtype and truncated versions of cdc18p is shown in Figure 4.3a. An additional construct was designed carrying a truncated version of cdc18p lacking the N-terminal 135 base pairs, but tagged at the amino terminus with the SV40 nuclear localisation signal (NLS). This was used to test whether changes in protein stability might be an indirect effect of the inability of a truncated cdc18p to be correctly targetted to the nucleus. This is an important control because in *S. cerevisiae*, the nuclear localisation of Swi5p has been shown to affect its stability (Nasmyth *et al.*, 1996), and alterations in the nuclear localisation of Cdc6p during the cycle are thought to contribute to the control of its activity (Piatti, *et al.*, 1996).

I analysed the effect of these truncations by ectopically expressing these mutant versions of *cdc18* from the *nmt* promoter, at intermediate levels which are not lethal, in an *nda3* mutant strain. Cells were then subjected to an *nda3* block and release protocol (as in Figure 4.2a). Although the endogenous cdc18p cannot accumulate in mitosis, removal of the N-terminal cdc2p consensus sites by truncation, in cdc18-d55, was sufficient to stabilise the protein when ectopically expressed in the *nda3* block (Figure 4.3b, lane 4). Furthermore, the level of cdc18p-d55 did not increase significantly on release into G1 (Figure 4.3b lanes 5-6). This truncated protein was also stable in HU as compared to the endogenous cdc18p (Figure 4.3c lanes 10 and 11). The less extensive truncation of *cdc18*, cdc18p-d5, behaved similarly and is discussed below. In order to ensure that the mitotic stability was not due to alterations in the inability of truncated forms of cdc18p to enter the nucleus, this experiment was repeated using cdc18p-d5 tagged with the NLS (Figure 4.3b lanes 7-9). (Hideo Nishitani confirmed by immuno-localisation, that while much of the ectopically expressed *cdc18-d55* or *cdc18-d5* protein is localised in the cytoplasm, all of the protein tagged with the SV40 NLS accumulates in the nucleus). Exponentially growing *nda3* cells, carrying *cdc18-d5NLS*, were arrested for 6 hours at 18°C and then released at 32°C for 15 minutes. In this gel, the band, with a mobility slightly greater than that of cdc18p itself and cross-

reacting with anti-cdc18p antibodies, was particularly prominent. The nuclear localisation signal did not dramatically alter the stability of the cdc18p-d5 protein. In addition the nuclear cdc18p-d55 remained immune to the periodic instability affecting the wildtype protein. From these results it can be concluded that the N-terminus of cdc18p confines the accumulation of the protein to a period of low kinase activity in G1, by targetting it for degradation in mitosis and in HU.

The TPSTP motif shared by Sic1p, rum1p and cdc18p is probably not important in the cell cycle oscillation in cdc18p stability.

rum1 transcripts are present throughout the cell cycle (Moreno and Nurse, 1994). However, like cdc18p, rum1p accumulates exclusively in G1 (Correa-Bordes and Nurse, 1995). As for cdc18p, this is thought to result from cell cycle specific degradation controlled by the *pop1* ubiquitin-proteasome pathway (Kominami and Toda, 1997). The *rum1* protein shares several features with cdc18p. It contains cdc2p consensus phosphorylation sites at its N-terminus and an N-terminal sequence TPSTP (Moreno and Nurse, 1994), which could be the target for MAPK phosphorylation. The budding yeast protein Sic1p, contains an identical TPSTP motif and is subject to similar regulated proteolysis (Schneider, et al., 1996; Schwob *et al.*,, 1994). The conservation of this TPSTP motif in multiple proteins with a regulatory role over G1 progression, suggests that it may be functionally important. This motif was removed as part of the d55 truncation of cdc18p, (see Figure 4.3a). By comparing the behaviour of a cdc18p containing or lacking this sequence, it may be possible to identify a role for this conserved motif in the control over protein stability. Both versions were compared in parallel in Figure 4.5.

The version of cdc18p, cdc18p-d5, which lacks the cdc2p consensus sites, but which still contains the TPSTP motif, behaved identically to the cdc18p-d55 mutant in an *nda3* block and release experiment (Figure 4.5a). Like the d55 truncation, cdc18p-d5 remained stable in metaphase and did not accumulate on release into anaphase and G1 (Figure 4.5a, left hand panel). Therefore, the presence of the TPSTP motif does not destabilise cdc18p in the mitotic arrest. This shows that this sequence in itself is insufficient to target cdc18p for proteolysis. Also, the overall level of the cdc18p-d5, driven by the weak or medium strength *nmt* promoter, was

indistinguishable from that of d55p driven by the same promoter (data not shown and Figure 4.5b, compare the levels of cdc18p-d55 and cdc18-d5 in the presence and absence of pheromone). Taken together these data imply that the TPSTP sequence is unlikely to play a role in control of *cdc18* turnover through the mitotic cycle, although it could act in conjunction with other elements residing within the N-terminus. Interestingly, Hideo Nishitani has shown that cdc18p-d55 is more potent than cdc18p-d5 at causing over-replication. Therefore, this motif may play a functional role in the regulation of cdc18p activity.

Rum1p is present in a mitotic arrest and does not accumulate further upon release into anaphase and G1.

The Westerns in Figure 4.5a were then probed for rum1p which also contains a similar motif. Interestingly, rum1p was present in cells arrested in mitosis at the *nda3* block, and did not accumulate further on release into anaphase. Thus, rum1p behaves like the truncated versions of cdc18p. The difference in the profile of *rum1* and *cdc18* proteins is interesting because several pieces of information suggest that the similar N-terminal portions of rum1p, cdc18p and Sic1p confer periodic instability on these proteins. In particular, while the N-terminus of rum1p is dispensable for its function, truncation of this portion of the protein leads to a more potent effect on over-replication possibly via increasing protein stability (Bodo Stern, unpublished data). It has also been shown *in vitro*, that rum1p is phosphorylated by CDKs in immuno-precipitates from *S. pombe* cells, and there are several indications that phosphorylation may mark the protein for degradation (Correa-Bordes and Nurse, 1995). This suggests that there are complex controls defining several patterns of proteolysis of proteins acting in G1.

The TPSTP motif could be the target for a MAPK. *spk1* is such a MAPK and is activated in response to the addition of pheromone (Toda, et al., 1991). A possible role for this rum1p/cdc18p motif could therefore be in the control over protein stability in a pheromone induced arrest, a condition which absolutely requires the presence of the *rum1* protein (Stern and Nurse, 1997). To test whether this motif plays a role in pheromone induced alterations in protein stability, *h- cyr1Δ sxa2Δ* cells, which are responsive to P-factor, were transformed with *rep41::cdc18-d5* and *rep41::cdc18-d55*

plasmids. Cells were then arrested in G1 by the addition of P-factor for 6 hours, and the levels of truncated or wildtype cdc18p and rum1p determined by Western blotting (Figure 4.4b). The level of cdc18p was compared with that observed in a *nda3* block and release experiment in Figure 4.4a. The addition of pheromone arrested close to 100% of cells in G1 (data not shown). As expected, rum1p accumulated in the pheromone arrest, (rum1p is barely detectable in exponential cultures of cells but accumulates in G1 arrested cells). In contrast, the endogenous cdc18p remained at low levels, similar to those seen before the addition of pheromone, in spite of the fact that *cdc18* mRNA accumulates in a pheromone induced arrest (see Figure 2.4 (Stern and Nurse, 1997)). In addition, the levels of cdc18p, lacking or containing the TPSTP sequence, were similar in cells during exponential growth and in a mitotic arrest. This shows that the destabilisation of the wildtype cdc18p in pheromone is also mediated by the N-terminus, but not the TPSTP motif. However, because pheromone inhibits CDK activity in G1, pheromone may destabilise cdc18p by a different mechanism to that used in mitosis. This experiment also emphasizes the fact that rum1p and cdc18p are subject to different cell cycle controls.

Perturbations of *cdc2* function alter cdc18p stability.

Based on the data presented so far, it seems likely that cdc18p is rendered unstable by cdc2p mediated phosphorylation of its N-terminus. It was already shown in Chapter 2 (Figure 2.6b), using the cyclin deletion strain, that while cells in G1, with or without CDK/cyclinB activity have similar levels of *cdc18* mRNA, they have dramatically different levels of cdc18p. In order to confirm this effect of CDK activity on cdc18p stability, I used *cdc2* ts-alleles to inactivate *cdc2* and monitored the effects on cdc18p levels. Firstly, cdc18p levels were monitored in wildtype and *cdc2-M26* cells using samples from the experiment shown in Figure 2.2, in which cells were arrested in G1 of the cell cycle by nitrogen starvation, and released at 36.5°C. Wildtype cells entered S-phase 150 minutes after the addition of nitrogen, whereas $cdc2^{ts}$ cells elongated but remained in G1 (Figure 2.2c). cdc18p was analysed by Western Blotting (Figure 4.6a). In the $cdc2^{ts}$ strain, cdc18p was shown to accumulate to a high level, much greater than that observed in the control. This suggests that cdc18p is stabilized in the absence of *cdc2* function. However, it should be noted that in this experiment, *cdc18*

mRNA accumulates to higher levels in the *cdc2* mutant strain than in the wildtype, probably because cells stuck at the *cdc2* arrest point are unable to down-regulate *cdc10* dependent transcription (Figure 2.2b). An additional experiment was carried out to determine whether, in an HU block, the low levels of cdc18p and the slight alteration in its gel mobility (Figure 4.2c) are dependent on cdc2p activity. *cdc2-33* and wildtype cells were arrested at the boundary of S-phase in HU, and then shifted to the restrictive temperature for 30 minutes, to inactivate cdc2p, in the continued presence of HU (Figure 4.6b). In the *cdc2-33* strain following the HU arrest, inactivating *cdc2*, caused an accumulation of the more rapidly migrating form of cdc18p. Whereas, in the wildtype control, the lower cdc18p band disappeared after the temperature shift, in spite of the persistance of the upper band. This suggests that the faster migrating form is de-phosphorylated cdc18p. In the future this will be tested directly, by phosphatase treating S-phase extracts.

Together these results imply that *cdc2* phosphorylates cdc18p (causing a gel shift), to target it for proteolysis. This could limit its action to G1 of the cell cycle, preventing re-replication. This cell cycle control is reinforced by the periodic transcription of *cdc18* driven by *cdc10* function, which drives the expression of *cdc18* from mitosis through until the subsequent S-phase. Since mutation of the CDK phosphorylation sites in *cdc18* does not block its ability to induce over-replication when highly expressed (in preparation H. Nishitani), their sole purpose may be to mediate CDK phosphorylation-dependent proteolysis of cdc18p.

Finally, the experiment shown in Figure 4.6b was repeated, and *cdc2-33* cells were shifted to the restrictive temperature while arrested in HU (Figure 4.6c). As in the previous experiment, the shift to the restrictive temperature caused a slight increase in the level of cdc18p in the *cdc2-33* strain, but not in the wildtype (data not shown). In this case, I also probed the Western for cig2p. Cig2p is present at low levels in exponentially growing cells but accumulates to very high levels in HU. However unlike cdc18p, cig2p is unaffected by the temperature shift in the $cdc2^{ts}$ mutant. This indicates that cig2p proteolysis is controlled very differently from that of cdc18p.

Cdc18p is able to function in early G1.

It appears that cdc18p is produced rapidly as cells leave mitosis. To determine whether the timing of this sudden appearance of cdc18p is of functional significance, I tested whether, in early G1 cells, *de novo* synthesis of *cdc18* is required for the subsequent S-phase. This was done using a *cdc18* switch-off strain expressing *cdc18* from the regulatable weak *nmt* promoter (Figure 4.7a). Exponentially growing *cdc18Δ nmt::cdc18* cells die by cutting from G1 when thiamine is added to the medium (Kelly *et al.*, 1993). Small G1 cells were obtained by nitrogen starvation, and thiamine added to half of the culture. 3 hours later, cells were re-fed with nitrogen to enable the resumption of growth. In the presence or absence of thiamine, cells entered S-phase, with identical kinetics. This shows that nitrogen starved cells have accumulated sufficient cdc18p prior to their arrest, (possibly during the exit from mitosis before the arrest in G1), to enable them to enter S-phase even after several hours in stationary phase. In light of this result, a control experiment was carried out to confirm the published observation that *cdc10* is required for entry into S-phase after nitrogen starvation (Fisher and Nurse, 1996)(Figure 4.7b). *cdc10-129* cells were arrested in G1 at 25°C and then released at 29 or 36°C by the re-addition of a nitrogen source. Cells entered S-phase at 29°C, a permissive temperature, but not at 36°C despite the accelerated growth at this temperature (Figure 4.7b, top panel). Therefore, although expression of *cdc18*, from *nmt-cdc18*, prior to the G1 arrest, probably as cells left mitosis, was sufficient for entry into the subsequent S-phase, *cdc10* function is not complete in nitrogen starved G1 cells. This observation suggests that, although *cdc18* may rescue *cdc10* in cycling cells, other targets of *cdc10* may be critical for S-phase in cells recovering from nitrogen starvation.

De novo synthesis of cdc18p is required after cells leave metaphase to bring about S-phase.

In an additional experiment, I tested whether expression of *cdc18* in mitosis is sufficient for it to function in the following S-phase. Work presented earlier in this Chapter suggests that cdc18p accumulates as cells leave metaphase. Therefore, if *cdc18* is expressed in mitosis, but transcription is inactivated before cells are able to enter G1, cdc18p will be unable to function, and cells will arrest in G1 without cdc18p, and then cut (enter the next mitosis directly from G1). Two identical strains were independently

constructed by crossing the *cdc18* switch off with an *nda3* mutant. The double mutants were subjected to an *nda3* block and release experiment (Figure 4.8). Cells were arrested in mitosis at 18°C and after 4 hours at the restrictive temperature, thiamine was added to half the culture, to prevent further expression of *cdc18*. Cells were then released from the block, after an additional 2 hours at 18°C. Cells expressing *cdc18* were able to pass through S-phase without a visible G1 period, but the presence of thiamine (which prevents transcription of *cdc18*) caused a large proportion of cells to arrest in G1. Many cells eventually cut (as observed by cells with low level propidium iodide staining in the FACS analysis). The cells giving rise to the residual G2 peak are probably, in the main, still in G2 or mitosis of the previous cycle. This experiment confirms, that for *cdc18* to function, it must be synthesized *de novo*, after metaphase, during the next cycle. By showing that the *cdc18* switch off strain works as expected, this experiment also provides an important control for the experiment shown in Figure 4.7.

Discussion

In this Chapter, I have explored the function of the mitotic expression of *cdc18*, the key target of *cdc10*. This reveals a potential role for the mitotic activation of *cdc10* dependent transcription in the control of the cell cycle. I found that, although *cdc10* is active in mitosis, it cannot perform its S-phase function in a metaphase block. In addition, I showed that *cdc18* must be synthesized *de novo* after mitosis, for S-phase to occur normally. These results can be explained in part by the observation that cdc18p is unstable in the presence of high kinase, in a metaphase arrest, and cannot accumulate. Upon release into anaphase, cdc18p rapidly accumulates. This occurs independently from transcriptional, and also, probably, translational controls over *cdc18*, and is therefore likely to be the result of regulated proteolysis. Interestingly, this burst of cdc18p synthesis at the boundary of mitosis and G1 may be sufficient for the following S-phase, because cells released from a nitrogen starvation arrest in G1, do not require further *cdc18* expression to complete a round of DNA replication.

I next looked at the mechanism controlling the mitotic instability of cdc18p. This is conferred on cdc18p by its N-terminus, which contains multiple S/T, P, X, R/K consensus sites for *cdc2*. This portion of cdc18p binds cdc2p ((Jallepalli and Kelly, 1996; Hideo Nishitani unpublished data). Truncation of cdc18p, removing these cdc2p consensus sites, generated a stable protein which persists at constitutive levels through the cell cycle. I also demonstrated a direct correlation between CDK activity and cdc18p stability by showing that cdc18p is stabilised by artificial reductions in cdc2p protein kinase activity, in both $cdc2^{ts}$ strains and in a cyclin deletion mutant (Chapter 2). In cells arrested in HU, inactivation of the ts-*cdc2* function enables the accumulation of a more rapidly migrating form of cdc18p, which may be the dephosphorylated form of the protein. In addition, a version of cdc18p mutated at all the *cdc2* consensus sites appears to be stabilised (Hideo Nishitani, unpublished observation). Together these results suggest that cdc2p phosphorylates cdc18p, at these consensus sites, to target it for proteolysis. These data are consistent with the observation that over-expression of the CDK inhibitor *rum1* also leads to the accumulation of cdc18p (Jallepalli and Kelly, 1996). The S/T, P, X, R/K consensus cdc2p sites in the N-terminus of cdc18p may therefore constitute a functional link

between *cdc2* and *cdc18* gene functions. When combined with the observation that *cdc18* mRNA is expressed from metaphase until S-phase, we can see how, the collapse in mitotic cdc2p kinase levels at the exit from mitosis, directly relieves cdc18p degradation, causing the dramatic accumulation of cdc18p as cells enter G1.

These observations strengthen the analogy between *cdc18* function in *S. pombe* and *CDC6*, its budding yeast homologue. *CDC6*, like *cdc18*, is transcribed in mitosis and the endogenous protein product accumulates in late mitosis or early G1 (Piatti, *et al.*, 1995; Detweiler and Li, 1997). Cdc6p is also subject to proteolysis via ubiquitin ligation catalysed by the *CDC4* complex (Piatti, *et al.*, 1996) which is homologous in sequence and function to *pop1* (Kominami and Toda, 1997; Schwob *et al.*,, 1994; Piatti, *et al.*, 1996). Importantly, Cdc6p has been shown to act at the G1/M boundary, at which time it is required to set up the "pre-replicative complex"(Piatti, *et al.*, 1995; Cocker, *et al.*, 1996). If the synthesis of *CDC6* is delayed, cells eventually enter mitosis from G1 (Piatti, *et al.*, 1996). Therefore, the expression of *CDC6* at the end of mitosis is essential for its function. The data presented here suggests that cdc18p acts in a similar fashion.

By analogy with Cdc6p, the presence of cdc18p in early G1 cells may mark the DNA for subsequent entry into S-phase. Therefore, it is possible that the CDK control over cdc18p instability constitutes a direct link between the end of mitosis and DNA replication "licensing" (Blow and Laskey, 1988). Later in the cycle, cdc18p is degraded, as renewed cdc2p protein kinase activity drives the initiation of DNA replication. This destroys the cell's capacity to re-replicate its DNA. Therefore, periodic transcription and CDK regulated proteolysis probably act, together, to confine cdc18p to the period from the end of mitosis until late G1. This control therefore plays an important role in ordering the successive rounds of S-phase and mitosis.

This chapter also touches on the general mechanisms used to bring about the regulated proteolysis of proteins that function in G1 of the cycle. It is interesting to note that, while rum1p and cdc18p are both targetted for proteolysis by a *pop1* mediated pathway, the profile of their accumulation through the cell cycle differs. cdc18p accumulates from late mitosis to late G1 and rum1p accumulates from metaphase to late G1. Since cdc18p, rum1p and Sic1p also share a short motif TPSTP in addition to N-terminal

cdc2/CDC28 target motifs, I also tested whether this sequence affects cdc18p destruction. This sequence did not appear to contribute to the oscillation in the level of cdc18p through the cycle. My work has also highlighted differences in the control of cig2p and cdc18p degradation. Cig2p accumulates with a superficially similar cell cycle profile to cdc18p in cycling cells, peaking in late G1 or S-phase. However, unlike cdc18p and rum1p, cig2p is present at low levels soon after mitosis (data not shown); in part because *cig2* mRNA may not accumulate until sometime in G1. Also cig2p accumulates to very high levels in HU (see Figure 4.7b). The levels of cdc18p, rum1p and cig2p are also controlled differently in cells responding to pheromone. In pheromone, rum1p is essential for the arrest and accumulates, cig2p is degraded (Stern and Nurse, 1997) and cdc18p levels remain relatively low despite the increase in *cdc18* mRNA (Stern and Nurse, 1997). It is possible that although rum1p and cdc18p are both targeted for proteolysis by *pop1*, that the signal for their recognition by the *pop1* complex may be a prior modification, e.g. phosphorylation. Therefore, differences in the timing of cdc18p and rum1p destruction may be regulated by the use of multiple kinases, which modify specific substrates at particular times during the cell cycle, targetting them for *pop1* mediated ubiquitination and proteolysis. These observations point to an unexpected degree of complexity in the control of proteolysis of "G1" proteins.

Rum1p is thought to serve a G1 function, inhibiting the activity of cdc2p/cdc13p and enhancing the turnover of cdc13p in G1 of the cycle. Therefore the fact that rum1p is present in metaphase arrested cells suggests that G1 events other than *cdc10* dependent transcription may also be initiated during mitosis. Sic1p, the functional homologue of rum1p in *S. cerevisiae*, is thought to play a minor role in in the exit from mitosis (Toyn *et al.*, 1997), so it is possible that rum1p has a similar function in late mitosis, (although this has never been observed). Interestingly, in *S. cerevisiae*, pheromone dependent transcription, (which inhibits START and controls conjugation from G1), is also initiated in cells arrested in metaphase (Oehlen and Cross, 1994). Perhaps metaphase cells are already committed to enter into G1, (as indicated by the experiment in Figure 4.1) in which case, it is logical that they initiate events which will come into play after the completion of mitosis.

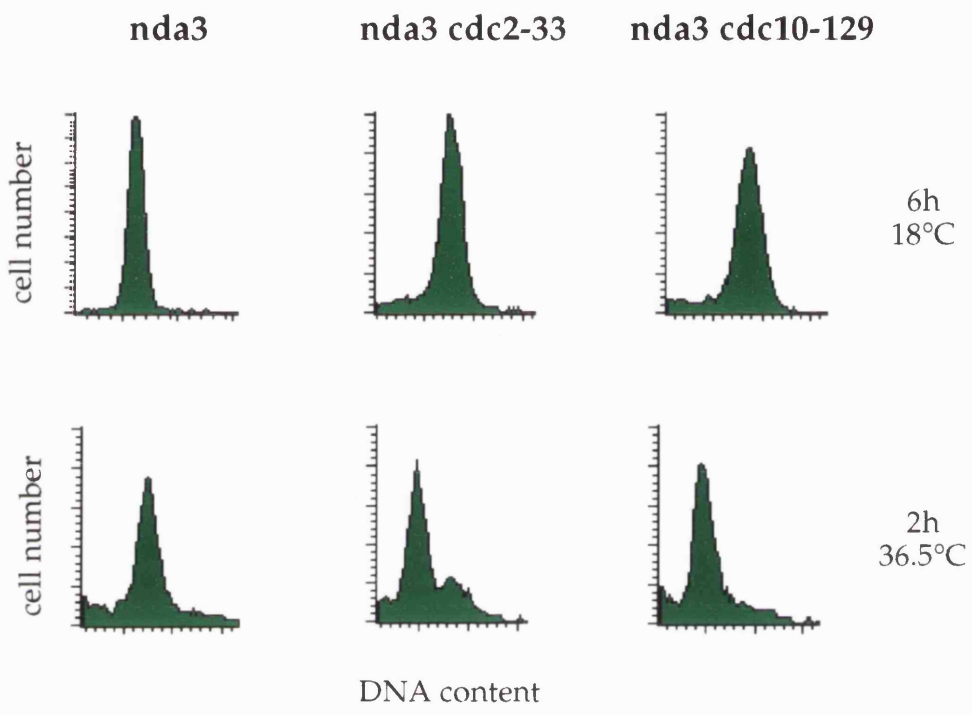


Figure 4.1

Although *cdc10* is active in mitosis, the S-phase function of *cdc10* has not been completed in metaphase.

An *nda3-km311* block and release experiment was carried out to assess whether the *cdc10* or *cdc2* are able to complete their S-phase functions in metaphase. *nda3-km311 cdc10-129, nda3-km311 cdc2-33* and *nda3-km311 cdc10+* strains were arrested in mitosis for 6hrs at 18°C and then released at 36.5°C. FACS samples were taken in mitosis and 120 minutes after release to determine the proportion of cells entering S-phase in the first cycle.

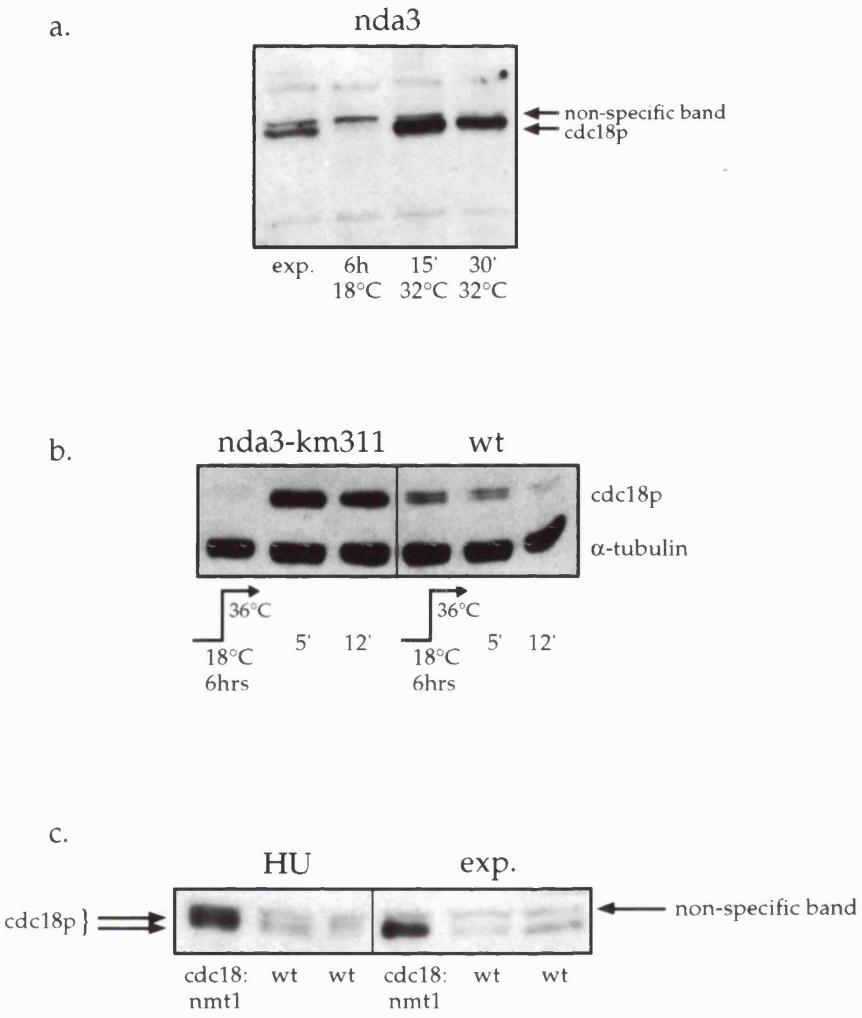


Figure 4.2

Although *cdc18* is expressed in mitosis and in S-phase, cdc18p cannot accumulate. A burst of cdc18p is observed after release from a metaphase arrest. **2a.** Exponentially growing *nda3-km311* cells were arrested in mitosis for 6 hours at 18°C and then released at 32°C, into anaphase and the next cycle. cdc18p levels were analysed by Western blotting. **2b.** This experiment was repeated, using both an *nda3* and wildtype strain, but with the modification that cells were released by shifting the cultures to 36°C. Samples were taken for Western analysis 5 and 12 minutes after the addition of pre-warmed media. **2c.** Wildtype cells and cells carrying *cdc18* expressed from the medium strength *nmt1* promoter, were arrested in HU for 4 hours. A Western analysis was used to compare cdc18p levels in exponentially growing cells and in those in the HU arrest. In each case, cdc18p was present at an intermediate level in the HU block and the protein was smeared towards higher molecular weights.

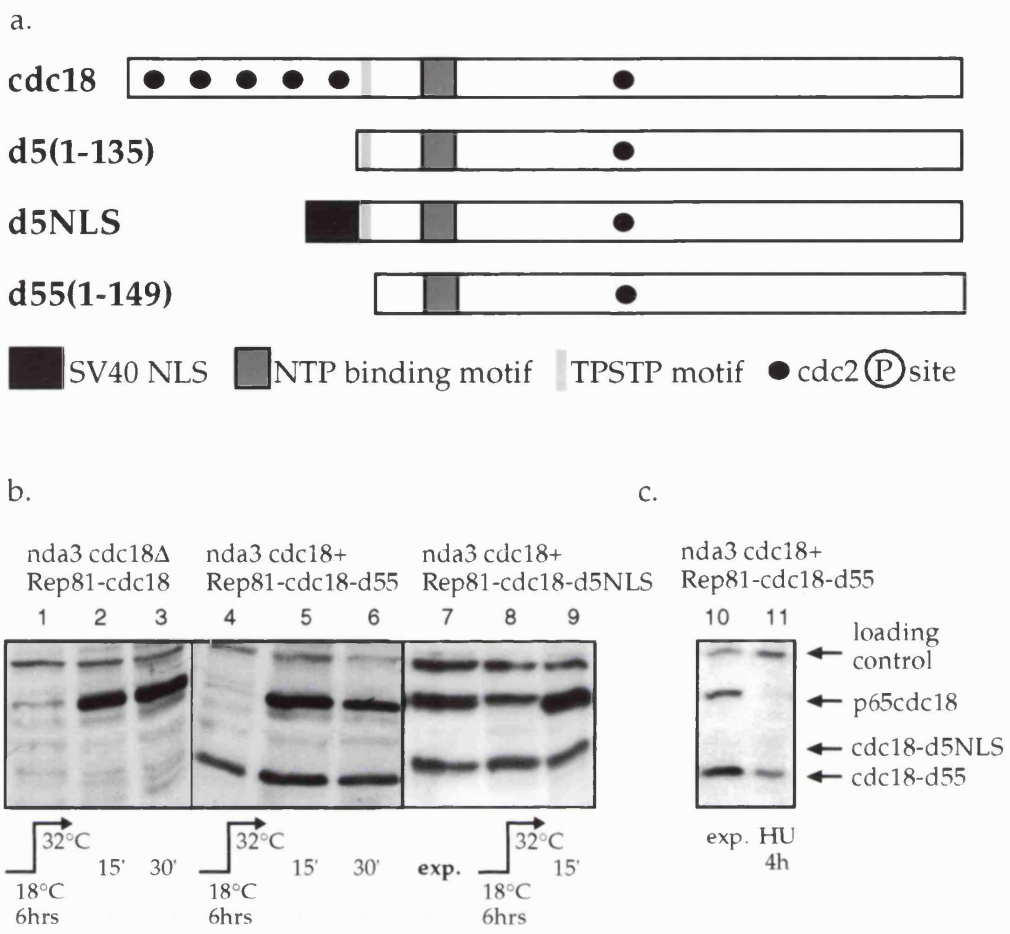
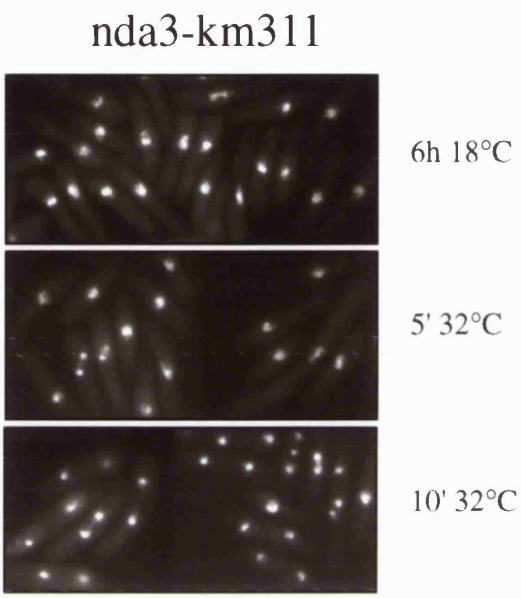


Figure 4.3

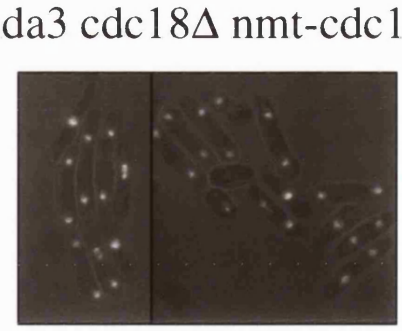
The low level of cdc18p in cells arrested in mitosis is the result of changes in protein stability mediated by the N-terminus of the protein.

The *nda3* block and release protocol used in the previous figure (4.2) was repeated to determine whether removal of the N-terminal portion of cdc18p, containing 5 consensus cdc2p sites, stabilised the protein in a mitotic and S-phase blocks. **3a.** A schematic is shown of the wildtype cdc18p, and the three truncated variants, cdc18p-d55 and cdc18p-d5, lacking the cdc2p consensus sites. Cdc18p-d55 also lacks the potential phosphorylation site, TPSTP. The SV40 nuclear localisation signal was also added to cdc18-d5NLS. **3b.** *nda3* cells deleted for the endogenous *cdc18*, carrying a plasmid expressing *cdc18* from behind the constitutive weak *nmt* promoter, were subjected to a mitotic block and release protocol (lanes 1-3), as were *nda3* cells expressing N-terminally truncated cdc18p, cdc18p-d55 (lanes 4-6) or cdc18p-d5 to which a nuclear localisation sequence was added (cdc18p-d5NLS) (lanes 7-9). Exponentially growing cells were arrested in mitosis for 6 hours at 18°C and then released, at 32°C, into the next cycle. In **3c**, *nda3* cells expressing cdc18-d55 were arrested in HU for 4 hours. Levels of cdc18p were determined by Western blotting.

a.



b.



10 minutes after release from a 6h *nda3* arrest

Figure 4.4

Synchronous anaphase in *nda3* block and release experiments.

nda3 cells and *nda3* cells carrying a deletion in the endogenous *cdc18* and an episomal copy of *cdc18* expressed from the *nmt* promoter, were subjected to an *nda3* block and release protocol. Cells were stained with DAPI to visualize nuclei. **4.4a.** A picture is shown of *nda3* cells in the arrest and 5' and 10' after the release at 32°C. **4.4b.** *nda3 cdc18Δ nmt-cdc18* cells are shown 10' after release from a similar mitotic block.

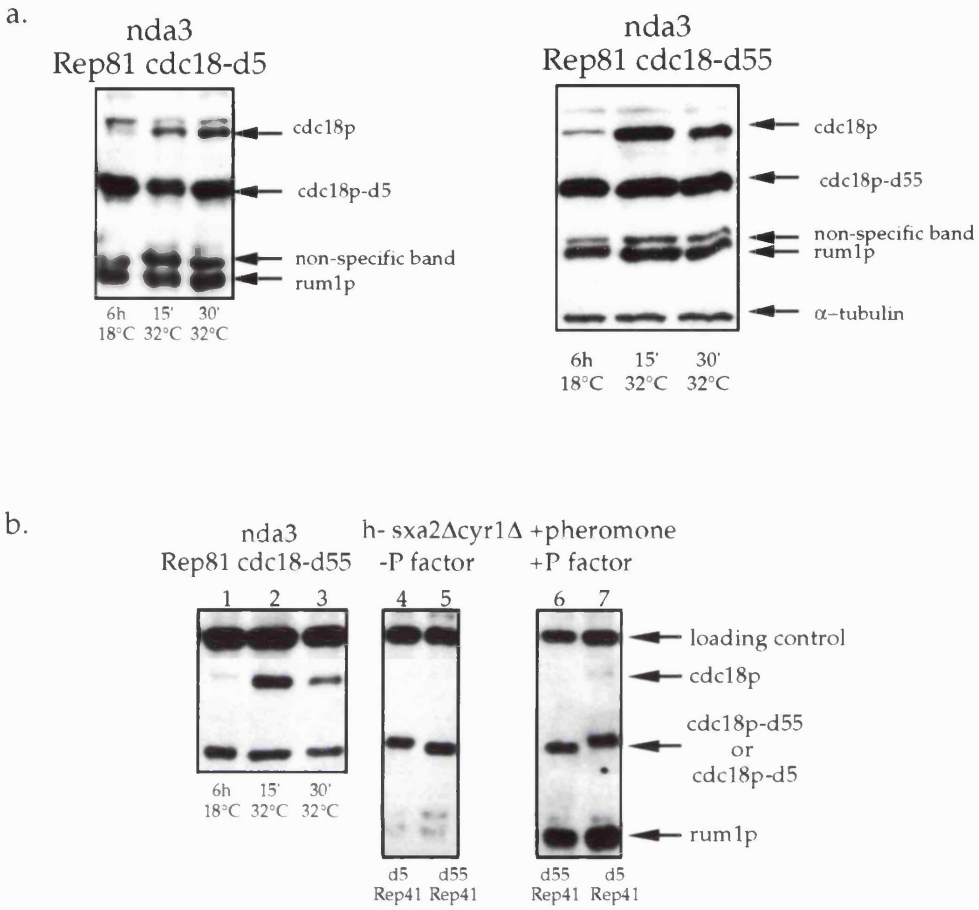


Figure 4.5

Rum1p and cdc18p have different profiles of expression during the cell cycle. Also rum1p is stable in pheromone, while cdc18p is not. The N-terminal portion of cdc18p mediates its instability in a pheromone arrest.

The N-terminal deletion cdc18-d55, lacks the 5 consensus *cdc2* sites and a potential phosphorylation motif, TPSTP, found in another G1 protein, rum1p. A smaller truncation, d5, leaves this is intact. Plasmids expressing either cdc18-d55 or cdc18-d5 were transformed into an *nda3* strain. Cells were first arrested at the metaphase block and then released into the cell cycle. Levels of wildtype and truncated cdc18p together with rum1p were assessed. α-tubulin was used as a loading control. **4.5a.** Westerns were carried out for both transformants. Cells were sampled in the mitotic block and 15 and 30 minutes after the release at 32°C. In a second experiment, **4.5b**, cdc18-d5 (lanes 4 and 7) and cdc18-d55 (lanes 5 and 6) were introdcued into an *h- sxa2Δ cyr1Δ* strain. Cells were then subjected to a pheromone induced G1 block. Samples from Figure 4.3b were included as a control (lanes 1-3). The Western was again probed for rum1p and cdc18p. (A cross-reacting band is used as a loading control in 4.5b).

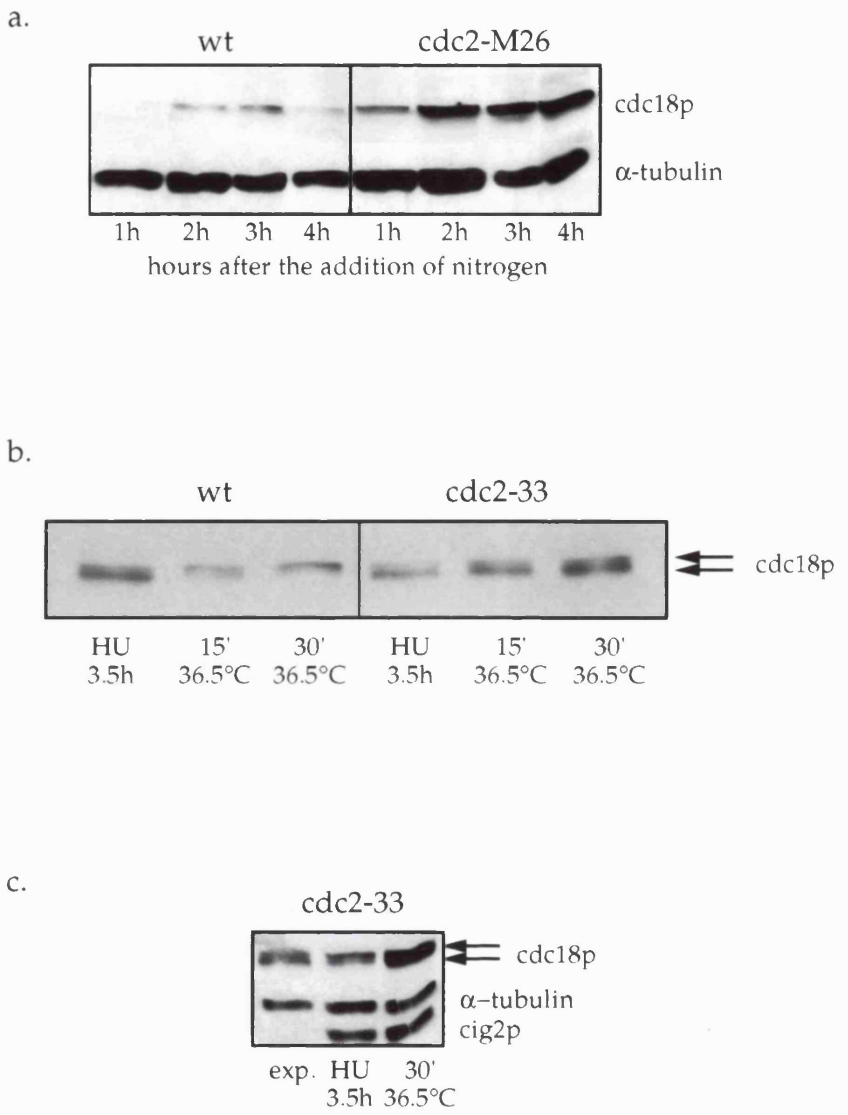


Figure 4.6

Perturbations in *cdc2* function increase the stability of cdc18p.

4.6a. Samples were taken from the experiment shown in Figure 2.2, in which wildtype and *cdc2-M26* cells were arrested in G1 by nitrogen starvation and re-fed at 36.5°C. cdc18p and α-tubulin levels were assessed by Western analysis in samples taken after the re-addition of nitrogen. **4.6b.** *cdc2-33* and wildtype cells were arrested in HU for 3.5 hours and then shifted to the restrictive temperature for 30 minutes. A Western was probed for cdc18p. **4.6c.** The experiment in 4.6b was repeated, and a Western probed for cdc18p, cig2p and α-tubulin.

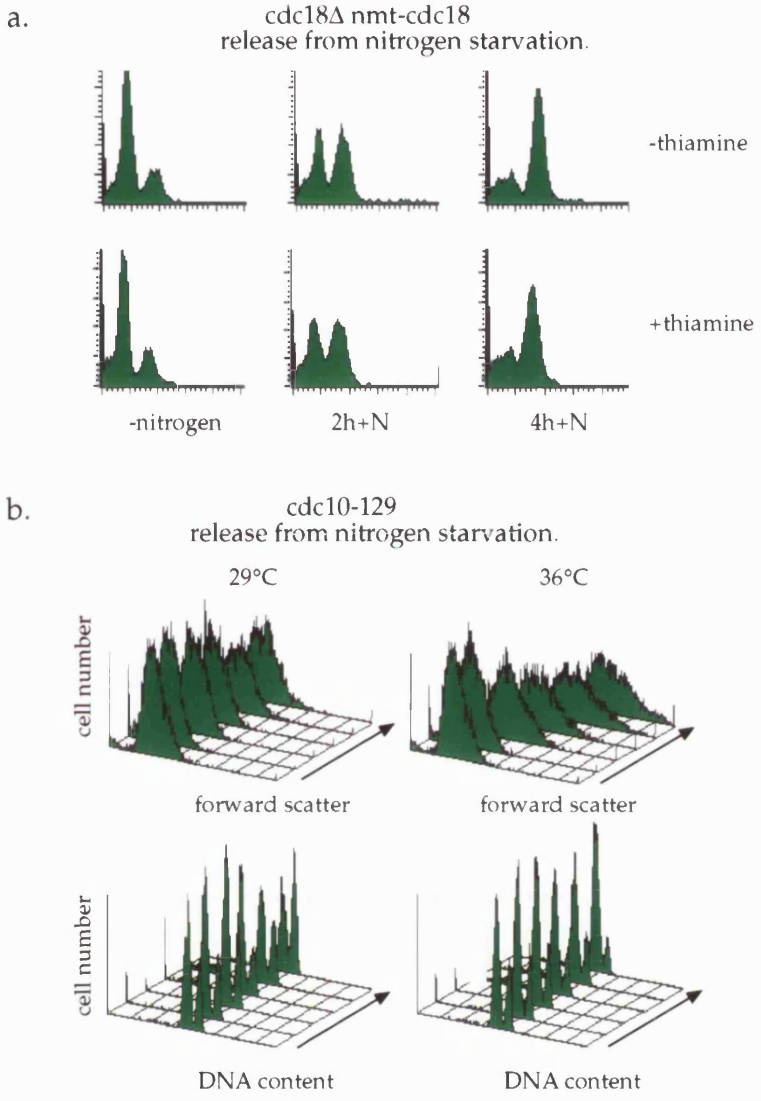


Figure 4.7

In cells released from a nitrogen starvation G1 arrest, *de novo* synthesis of cdc18p is not required for S-phase, although *cdc10* function is.

7a. Cells deleted for the endogenous copy of *cdc18*, but carrying *nmt-cdc18* on a plasmid, were arrested in G1 by nitrogen starvation. Thiamine was then added to half the culture, and after a further 3 hours, nitrogen was re-added enabling cells to proceed towards S-phase, with or without *cdc18* expression. Samples were taken for FACS analysis at various time-points thereafter.

7b. *cdc10-129* cells were arrested in G1 at 25°C by the removal of nitrogen from the media and re-fed at a permissive or restrictive temperature, 29 or 36°C. Samples were taken for FACS analysis every 30 minutes to determine whether cells entered S-phase.

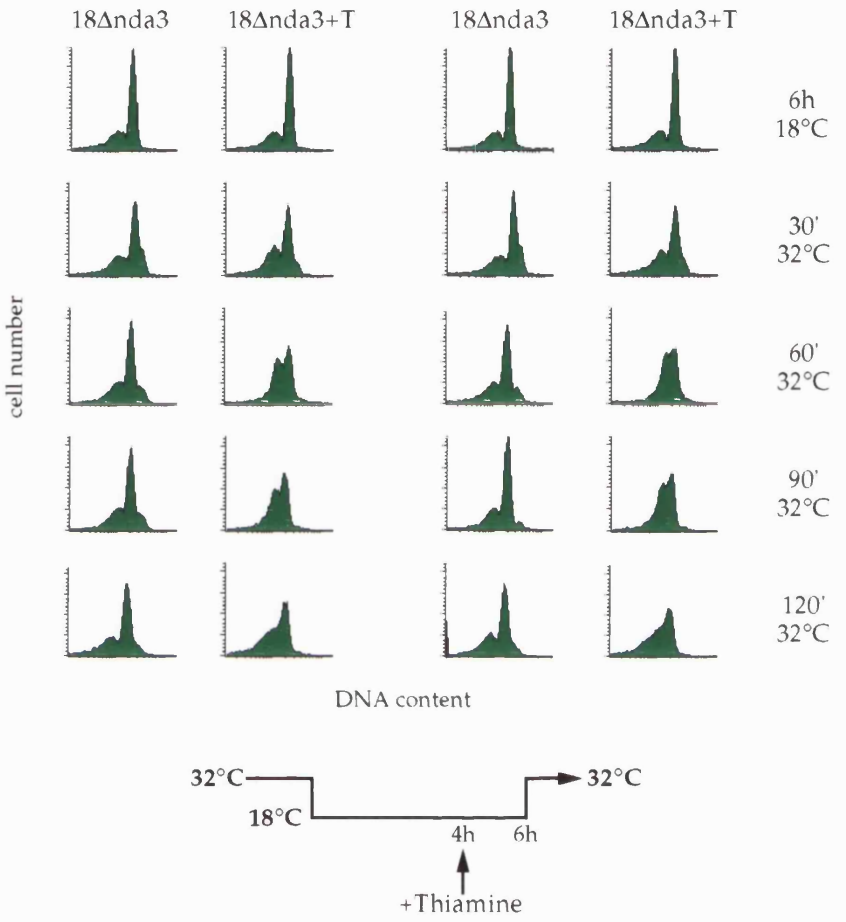


Figure 4.8

After mitosis, cells require *de novo* synthesis of *cdc18* for the subsequent S-phase.

nda3-km311 cdc18Δ cells kept alive by expression of *cdc18* from the *nmt* promoter were shifted to 18°C. After 4 hours at this temperature thiamine was added to prevent further transcription of *cdc18*. After an additional two hours at 18°C, cells were shifted to 32°C to release them into the subsequent cycle. DNA content was analysed by FACS analysis to observe cells arresting in G1. At later time-points cut cells appear.

CHAPTER 5

Components of the cdc10p transcriptional complex and periodic transcription.

Introduction

Having established a general picture for the timing and control of *cdc10* dependent transcription, I next assessed the role of the components of the transcriptional complex in setting up periodic S-phase transcription. This analysis may shed light on which proteins within the complex read the cell cycle time. It also provides an alternative approach with which to identify the signal that communicates passage through the cell cycle to the transcriptional machinery, generating periodic transcription. In this chapter, I first describe the effects of mutation and over-expression of components of the complex on periodic S-phase transcription. I also present a limited biochemical analysis of the bandshift complex, DSC1, which contains cdc10p and which binds to the *cdc18* promoter in cell extracts. From this data, I will attempt to determine the significance of DSC1 for the *in vivo* function of *cdc10*.

An analysis of the periodicity of *cdc10* dependent transcription in the absence of components of the complex.

I began by testing the effect of deleting genes implicated in the control of periodic *cdc10* dependent transcription (*cdc10*, *res1*, *res2*, *rep1* or *rep2*), each in turn. All these genes may, under certain circumstances, contribute to the function of the transcriptional complex (Aves, *et al.*, 1985; Caligiuri and Beach, 1993; Tanaka, *et al.*, 1992; Marks *et al.*, 1992; Miyamoto, *et al.*, 1994; Zhu, *et al.*, 1994; Nakashima, *et al.*, 1995; Sugiyama, *et al.*, 1994). The *cdc10* deletion is inviable, so the double mutant *cdc10Δ sct1-1* was used, in which a point mutation in *res1* (*sct1-1*) suppresses the lethality of the *cdc10Δ* (Marks, *et al.*, 1992; Caligiuri and Beach, 1993). *sct1-1 cdc10*$^{+}$, *res2Δ rep2Δ* and *cdc10-C4* strains were also included in the study (Figure 5.2a and 5.2b). The deletion of *res1*, *cdc10* and *rep2* has a strong affect in mitotically growing cells, whereas *res2* and *rep1* deletion cells behave relatively normally during the mitotic cycle, although the *res2* and *rep1* mutations have a strong meiotic phenotype. For this reason, cdc10p/res1p is thought to mediate the expression of S-phase genes in the mitotic cycle and res2p/cdc10p primarily in the meiotic cycle (Miyamoto, *et al.*, 1994). However, the physical and genetic interaction between res2p and rep2p suggest a possible mitotic role for *res2* (Miyamoto, *et al.*, 1994).

Cells harbouring deletions in *cdc10*, *res1*, *res2* or *rep2* were grown at 30°C, where they are viable, and subjected to a hydroxyurea induced arrest followed by release. RNA was then analysed by Northern Blotting to assess *cdc18* message levels (Figure 5.1a). FACS analysis (data not shown) confirmed that in all cases, at least 90% of cells were arrested with a G1 DNA content, 4 hours after the addition of HU to cultures, and that greater than 95% of cells were in G2 an hour after the removal of HU. The Northern was quantified by phosphor-imager analysis (Figure 5.1b), and the bar chart includes data from wildtype cells subjected to the same protocol (samples were analysed on the same Northern blot).

During log-phase growth, the levels of *cdc18* transcript in the *res1Δ* were similar to the low levels seen in wildtype cells. Exponentially growing *rep2Δ* and *cdc10Δ sct1-1* cells exhibited even lower *cdc18* transcript levels (Figure 5.1b columns 1, 4, 10 and 13), whereas in the *res2Δ* strain, *cdc18* transcript levels were elevated (Figure 5.1b column 7). After treatment with

HU for 4 hours, cells arrest in early S-phase. In the wildtype, *cdc18* transcript levels were elevated during S-phase. However, no significant elevation in the level of *cdc18* mRNA was seen in *res1Δ* or *cdc10Δ sct 1-1* cells and only a small elevation was observed in *rep2Δ* cells. These data suggest that *res1, cdc10* and *rep2* are required to achieve wildtype levels of *cdc18* expression (Figure 5.1b columns 2, 5, 11 and 14). In the *res2Δ* strain, *cdc18* mRNA levels were close to the peak level seen in S-phase wildtype cells, both during and after release from the HU block (Figure 5.1b columns 8 and 9). In wildtype cells, within lh of removing HU as S-phase was completed, *cdc18* transcript levels fell dramatically (Figure 5.1b, column 3). In *res1Δ* and *cdc10Δ sct 1-1* cells, *cdc18* expression was unchanged after HU was removed, while in the *rep2Δ, cdc18* mRNA returned to its initial basal level within an hour of the release (Figure 5.1b columns 6, 12 and 15). As a control for *cdc10Δ sct1-1,* the single mutant *sct1-1* was subjected to the same procedure. The restoration of *cdc10* function reinstated periodic S-phase transcription in this strain (Figure 5.2a). However, there was some delay in the down-regulation of *cdc18* message as cells left the HU block, suggesting that the *sct1-1* mutation in the *res1* gene may cause a partial deregulation of *res1* function (see Figure 5.5). This experiment confirms that the *sct1-1* mutation is not responsible for the aperiodic behaviour of *cdc18* transcription in the *cdc10Δ sct1-1* strain. I also subjected a culture of *cdc10-C4* cells grown at 25°C to the HU block and release protocol (Figure 5.2b). *cdc18* mRNA levels were elevated and constitutive through the experiment. This confirms the published observation that, at low temperatures, the *cdc10-C4* mutation deregulates the periodic expression of target genes (McInerny, *et al.*, 1995).

These results show that *cdc18* transcript levels are reduced and constant during the HU block and release in *res1Δ* and *cdc10Δ sct1-1* strains. *cdc18* transcription is elevated and constant in *res2Δ* and *cdc10-C4* cells, and is reduced but periodic in *rep2Δ* cells. In the double mutant, *rep2Δ res2Δ, cdc18* transcript levels were high and constant (Figure 5.2a). This bolsters genetic data which suggested that rep2p has no role in the absence of res2p (Nakashima, *et al.*, 1995). I also confirmed previous work suggesting that *rep1,* a *rep2* homologue, has no role in S-phase transcription during the mitotic cell cycle (Sugiyama, *et al.*, 1994) (Figure 5.2c). To do this, the *rep1* deletion was introduced into a *cdc25-22* strain. I then subjected these cells to a *cdc25* block and release protocol to assess the periodicity of *cdc10* target

message levels through a synchronous cycle. A portion of the culture was also arrested in hydroxyurea for 4 hours at 25°C (Figure 5.2c, lane 1). Periodic transcription of *cdc18* was not perturbed in a *cdc25* block and release*cdc25* by the deletion of *rep1* (compare with Figures 3.1 and 3.2). This suggests that rep1p has no role in the mitotic cycle. (This protocol was not used to assess periodicity of transcription in *res1Δ* or *cdc10Δ* mutants, because deleting these genes in a *cdc25-22* background caused cells to become sick, probably as a result of combining cs and ts mutations).

Periodic S-phase transcription through a synchronous cell cycle, in cells deleted for putative components of the *cdc10* complex.

The role of the various components of the transcriptional complex in controlling the periodicity of S-phase transcription was further investigated using synchronous cultures of wildtype fission yeast and strains deleted for *cdc10, res1, res2* (Figure 5.3a-d) and *rep2* (Figure 5.3g). The level of cell synchrony in each culture is indicated by the septation index (shown in Figure 5.3e, and 5.3h for the *rep2Δ*). Similar results were obtained using *cdc18, cdc22, cdt1* and *cig2* mRNA levels as a measure of *cdc10* dependent transcription (data not shown). In wildtype cells synchronised by elutriation, the levels of *cdc18* transcript are periodic during the cell cycle, being maximal around the peak in septation (Kelly, *et al.*, 1993) (Figure 2a). In the *res1Δ* strain (Figure 2b), *cdc18* transcripts were present at low levels throughout the cell cycle. Therefore, res1p is required to activate *cdc10* dependent transcription during the cell cycle and, in its absence, periodicity is abolished, and the absolute level of transcription is reduced. Deleting *res2*, also abrogates the *cdc18* mRNA oscillation through a synchronous cell cycle (Figure 5.3c). So, res2p may be required to periodically repress transcription during the cell cycle. In the *cdc10Δ sct1-1* strain (Figure 5.3d), *cdc18* transcript levels were constant but very low throughout the cell cycle. Therefore, although in the *cdc10Δ sct1-1* strain, *cdc10* targets are expressed at low levels, cdc10p is absolutely required for the periodicity of this transcription. Finally, I followed the expression of *cdc18* in *rep2Δ* cells through a synchronous cell cycle. RNA isolated from the elutriated *rep2Δ* culture was run independently on 2 gels. The first is shown in the Northern blot in Figure 5.3f and includes samples from the first 20 timepoints. (The second to last lane may be an aberrant point). The second blot, with 24 samples, was quantified and is plotted in Figure 5.3g. The septation index is included as a measure of synchrony in the culture. These

data confirm the previous observation that, in a *rep2Δ* strain, *cdc10* dependent transcription is still periodic during the cell cycle, although transcripts of target genes are present at reduced absolute levels.

Finally, the effect on the mitotic transcription of *cdc18* was assessed in cells deleted for *res1* and *res2,* using ectopic *nmt* driven expression of *cdc13Δ90* (from an integrated plasmid) to arrest cells in mitosis (Figure 5.4). Cells were grown at 30°C in minimal media containing thiamine and then thiamine was washed out. Samples were taken 15 hours after the induction of the *nmt* promoter, as cells begin to arrest in mitosis as a result of the high levels of the mitotic kinase. *cdc18* mRNA levels were analysed by Northern blotting (see Figure 3.6b for comparison). No alterations in *cdc18* mRNA levels were observed as cells entered the telophase arrest. This experiment indicates that mitotic transcription requires *res1,* together with *cdc10,* for its activity and confirms that deletion of *res2* eliminates all periodicity of expression of *cdc10* targets through the cell cycle.

An over-expression analysis of components of the *cdc10* complex.

I next investigated the effect, on periodic S-phase transcription, of the ectopic expression of *res1, res2, cdc10 , rep1* and *rep2* (Figure 5.5). *cdc25-22* strains were transformed with multicopy plasmids containing either *res1, res2, cdc10 , rep1* and *rep2* under control of the thiamine repressible *nmt* promoter (Maundrell, 1993). *res1, res2, rep1* or *rep2* expression was driven by the full strength *nmt* promoter, while the medium strength promoter was used to express *cdc10.* Cultures were split in two, and expression from the *nmt* promoter was induced in half the cells by growth in the absence of thiamine, for 20 hours at 25°C. Both induced and uninduced cultures were then shifted to 36°C, for 4 hours, to inactivate the $cdc25^{ts}$ function. This arrests cells in G2 where *cdc18* transcript levels are normally low. Cells were then released at 25°C into mitosis and a subsequent cell cycle.

In the presence of thiamine, which prevents ectopic expression from the *nmt* promoter, (marked OFF in Figure 5.5), *cdc18* transcript levels were low in G2, increased to peak levels on release into mitosis and decreased after entry into S phase. However, in G2 arrested cells expressing *res1* from the *nmt* promoter (marked ON in Figure 5.5a), *cdc18* transcription was activated to maximal levels. Following release of the *res1* expressing cells into mitosis and the subsequent cell cycle, *cdc18* transcript was maintained at

high levels. This result agrees with published data, showing that over-expression of res1p can drive expression of *cdc10* targets (Ayte, *et al.*, 1995). However, my results differ from those of Ayte *et al.*, in that I observed no significant G1 arrest after release from the *cdc25* block (data not shown). This difference may be explained by the shorter time-course of induction in my experiment. Thus, I was able to separate direct effects on transcription from blocks in cell cycle progression. Other *cdc10* target transcripts, *cdc22*, *cdt1* and *cig2* (data not shown) were similarly affected. This data suggests that *res1* plays an important role in activating periodic transcription during the cell cycle. Ectopic expression of *cdc10* (Figure 5.5a) (Ayte, *et al.*, 1995; McInerny, *et al.*, 1995) *rep1*, *rep2* (Figure 5.5b) or *res2* (Figure 5.5c) had no strong activating or repressing effect on the periodic transcription of *cdc10* targets, (*cig2* and *cdt1* mRNAs are shown in the over-expression of *res2* in Figure 5.5c and *cig2* mRNA levels in the over-expression of *res1*, *res2* and *cdc10* in 5.5d). It is possible however that if expressed at higher levels, *cdc10*, *res2*, *rep1* and *rep2* could perturb S-phase transcription.

I next investigated whether ectopic expression of the C-terminus of *cdc10* could alter the expression of *cdc18* (Figure 5.6b). This was prompted by the observation that deletion of the *cdc10* C-terminus, results in hyperactivation of *cdc10* dependent transcription at low temperatures (McInerny, *et al.*, 1995). For this reason, it was thought possible that expression of the *cdc10* C-terminus alone could titrate out an inhibitor of cdc10p and induce an increase in the levels of *cdc10* target transcripts. The portion of the C-terminus involved could then be used as a biochemical hook to isolate the inhibitor. To assess the the effect *in vivo*, I compared the effect of three constructs (constructed and donated by Reymond and Simanis, 1993) which express portions of the *cdc10* gene to moderate levels. These constructs, (schematically depicted in Figure 5.5e), had little effect on levels of *cdc18* mRNA during exponential growth, or after 4 hours in an HU block. A sample from the same Northern blot, probed for *cdc18*, *cig2* and a control message, is included, showing the effects of ectopic expression of *res1*, *res2* and *cdc10* after 24 hours. It is possible that the C-terminal fragments of *cdc10* must be expressed at higher levels to disrupt S-phase transcription. Using a similar approach, ectopic expression of the C-terminus of *cdc10* has been shown to de-repress *cdc10* dependent transcription in exponential cells (McInerny, *et al.*, 1995).

In a final set of experiments, I used the observation that S-phase transcription is derepressed in G2 by the *res2Δ* mutation, to take another look at relationship between *cdc10* and *cdc2*. In particular, I asked whether *cdc2* activity is required for the expression of S-phase genes in the *res2Δ* background. To do this, I introduced the *res2* into a *cdc2-33* background. In the first experiment (Figure 5.7a), *cdc18* mRNA levels were assessed in *res2Δ cdc2-33* cells at 25°C, where they are viable, and after a shift to 36°c for 2 and 4 hours. A control sample is included in the Northern blot to show the level of *cdc18* message in wildtype cells arrested in HU. In a second experiment, I used a *cdc25 res2Δ* as a control for the effects of cell mass and a G2 arrest on S-phase transcript levels in the *res2Δ*. *res2Δ*, *res2Δ cdc2-33* and *res2Δ cdc25-22* cells were grown at 25°C, half the culture was shifted to 36°C and half to 18°C. *cdc18* transcription was monitored after 2 and 4 hours at 36°C, and after 4 hours at 18°C. It is clear that in *res2Δ* cells, in both *cdc2* and *cdc25* cell cycle blocks, *cdc18* mRNA is actively transcribed. Therefore, this experiment tentatively supports the thesis that *cdc10* does not require *cdc2* activity for active transcription, in this case, even after 4 hours at the restrictive temperature. It is striking, that in both the *cdc25* and *cdc2* double mutants, the expression of *cdc18* is increased. This may be the result of the increased cell size in these strains. Interestingly, this effect is more pronounced with *cdc18* mRNA than with *cdc22* transcripts, which are hardly affected by the treatment (also observed by José Ayte personal communication). Finally, this experiment suggests that the cold sensitivity of the *res2Δ* strain, results from a reduction in S-phase transcription at 18°C.

Analysis of the composition and cell cycle behaviour of DSC1

To provide a biochemical correlate for our analysis of *cdc10* dependent transcription, Jérôme Wuarin and I investigated the behaviour of DSC1. This bandshift activity has been shown to binds to MCB containing promoters, and contains cdc10p and res1p (Lowndes, *et al.*, 1992b; Ayte, *et al.*, 1995). The DSC1 bandshift was obtained by incubating cell extracts with a radio-labelled fragment of the *cdc18* promoter containing the putative palindromic MCB repeats (162 bp, as shown in Figure 5.8c). The bandshift was shown to contain cdc10p (Figure 5.8a) and to be sensitive to cold competitor DNA (data not shown). It is therefore likely to represent the same complex previously identified as DSC1.

To determine which gene functions are required to generate the DSC1 bandshift activity, its presence was monitored in extracts made from wild-type, *res1Δ*, *res2Δ*, *cdc10Δ sct1-1* and *rep2Δ* cells (Figure 5.8b). We found that all four genes were required to generate the DSC1 band shift activity, although a very faint bandshift, of equivalent mobility to DSC1, was seen in *rep2Δ* cells. This implies that, in the absence of *rep2Δ* DSC1 may form, but only inefficiently. It is noteworthy that DSC1 is absent in *res2Δ* cells in which S-phase transcription is high. DSC1 is also absent in the *cdc10-C4* mutant which exhibits high levels of S-phase transcription (Lowndes, *et al.*, 1992b; Reymond and Simanis, 1993; McInerny, *et al.*, 1995). Thus, the presence of DSC1 does not correlate with the activity of *cdc10* dependent transcription.

A preliminary analysis of the behaviour of DSC1 through the mitotic cell cycle, using a *cdc25* block and release protocol, was described in Chapter 3, (Figure 3.3d). To extend this analysis, Jérôme Wuarin and I performed gel-shift experiments with extracts from cells arrested in the mitotic cycle; in G1 where *cdc10* dependent transcription is active, and in G2 where it is inactive. Cell cycle arrests at the G1/S boundary were achieved using either hydroxyurea, a *cdc22-M10* or a *cdc2-M26* mutant strain. In the latter case, *cdc2-M26* cells (from the experiment shown in Figure 2.2) were arrested in G1 by nitrogen starvation and then released at the restrictive temperature and sampled after 3 hours. Arrest at the G2/M boundary was achieved by shifting *cdc25-22* and *cdc2-33* cells to the restrictive temperature for 4 hours, (*cdc2-33* cells were first shifted to 34°C for 2 hours, enabling cells arrested in G1 to leak through into G2, and then to 36.5°C for an additional 2 hours leading to a complete cell cycle arrest in G2).

DSC1 was present in exponentially growing wildtype cells and in cells blocked in G2 in a *cdc25* or *cdc2* arrest. However, DSC1 was consistently undetectable in cells arrested at the G1/S boundary with high levels of *cdc10* dependent transcription (Figure 5.9). This confirms the data shown for the *cdc25* block and release (Figure 3.2b) which suggest that DSC1 appears after the peak of *cdc10* activity, possibly as it becomes inactivated at S-phase. These data are consistent with published work in which the cell cycle timing of the appearance of DSC1 was observed (Reymond *et al.*, 1993), but indicate that, contrary to previous conclusions, the presence of the DSC1 bandshift activity correlates with inactive *cdc10* dependent transcription.

Although the significance of this finding is not clear, it is possible that the *in vitro* activity of DSC1 in G2, represents a form of the complex which represses transcription *in vivo*, in G2 of the cell cycle. This notion is given some credence by the observation that res2p is both necessary for the formation of DSC1, and required for the repression of S-phase transcription in G2 cells.

The MCB element from the *cdc18* promoter can activate the transcription of a reporter gene in an *nda3* block but may not be able to repress transcription in G2.

The observation that *res2* is required for repression of *cdc10* dependent transcription in G2 cells, possibly as a part of the DSC1 complex, prompted me to attempt to identify whether the G2 complex is inactive or whether it can actively repress transcription in the context of an active promoter. I therefore introduced 162 bp from the *cdc18* promoter containing all the putative MCB sites, in its native orientation into the *nmt1* promoter (Figure 5.9a). I placed it between the putative *nmt1* promoter trans-activating elements (Maundrell, 1990; Maundrell, 1993) and the transcription start site. The open reading frame of the mutated *GFP* (the Green Fluorescent Protein, (Prasher, et al., 1992)) was used as a reporter gene (Figure 5.9a). I introduced this as part of a multicopy plasmid into *cdc10-V50* and *nda3-km311* cells (Figure 5.9, b and c). In these constructs, *GFP* was only transcribed in the absence of thiamine. Arresting *cdc10* mutants, containing the reporter plasmid, in HU, increased the level of expression of the reporter 3 fold. I hoped to be able to show that the inactivation of *cdc10* would decrease transcription in G1, and increase the expression of *GFP* in exponential cells (which are mainly in G2) by removal of an inhibitory *cdc10* complex. Shifting *cdc10-V50* cells to the restrictive temperature reduced the level of *GFP* 3 fold in both exponentially growing cells and in cells arrested in HU. This observation may be complicated by the contribution of cells in the exponential population, with active *cdc10*. In Figure 5.9b, I investigated expression of *GFP* in *nda3* mutant cells. I found that arresting cells in either mitosis or in S-phase, in HU, induced the expression of *GFP*. This suggests that this short region of the *cdc18* promoter, which contains the MCB elements, may be sufficient to drive periodic *cdc10* dependent transcription in S-phase and mitosis, outside of its native context. A much more detailed analysis of the *cdc18* promoter is required before any strong conclusions can be made about the region

responsible. In the future these preliminary experiments could be extended to test, definitively, whether *cdc10* can inhibit polII mediated transcription in G2 of the cycle and to define the elements within the *cdc18* promoter which drive periodic expression.

Discussion

The experiments in this chapter have uncovered diverse roles for putative components of the *cdc10* transcriptional complex. In this discussion I will focus on each in turn. Firstly, I showed that, in the absence of res2p, transcription of target genes is high and constant throughout the cell cycle. (Since the *res1* and *res2* deletions are synthetic lethal (Miyamoto, *et al.*, 1994), the constitutive expression in the *res2Δ* probably requires *res1*). This identifies an unexpected role for res2p in the periodic repression of S-phase transcription during the G2 phase of the mitotic cell cycle. In the *res2Δ*, the constitutive expression of S-phase target genes has only a mild effect on cells in the mitotic cell cycle, so it was previously thought that res2p acted primarily during the meiotic cell cycle, where it is essential (Miyamoto, *et al.*, 1994; Zhu, *et al.*, 1994). Since, rep2p is required for active transcription, but only in the presence of res2p, both rep2p and res2p may be part of the active cdc10p complex during the mitotic cell cycle. If res2p were in both the hypothetical inactive and active cdc10 complexes, this might explain why over-expression of *res2* cannot inhibit *cdc10* dependent transcription. Very recently, res2p was also been shown to be present in a bandshift complex together with cdc10p and res1p, throughout the cycle (Zhu, *et al.*, 1997). These data suggest that the presence of res2p in the complex may not define the difference between its active and inactive states. Res2p is also required to form the DSC1 band shift activity, which appears in G2 and correlates with inactive transcription. Thus, it is possible that res2p brings about repression of transcription in part through the action of the DSC1 complex. However, I was unable to determine whether cdc10p can repress the activity of a promoter element adjacent to the MCB in G2 of the cell cycle.

I also found that *cdc2* activity is not required for high levels of transcription in the *res2Δ*. In fact, introducing *cdc2-33* mutations into the *res2Δ* background increase the expression of targets of the S-phase transcriptional machinery throughout the cycle. (This may be primarily the result of the alteration in cell size in this strain, as a similar effect is also observed in *cdc25-22* cells). The continued expression of S-phase genes in the *res2Δ cdc2-33* strain at 36.5°C, reinforces the idea (from Chapter 2) that *cdc10* dependent transcription does not require activation by CDKs.

cdc10 is essential for entry into S-phase because it is a critical component of the active S-phase transcriptional machinery (Nurse and Bissett, 1981; Kelly, *et al.*, 1993). However, a mutation in the *res1* gene, known as *sct1-1* (Marks, *et al.*, 1992; Caligiuri and Beach, 1993), rescues the lethality of the *cdc10Δ* mutation, (although cells remain severely compromised for G1/S progression). In the *sct1-1 cdc10Δ* strain, "S-phase" transcripts are expressed throughout the cycle, at low levels, whereas S-phase transcription is more or less wildtype in the *sct1-1 cdc10*$^{+}$ strain. This demonstrates that cdc10p is essential for the periodicity of S-phase transcription. Interestingly, a C-terminal truncation of *cdc10,* in the *cdc10-C4* mutant, results in constitutive high level transcription of targets, as in the *res2Δ* strain (McInerny, *et al.*, 1995). This C-terminal portion of *cdc10* has been shown to mediate its interaction with *res2* (Sturm and Okayama, 1996: Zhu, *et al.*, 1997). Although the *C4* mutation is recessive, it is difficult to tell whether it reveals a latent activity present in *cdc10,* or results in a spurious, gain of function. However, because the *res2Δ* results in a similar phenotype, it seems likely that these perturbations de-repress inactive *cdc10* in G2 cells. One possibility is that wildtype cdc10p is able to bind DNA in the G2 portion of the cycle together with res2p which masks its capacity to activate transcription. Ectopic over-expression of cdc10p does not affect the periodicity or level of transcription, an observation supported by work from other groups (Ayte, *et al.*, 1995). This suggests that cdc10p may act as a scaffold to support both the active and inactive complexes.

Res1p, in contrast to res2p, may activate S-phase transcription. Res1p is required to for high level expression of all *cdc10* target genes; in its absence S-phase transcription is relatively inactive and constant throughout the cell cycle. Also, importantly, ectopic expression of res1p during G2 is sufficient to activate transcription to a high level (also see Ayte, *et al.*, 1995). This suggests that res1p may control the periodic activation of S-phase transcription during the cell cycle. Thus, it seems likely that res1p and cdc10p act together to promote activation of periodic S-phase transcription, and that in an altered G2 complex, res2p represses this transcription.

In the absence of rep2p, transcription is still periodic, but is much reduced in magnitude; even the low, G2 level of gene expression may be reduced. Therefore, although rep2p is required to elevate the absolute level of activity, it may not to contribute to the periodicity of S-phase transcription.

Such a general activating role is consistent with its having a strong transcriptional activation domain (Nakashima, *et al.*, 1995 and Pete Stacey personal communication). However, *rep2* over-expression appears to have no effect on S-phase transcription so its presence may not define the difference between the active and inactive complexes. Rep2p is also not required to activate transcription when the res2p repressor is absent. This suggests that the role of rep2p may be to counteract the res2p repressor. Finally, I confirmed earlier reports that rep1p does not function as a part of the S-phase transcriptional control during the mitotic cell cycle (Sugiyama, *et al.*, 1994), and as with *rep2*, ectopic expression of *rep1* has no effect.

In a final set of experiments, Jérôme Wuarin and I studied the *cdc10* gel-shift complex, using a radiolabelled fragment of the *cdc18* promoter. Firstly, we confirmed that the bandshift activity observed is equivalent to the DSC1 complex observed in other studies, by showing that the bandshift complex is lost in the presence of specific, but not non-specific, competitor DNA, and that it contains cdc10p, using an antibody to super-shift the complex. We found that *cdc10*, *res1*, *res2* and possibly *rep2*, are all required to form the DSC1 band shift activity in exponential cells. In a series of cell cycle experiments, DSC1 was shown to be present at high levels in G2 cell extracts, but barely detectable in extracts from cells arrested in G1 (in the absence of *cdc2* or in a HU/*cdc22* induced arrest). In a synchronous cell culture, in Chapter 3, DSC1 seemed to disappear transiently upon release from a *cdc25* block, reforming after the peak of *cdc10* dependent transcription close to the onset of S-phase (Figure 3.2c). While the latter data is much less convincing, it again correlates DSC1 with inactive *cdc10* dependent transcription. Our results agree closely with data from a published, more comprehensive analysis of the timing of the appearance of DSC1 through the cell cycle (Reymond *et al.*, 1993). However, our earlier re-assessment of the timing of *cdc10* dependent transcription brings a new twist to these earlier observations, and suggest an alternative interpretation: that the presence of the DSC1 bandshift correlates with repressed transcription. (This is also true in *cdc2* induced re-replication experiments (Reymond *et al.*, 1993)). This resolves the paradoxical persistance of DSC1 in extracts from G2 cells and explains why the DSC1 bandshift is undetectable in extracts from *cdc10-C4* cells. Thus, cdc10p, res1p and res2p may be part of an *in vivo* complex, related to DSC1, which binds to MCB sites in G2 cells, but which is unable to activate transcription. In budding yeast, and in

mammalian cells, the periodicity of transcription required for S-phase is also controlled in part by the interconversion of the active and inactive complexes. Therefore, control of periodic expression of S-phase genes by both transcriptional activation and repression may be conserved.

Finally, an intriguing implication of the data in this Chapter, is that both the active and inactive forms of the cdc10p complex may contain cdc10p, res1p, res2p. Also, each of these components is essential for the measurement of cell cycle time, and the periodic expression of S-phase genes. However, as yet, the mechanism controlling periodic transcription remains unexplained and a little mysterious.

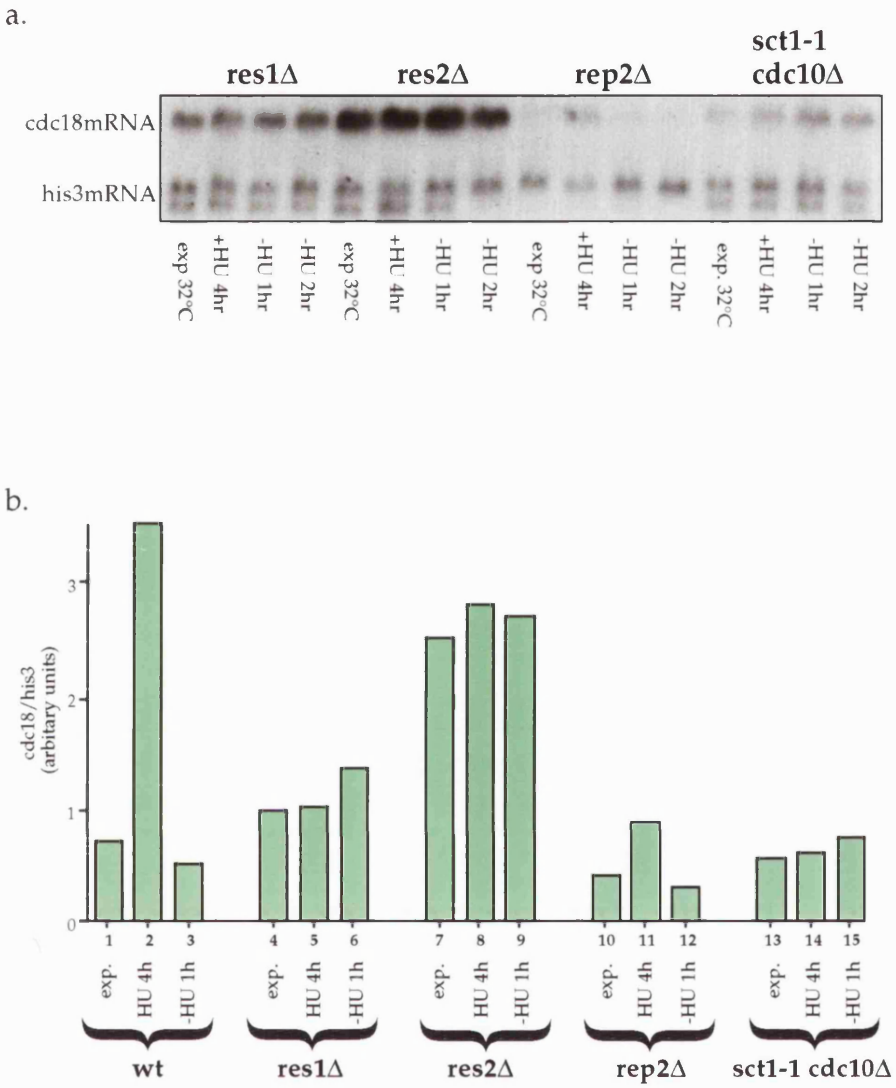


Figure 5.1

The role of components of the cdc10p complex in the control of periodic transcription in mitotic cells.

res1Δ, res2Δ, rep2Δ and *sct1-1 cdc10Δ* strains were grown at 30°C, where they are all viable, and arrested at S-phase by the addition of 11mM hydroxyurea for 4hr. HU was then washed-out, enabling cells to pass through S-phase and into G2 (confirmed by FACS analysis, data not shown). In **5a**, samples were taken for Northern analysis and probed for *cdc18* mRNA, *his3* serving as a loading control. **5b.** The Northern Blot containing samples from experiments shown in 5a and a wildtype control (run on the same gel but not included in 5a) were quantified by phosphor-imager analysis (using an arbitrary scale).

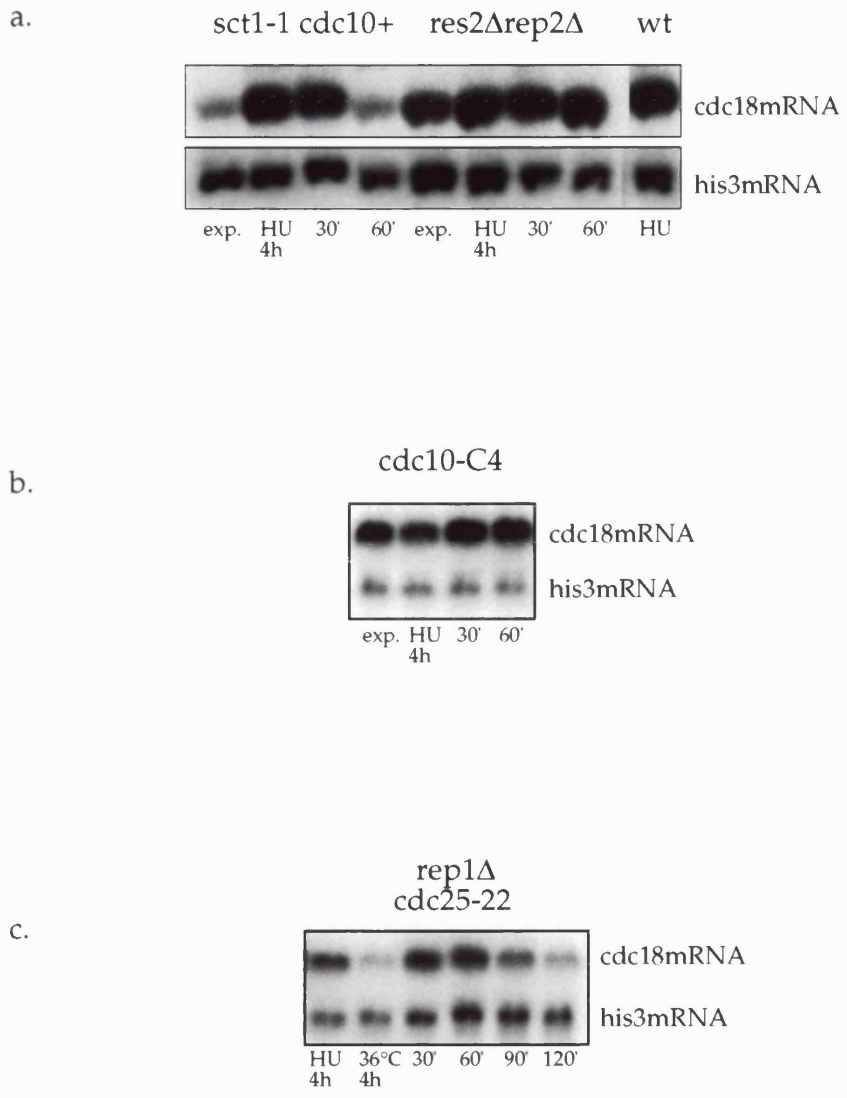


Figure 5.2

***cdc10+* restores periodic transcription to the *sct1-1 cdc10Δ* strain, and *rep2* requires *res2* to affect S-phase transcription.**

2a. The double mutant *res2Δ rep2Δ* and *sct1-1 cdc10+* were subjected to a HU block and release experiment (as in Figure 5.1). A Northern blot was used to assess levels of *cdc18* and *his3* message. A wildtype sample, arrested in HU for 4 hours, was included in the blot as a control. **2b.** In a similar HU block and release experiment at 25°C, levels of *cdc18* mRNA were measured in a *cdc10-C4* mutant. **2c.** To observe periodic transcription in the *rep1Δ* strain, a *rep1 cdc25-22* double mutant was used. Cells were arrested in HU for 4 hours at 25°C and also arrested in G2 for 4 hours at 36°C after which they were released into a synchronous cell cycle. *cdc18* and *his3* mRNA levels were monitored by Northern blotting in samples from cells arrested in HU and during the *cdc25* block and release. (Compare with Figure 3.1).

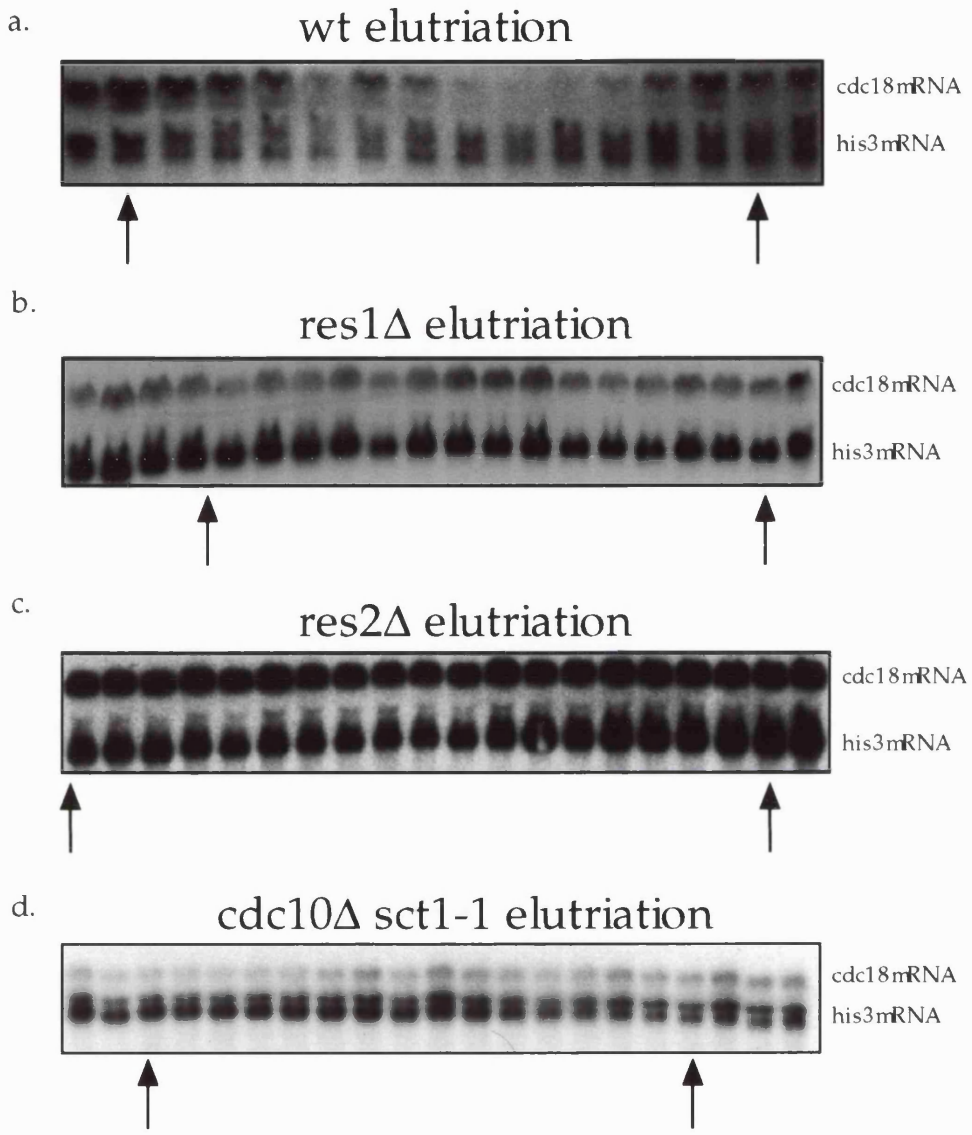


Figure 5.3

The role of components of the *cdc10* complex in the control of periodic transcription through a synchronous cell cycle.

Cultures of wildtype **3a**, *res1Δ* **3b**, *res2Δ* **3c** and *sct1-1cdc10Δ* **3d** cells were elutriated at 30°C. Cells were then followed through a synchronous round of cell division. The level of synchrony for each culture is indicated by the septation index (see Figure 5.3e). Cell number and FACS data confirmed the cell synchrony (data not shown) and Northern Blots were probed for *cdc18* and *his3* message to assess the periodicity of *cdc10* dependent transcription. Arrows indicate peaks of septation.

5.3e

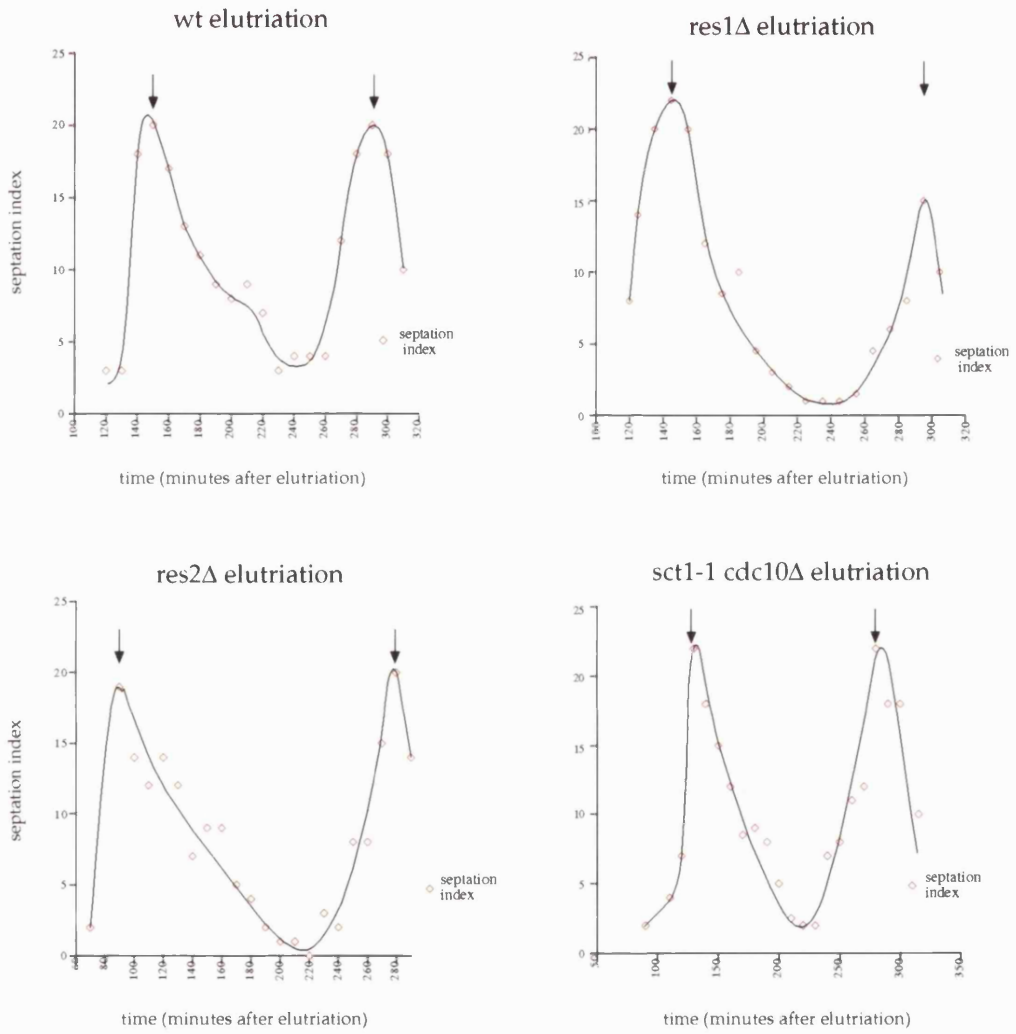


Figure 5.3e

The role of components of the cdc10p complex in the control of periodic transcription through a synchronous cell cycle.

Cultures of wildtype **3a**, *res1Δ* **3b**, *res2Δ* **3c** and *sct1-1cdc10Δ* **3d** cells were elutriated at 30°C. Cells were then followed through 2 synchronous rounds of cell division. The level of synchrony for each culture is indicated by the septation index.

5.3f.

5.3g.

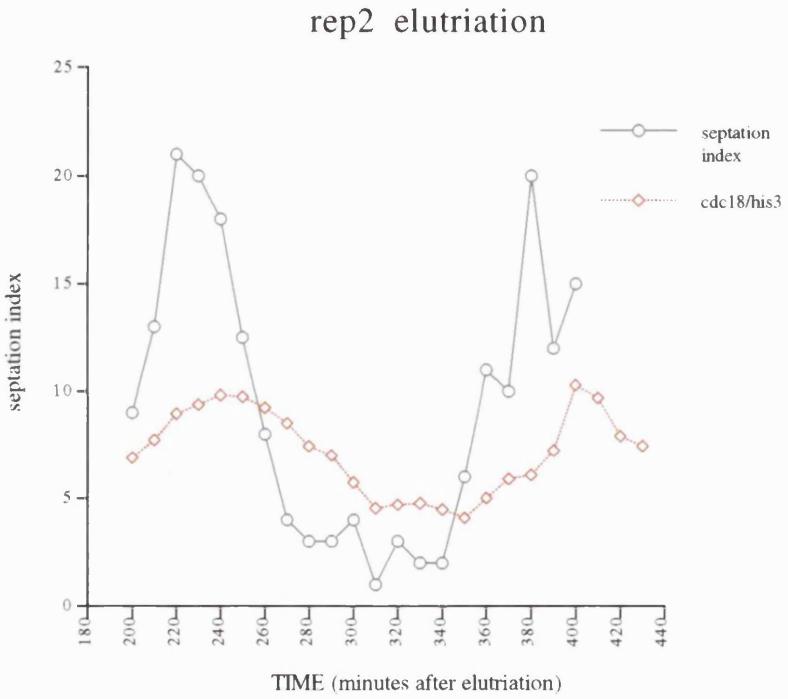


Figure 5.3 f & g

The role of rep2p in the control of periodic transcription through a synchronous cell cycle.

A culture of *rep2Δ* cells was elutriated at 30°C. Cells were then followed through 2 synchronous rounds of cell division. The level of synchrony is indicated by the septation index shown in Figure **3g**. Cell number and FACS data confirmed the cell synchrony (data not shown). Two Northerns were used to assess *cdc18* mRNA levels. The first with 20 time-points is shown in **3f**. The second, shown in **3g**, contained 24 samples and was quantified by phosphor-imager analysis to confirm the result.

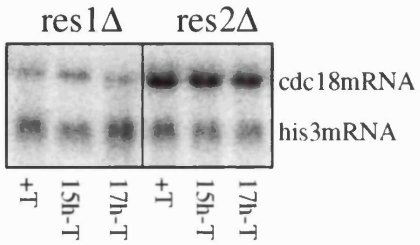


Figure 5.4

The activation of *cdc18* expression in cells arrested in mitosis by ectopic expression of *cdc13Δ90* requires *res1*.

*res1*Δ and *res2*Δ mutations were crossed into a strain containing an integrated copy of *cdc13Δ90* behind the *nmt* promoter. Cells were grown to exponential phase and *nmt* driven expression induced by washing cells free of thiamine. Samples were taken after 15 and 17 hours in the absence of thiamine. DAPI staining of nuclei confirmed that cells were arrested in mitosis. A Northern blot probed for *cdc18* and *his3* mRNA.

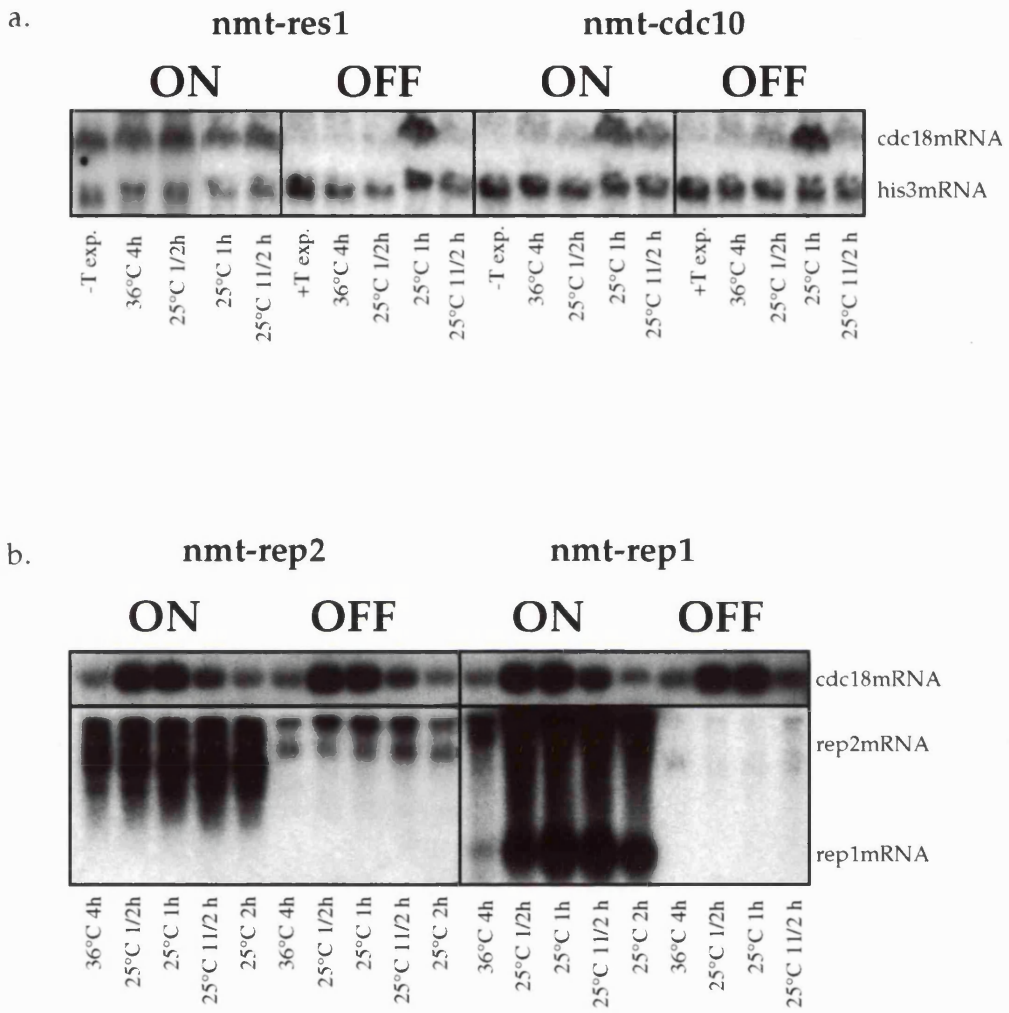


Figure 5.5

The effects of over-expression of putative components of the cdc10p transcriptional complex in cells synchronised in G2 and released into a synchronous cell cycle.

cdc25-22 cells containing *res1*, *cdc10* **(5.5a)**, *rep1* or *rep2* **(5.5b)** behind the *nmt1* promoter were grown to exponential phase in the presence of thiamine and washed 4 times to induce gene expression. After growth for 20h at 25°C, cells were shifted to the restrictive temperature for 4 hours. Cells were then rapidly cooled to 25°C allowing synchronous entry into the mitotic cycle (confirmed by the septation index, data not shown). Northern Blots were probed for *cdc18* and *his3* messages. *rep1* and *rep2* message levels were assessed in 5.5b, because while over-expression of *res1* or *res2* has a dramatic cell lethal phenotype, ectopic expression of *rep1* or *rep2* does not.

5.5c

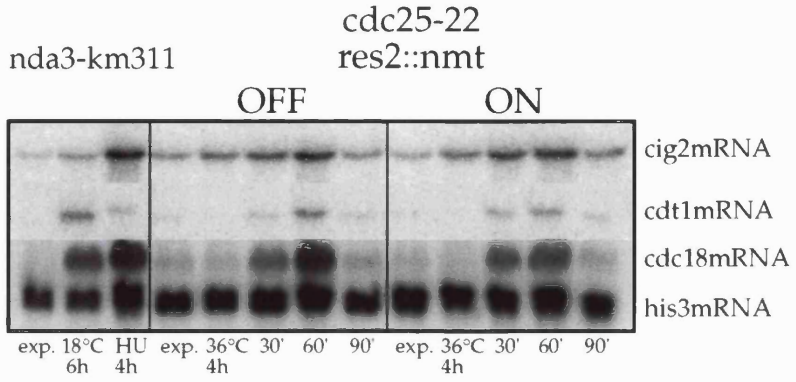
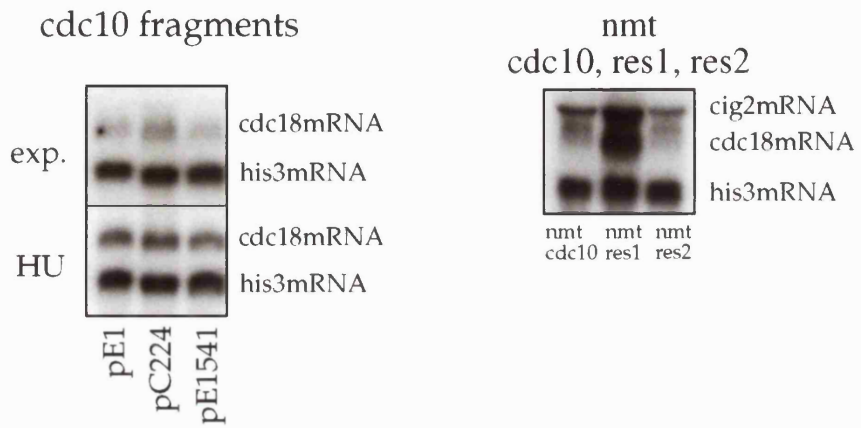


Figure 5.5c

The effects of over-expression of *res2* in a *cdc25* block and release.

cdc25-22 cells containing *res2* behind the *nmt1* promoter were subjected to a *cdc25* block and release protocol in the presence and absence of thiamine. To asses *cdc10* dependent transcriptional activity, a Northern Blot was probed for *cdc18*, *his3*, *cig2* and *cdt1* mRNAs.

a.



b.

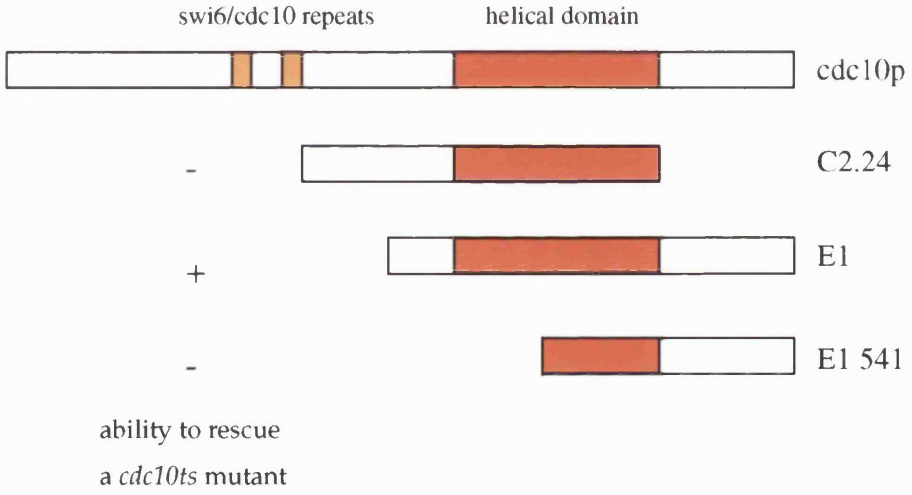
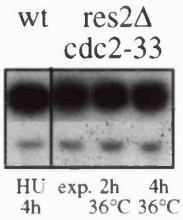


Figure 5.6

Ectopic expression of fragments of *cdc10*, the full length *cdc10, res1 and res2* in wildtype cells. **6a.** The three *cdc10* constructs, pE1, pC224 and pE1541 were transformed into cells and *cdc18* message levels measured in exponential cells and those arrested in HU for 4 hours, using *his3* as a control. This was compared with the effect of expression of full length *cdc10, res1* and *res2* from the *nmt1* promoter for 24 hours at 25°C run on the same gel. **6b.** A schematic of the fragments of cdc10p expressed in wildtype cells in 5d is shown. Only the fragment expressed from pE1 rescues a *cdc10* ts-mutant (Reymond et al. 1993b).

a.



b.

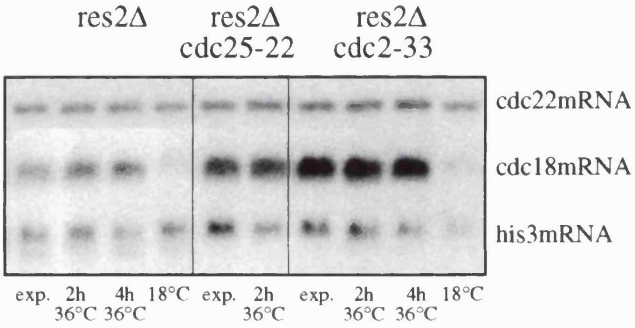


Figure 5.7

cdc2 activity is not required for the expression of _cdc18_ in _res2Δ_ cells.

In two experiments, I compared expression of *cdc18* in *res2Δ*, *res2Δ cdc2-33* and *res2Δ cdc25-22* strains. Firstly, in **7a**, *res2Δ cdc2-33* cells were grown at 25°C and shifted to 36°C for 4 hours. Wildtype cells arrested in HU for 4 hours were included as a control. This experiment was then repeated, in **7b**, with the inclusion of *res2Δ cdc25-22* control cells. Samples from the *res2Δ* and *res2Δ cdc2-33* cultures were also shifted to 18°C for 4 hours to test the effect on *cdc10* dependent transcription of the cold sensitivity of *res2Δ*. Northerns were probed for *cdc18*, *cdc22* and *his3* messages.

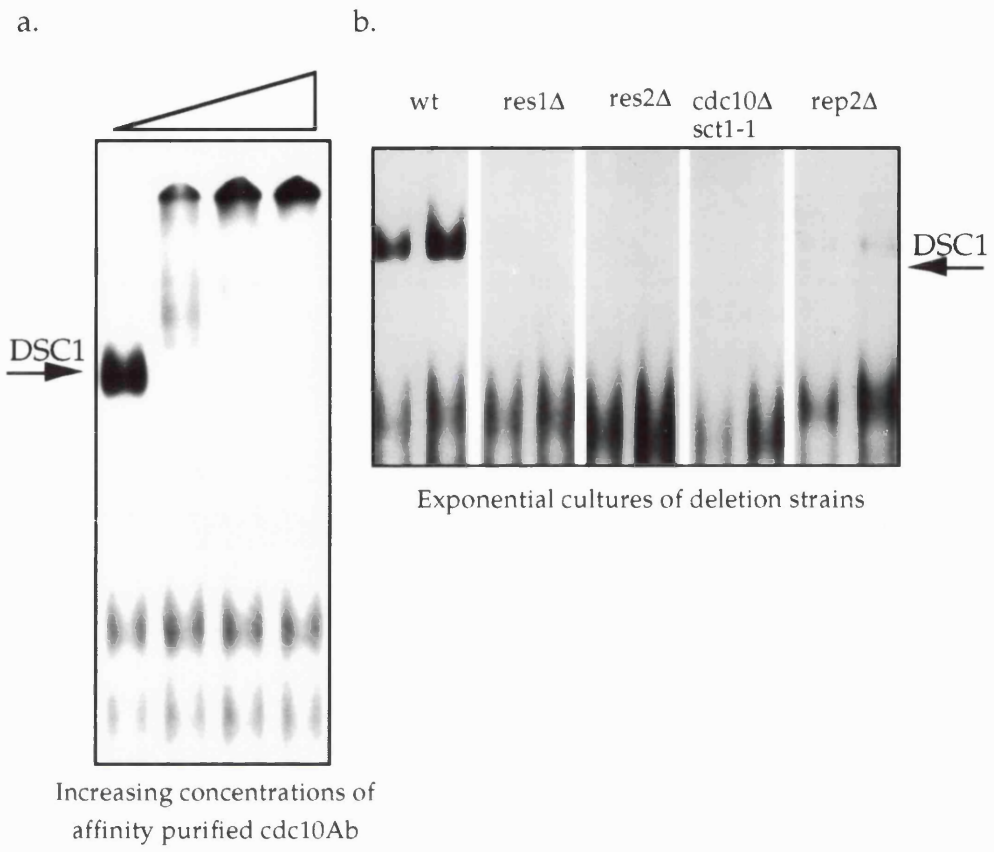


Figure 5.8

Components of the cdc10p complex required for the formation of DSC1.

A band-shift analysis was carried out by adding a radio-labelled MCB containing element, from the *cdc18* promoter, as a probe to cell extracts. In **8a**, wildtype cell extracts were incubated with increasing concentrations of affinity purified, polyclonal rabbit cdc10Ab. **8b.** Gel shift assays were performed with cell extracts from exponential cultures, grown at 30°C, from *res1Δ, res2Δ, cdc10Δ* (in a *sct1-1* background) and *rep2Δ* mutants. A wildtype control was included. Samples were prepared in duplicates, at two different concentrations of cell extract (20µg and 40µg) and loaded in adjacent lanes.

5.8c.

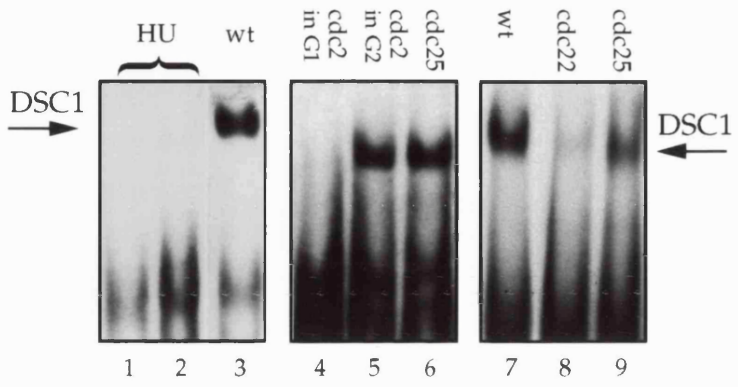


Figure 5.8c

The periodic appearance of DSC1 through the cell cycle.

8c. Gel-shift assays for cells arrested at various points in the cell cycle were carried out. Extracts were taken from exponential growing wildtype cells and cells arrested at the onset of S-phase by the addition of HU for 4h. Samples were loaded on the same gel. Two bandshift assays were conducted using 20µg and 40µg of the HU extract. On a second gel (lanes 4-6), bandshifts were carried out using 40µg of cell extracts from *cdc2-M26* cells arrested in G1, 3 hours after release from nitrogen starvation (from the experiment shown in Figure 2.2) and from cells arrested in G2 using mutations in *cdc2* and *cdc25*, in which *cdc10* dependent transcription is inactive (data not shown). Finally, (lanes 7-9) *cdc25-22* and *cdc22-M10* mutants were shifted to 36°C for 4hours to arrest cells in G2 and at G1/S respectively. Extracts were used in a bandshift assay alondside a wildtype cell extract as a positive control.

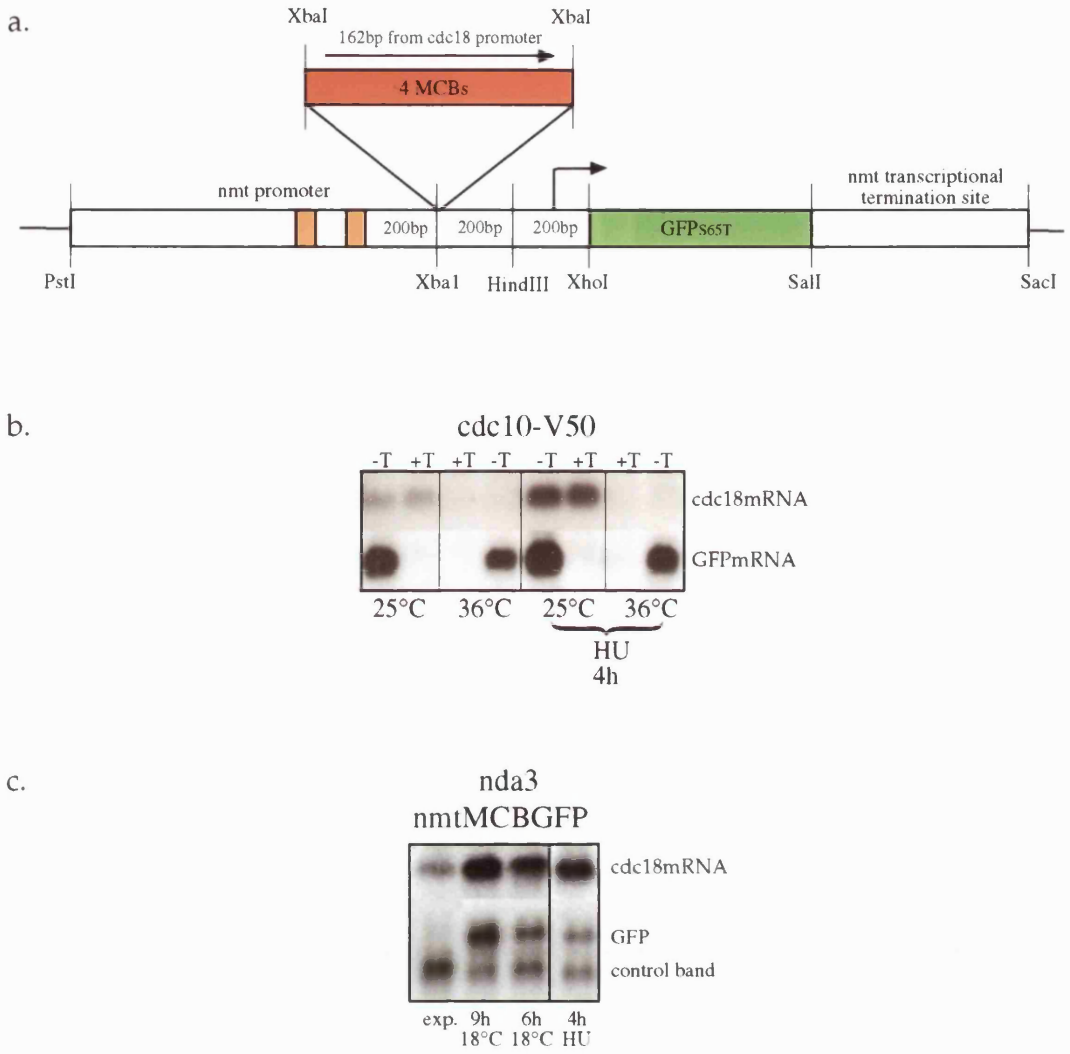


Figure 5.9

A portion of the *cdc18* promoter can drive expression of *GFP* in an *nda3* block. **9a.** A fragment of the *cdc18* promoter containing the MCB boxes was ligated into an XbaI site inthe weak *nmt* promoter close to the transcription start site. **9b.** The construct was introduced into *cdc10-V50* cells which were grown in the presence or absence of thiamine. Thiamine completely inhibited the expression of *GFP*. Cells were grown at 25°C, HU was added to half the culture and cells were shifted to 36°C for 30 minutes to inactivate *cdc10*. A Northern blot was probed for *cdc18* and *GFP* and quantified by phosphor-imager analysis. In the HU arrest, the levels of *GFP* mRNA were 3 fold higher than in exponential cells. Inactivating the *cdc10* function in HU arrested cells, reduced the level of *GFP* transcript 3 fold. **9c.** The *nmt-MCB* construct was also transformed into *nda3-km311* cells. Cells were grow, in the absence of thiamine, at 32°C and arrested at 18°C or in HU. *GFP* and *cdc18* mRNAs were detected by Northernblotting.

CHAPTER 6

Periodic expression of genes from mitosis until S-phase in *S. cerevisiae.*

Introduction

I began my thesis work by testing the widely held view that *cdc10* dependent transcription in *S. pombe* is controlled in a similar fashion to S-phase transcription in budding yeast (Johnston, 1992). Experiments in Chapters 2 and 3, show that regulation of *cdc10* dependent transcription in fission yeast is likely to be regulated differently from that of *SWI4/SWI6* and *SWI6/MBP1* in *S. cerevisiae.* Firstly, *cdc10* is active independently of *cdc2* in G1, and secondly *cdc10* dependent transcription is initiated as cells enter mitosis, not in late G1. Nevertheless, in Chapter 4 it became clear that there is an underlying similarity between the expression of *cdc18* and *CDC6* in the two yeasts which is likely to be of functional significance in governing order of the cell cycle. In this Chapter I investigate whether, by analogy with *cdc10*, Mbp1p/Swi6p mediated transcription in *S. cerevisiae* is also active in mitosis, and whether like *cdc18, CDC6* is first expressed in metaphase, not as currently thought at the M/G1 transition.

Summary of S-phase transcription in *S. cerevisiae.*

As discussed in the Introduction, in budding yeast, periodic S-phase transcription is brought about by the transcriptional complex Mbp1p/Swi6p, and is activated by the Cln3p/Cdc28p activity late in G1. Both components of the complex share homology with *cdc10/res1/res2* (Miyamoto *et al.*, 1994). Mbp1p/Swi6p also operates through promoter elements similar to those acted upon by cdc10p (Lowndes *et al.*, 1991; Johnston, 1992; Lowndes *et al.*, 1992) to drive the expression of several genes, homologous to *cdc10* targets. In budding yeast, important targets of Mbp1p/Swi6p include genes required for S-phase itself (Johnston and Lowndes, 1992) and, in addition, the *CDC6* gene which encodes the *S. cerevisiae cdc18* homologue. This transcription complex has been shown to be essential for the periodic inactivation of *CDC6* transcription through the cycle (Piatti *et al.*, 1995). Initially, *CDC6* was thought to be a target of Swi6p/Mbp1p and expressed in late G1 (Zhou and Jong, 1990). However, more recently, a shift in the perspective of workers in the field came with the realisation that events at the exit from mitosis are likely to be important for the control of S-phase. This led to the recognition that *CDC6* is in fact first expressed earlier in the cycle at telophase (Zwerschke *et al.*, 1994; Piatti, *et al.*, 1995). Initially, this was thought to be the result of *SWI5* dependent transcription (Piatti, *et al.*, 1995). Subsequently, *CDC6* was also shown to be under the regulation of Mcm1p (McInerny *et al.*, 1997). It has been concluded from these observations, that *CDC6* transcription is activated twice during the cell cycle, firstly at the end of mitosis and then again at the end of G1 (Piatti, *et al.*, 1995; Detweiler and Li, 1997).

During this period, I discovered that, in fission yeast, *cdc18* is transcribed from metaphase to S-phase by the S-phase transcriptional machinery. This suggested the possibility that in *S. cerevisiae*, by analogy with *S. pombe*, the expression of *CDC6* may also begin early in mitosis, rather than at telophase, and continue through into G1. (When gene expression in *S. cerevisiae* is concerned, telophase is often considered, whether rightly or wrongly, to be synonymous with early G1). If true, this would go part of the way in reconciling the data from *S. cerevisiae* and *S. pombe.* I therefore decided to investigate the timing of *CDC6* expression in a synchronous culture and in cells arrested early in mitosis, in metaphase and telophase blocks. Since the *CDC6* gene is a target of Mbp1p/Swi6p and contains MCB elements in its

promoter, I also investigated whether MCB elements, under the control of the Mbp1p/Swi6p transcriptional complex, could drive mitotic gene expression, using the same synthetic triple MluI promoter used previously (Figure 3.8) in a *S. cerevisiae* vector (Lowndes, *et al.*, 1991).

In this Chapter, I also present a preliminary analysis of the expression of several other *S. cerevisiae* genes, with varying G1 expression profiles, in mitosis. These include: *CDC46*, an MCM family member with a similar promoter to that of *CDC6* (Hennessy *et al.*, 1990); *SIC1*, a CDK inhibitor like *rum1*, which may play a role in accelerating the exit from mitosis and is a target of Swi5p (Knapp *et al.*, 1996; Toyn *et al.*, 1997); *CLB5*, an S-phase B-type cyclin (Epstein and Cross, 1992; Kuhne and Linder, 1993; Schwob *et al.*, 1994), with both Swi5p target sites and MCB elements in its promoter; *CDC9*, DNA ligase, the promoter of which contains MCB elements (White *et al.*, 1991); *HO*, an endonuclease required for mating type switching, which is expressed in mother cells in G1, by a combinatorial control generated using Swi5p (Nasmyth *et al.*, 1990) and Swi4p/Swi6p transcriptional complexes (Breeden and Nasmyth, 1987), and *CLN2*, a G1 cyclin, which is transcribed by Swi4p/Swi6p probably from classical SCB elements (Wittenberg *et al.*, 1990; Nasmyth and Dirick, 1991), as defined in the *HO* promoter, and via novel elements (Cross *et al.*, 1994; Stuart and Wittenberg, 1994).

In *S. cerevisiae, CDC6* and *CDC46* are expressed in mitosis in a synchronous culture.

In the first experiment, I looked at the timing of activation of *CDC6* expression in a synchronous population of budding yeast cells. Wildtype *S. cerevisiae* were treated with α-factor for 150 minutes to arrest cells in G1 and then washed to enable re-entry into the cycle. As cells entered S-phase and the subsequent mitosis, RNA samples were taken every fifteen minutes (Figure 6.1a). *CDC6* expression began after 75 minutes, coinciding with the peak of anaphase (as measured by DAPI staining cell nuclei), before the re-accumulation of cells in G1 (as observed by FACS analysis in Figure 6.1b). This expression preceded the accumulation of *HO* and *CLB5* transcripts by 15-30 minutes. This suggests that *CDC6* expression is initiated in mitosis, possibly during anaphase. *CDC46* expression was also analysed. Although *CDC46* transcripts exhibited less oscillation than those of *CDC6* and were present in the α-factor arrest, *CDC46* expression also appeared to peak in mitosis, even before *CDC6*. Strangely though, *CDC46* mRNA levels transiently dipped at the 75 minute timepoint.

CDC6, CDC46 and *SIC1* are expressed in mitotic arrested cells.

Next I decided to see whether *CDC6* mRNA and other G1 transcripts would accumulate in cells arrested in mitosis (Figure 6.2). In the first instance the mitotic arrests were achieved using ts-mutants: *cdc14, cdc15-1, cdc15-2, cdc16, cdc20* and *cdc23*. *cdc15* and *cdc14* mutants arrest cells in telophase with high levels of the mitotic kinase and an elongated spindle. *CDC14* encodes a phosphatase which is required to exit mitosis (Wan *et al.*, 1992), *CDC15* is a kinase which is required to destroy the mitotic kinase and to disassemble the spindle at the end of mitosis (Schweitzer and Philippsen, 1991). The *CDC15-1* allele arrests at metaphase and the *CDC15-2* allele at anaphase. Cdc16p, Cdc23p and Cdc27p (the latter was not used in this study) are components of the APC or cyclosome (King *et al.*, 1996) (*CDC27* and *CDC16* are homologous to *nuc2* and *cut9* in *S. pombe* respectively). As a result, cells carrying mutant alleles of these genes arrest at the restrictive temperature in metaphase with the nucleus at the bud neck, with high kinase, unable to undergo the metaphase/anaphase transition. Mutations in *cdc20* arrest cells at a similar point in the cell cycle. *CDC20* is a β-transducin like protein (Sethi *et al.*, 1991), homologous to *fizzy* in *Drosophila* (Dawson *et al.*, 1995), and is thought to regulate the activity of

the APC. DAPI stained *cdc16*, *cdc20* and *cdc15-2* cells arrested for 4 hours at 37°C are presented in Figure 6.2b. (The *cdc15-2* sample contains a mixture of cells arrested in metaphase and telophase).

In assessing the extent of gene expression, it is important to note that, because the G2 period of the cell cycle in *S. cerevisiae* is very short, the levels of "G1 transcripts" are elevated in exponentially growing populations of cells. In budding yeast cultures arrested at metaphase using *cdc15-1*, *cdc16* and *cdc20* mutations, or at telophase in *cdc15-2*, *CDC6* was expressed to relatively high levels, equivalent to, or at greater than those seen in exponentially growing populations of cells (Figure 6.2a). Interestingly, *CDC6* expression was particularly high in a *cdc20* arrest. *CDC46* and *SIC1* transcript levels were also high in cells arrested in mitosis, although *CDC46* was expressed to a similar extent in *cdc15-1*, *cdc15-2*, *cdc16* and *cdc20* arrests. In the cell cycle arrest induced using *cdc14* and *cdc23* ts-mutants, *CDC6* transcript levels, and those of all other polII targets looked at, were substantially diminished. This suggests a general inhibition mRNA accumulation which could either be under the direct control of the *CDC23* and *CDC14* gene products or alternatively, the consequence of subtle differences in the arrest points of different ts-mutants. It is particularly strange that mutations in different components of the APC can have such different effects on transcription. However, it is likely that members of this complex have distinct roles and different cell cycle arrest phenotypes. In *S. pombe*, mutations in different subunits of the APC have quite different phenotypes e.g. *nuc2-663* mutants arrest in metaphase and are sterile (Kumada *et al.*, 1995), whereas *cut9* mutants are fertile and arrest at metaphase, but initiate several post anaphase events (Samejima and Yanagida, 1994).

To verify the observation that in budding yeast, as in fission yeast, G1 genes are expressed in a metaphase arrest, I compared the expression of *CDC6*, *SIC1*, *CDC46* and *CDC9* in cells arrested in metaphase as the result of nocodazole (Figure 6.3). Nocodazole disrupts microtubules and arrests cells at metaphase as the result of a spindle checkpoint, in an analogous fashion to the *nda3* induced arrest in *S. pombe* (Murray, 1994). For comparison, the Northern blot included samples from *cdc15-2* cells arrested at anaphase/telophase. Also, samples of S-phase cells were included, from wildtype cells in the pheromone induced block and release experiment,

(shown in Figure 6.1a) as they passed from G1 into S-phase (Figure 6.3, lanes 10-12)and from wildtype cells arrested in 1M HU (Figure 6.3, lane 6). Finally, RNA from a *cdc23* arrest (lane 7) was included. As before, in both metaphase and telophase arrested cells, *CDC46* and *SIC1* transcripts accumulated to high levels. *CDC6* transcript levels appear to behave similarly, but the data is less convincing. The levels of these transcripts were higher in mitosis than on entry into S-phase, both after recovery from a pheromone induced G1 arrest and in a HU arrest. In cells arrested at metaphase in nocodazole, *CDC6*, *CDC46* and *SIC1* mRNA levels were elevated to levels similar to those seen in a *cdc20* induced arrest (Figure 6.3, compare lanes 3, 7 and 8). In contrast, expression of *CDC9* (which also contains MCB elements in its promoter) reached highest levels as cells entered S-phase from G1. However, *CDC9* expression is low in the HU arrest, suggesting that in *S. cerevisiae*, the peak of G1 transcription is over at the onset of S-phase. These first experiments suggest that *CDC6*, *CDC46* and *SIC1* genes are first expressed early in mitosis.

Several groups have published data indicating that *CDC6*, *CDC46* and *SIC1* mRNA levels are elevated at the boundary of M/G1, e.g. in a *cdc15-2* arrest (Piatti, *et al.*, 1995; Knapp, *et al.*, 1996; Toyn, *et al.*, 1997; McInerny, *et al.*, 1997). However, although *cdc15* mutants are often considered to arrest at the M/G1 boundary (Piatti, *et al.*, 1995), cells in the block maintain intermediate to high levels of mitotic kinase activity (Surana *et al.*, 1993). The data presented here, suggests that *CDC6* expression (and the expression of other early G1 transcripts), may begin even earlier than currently thought, in metaphase arrested cells. This finding is perhaps not so unusual as the analogous one, (of mitotic *cdc18* expression) in *S. pombe*, because budding yeast are not thought to condense their chromatin during mitosis. Furthermore, in *S. cerevisiae*, another G1 event, pheromone dependent transcription, has been shown to be activated in cells arrested in metaphase (Oehlen and Cross, 1994). This latter piece of data was not presented in the paper, probably owing to its unusual nature. Finally, although cells have been reported to re-replicate in a *cdc16* arrest (Heichman and Roberts, 1996), no increase in DNA content was observed in this experiment (see Figure 6.4a, and data not shown). However, other workers in the field were also unable to repeat this finding (K. Nasmyth personal communication).

MCB elements can activate mitotic transcription in *S. cerevisiae.*

To establish whether MCB promoter elements contribute to the mitotic transcription of *CDC6* and *CDC46,* I repeated the mitotic arrests using cells carrying a *lacZ* reporter gene behind the synthetic triple MluI promoter (Lowndes, *et al.*, 1991). I transformed wildtype *S. cerevisiae* and *cdc16* mutants with the reporter plasmid, pLGΔ178.3M. Cells were grown in minimal media in the absence of histidine to select for the plasmid. First, cells containing the plasmid were pre-synchronised in G1 by the addition of α-factor, then, after 150 minutes at 26°C, when essentially all the cells were arrested in G1 (Figure 6.4a/b), pheromone was washed from the media and the culture shifted to 37°C (the restrictive temperature for the *cdc16*). Cells were thereby synchronously released into S-phase and the subsequent cycle (Figure 6.4a/b). *CLN2* and *CLB5* transcripts were used to assay G1/S transcription. This began 80 minutes after the removal of pheromone, as measured by FACS analysis (Figure 6.4c/d). *LacZ* transcripts expressed from the artificial MluI promoter were elevated in pheromone (as previously reported in Lowndes, *et al.*, 1991), as is *CDC46* expression. As cells enter mitosis, between 110 and 120 minutes after the release, *lacZ* expression peaked, in both the wildtype and the *cdc16* strain (Figure 6.4a/b). It is evident from the lack of a second G1/S peak of *CLN2* expression and the FACS data (Figure 6.4c/d), that *cdc16* cells arrested in mitosis. Transcripts levels of *CDC46* and *CDC6* genes (although *CDC6* expression is less clear) reached similar peak levels in both the wildtype and the mitotically arrested culture, confirming the earlier work. However, in this experiment *SIC1* expression was not elevated to the same extent in the mutant and the wildtype. It is not clear why this is the case, but it suggests subtle differences between the expression of *SIC1* and *CDC46/CDC6* genes. In conclusion, this experiment suggests that MCB promoter elements can drive gene expression in a metaphase arrest. The *CLB5* gene, which contains 3 MCB elements in its promoter was also expressed to some extent in the mitotic block.

To confirm this conclusion, nocodazole was also used to arrest exponential cultures of cells in mitosis. Nocodazole was added to *cdc16* and *cdc23* cells carrying the synthetic MCB reporter plasmid (Figure 6.5). Cells were sampled after 4 hours growth in the presence of nocodazole. Samples from cultures arrested at 37°C were included as controls. *LacZ* expression was

elevated in both nocodazole arrested cultures and in the *cdc16* arrest. However, in agreement with the earlier observation, arresting cells in mitosis using the *cdc23* mutation prevented polII transcription.

Discussion.

In this Chapter I investigated the possibility that transcription of *CDC6*, like that of *cdc18*, is initiated early in mitosis. *S. cerevisiae* cells were arrested at metaphase, firstly by using cells carrying temperature sensitive mutations in *CDC16*, *CDC20* and *CDC23* genes and secondly, by the addition of nocodazole. In nocodazole and in the *cdc16* and *cdc20* cell cycle arrest, *CDC6* and *CDC46* genes were expressed to high levels, as was a reporter gene, *lacZ*, driven by the triple MluI synthetic promoter element. Interestingly, the levels of all polII targets appeared to be diminished in both the *cdc23* and *cdc14* arrests. The mitotic expression of *CDC6* and *CDC46* was also confirmed in synchronous cell cultures. Although transcription of these genes was thought to be initiated at telophase (Zwerschke, *et al.*, 1994; Piatti, *et al.*, 1995; McInerny, *et al.*, 1997), the levels of *CDC6* and *CDC46* in metaphase arrested cells, exceded that seen in a *cdc15-2* arrest, and in cells passing from G1 into S-phase. Both these genes carry MCB elements in their promoters so it is possible that this motif could drive their mitotic expression. However, *CDC9*, which also contains MCB elements, is only expressed at high levels at the G1/S boundary (White, *et al.*, 1991). Therefore, further work is required to dissect the mechanism of mitotic MCB driven expression.

In budding yeast, most of the known targets of Mbp1p/Swi6p are genes required for S-phase itself (Johnston and Lowndes, 1992) including*CDC6*, encoding the *S. cerevisiae cdc18* homologue (Zhou and Jong, 1990). In addition, MCB elements, responsive to Mbp1p/Swi6p, are contained within the promoters of several genes, which like *CDC6* have been shown to be expressed early in the cycle, e.g. *CDC46 SWI4, EGT2*, (Gordon and Campbell, 1991; McIntosh, *et al.*, 1991). It is not clear what function these MCB elements play in the early expression of these genes. (They are not critical for the early expression of *SWI4* (Foster *et al.*, 1993)). In each case, the early expression of these genes is presumed to be the result of transcription mediated by Swi5p or Mcm1p. In a very recent study, *CDC6, CDC46, CDC47, CLN3* and *SWI4* promoters were shown to possess an element termed the ECB, through which Mcm1p (probably as a component of a larger complex) may mediate mitotic expression (McInerny, *et al.*, 1997). In this study, the authors conclude that this box mediates expression at the exit from mitosis.

However, the data presented in this Chapter shows that early "G1 expression" occurs in metaphase cells.

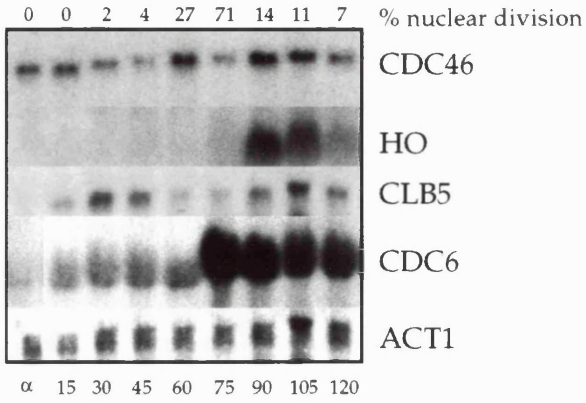
The *CDC6* promoter contains both MCB and ECB elements and a Swi5p target site, all of which can impart periodicity (Piatti, *et al.*, 1995; McInerny, *et al.*, 1997). Also, both Swi5p and Mbp1p/Swi6p are required for the periodic transcription of *CDC6* (Piatti, *et al.*, 1995). These observations have led to the postulate that *CDC6* is expressed in a double burst: the first at telophase and the second in G1, each mediated by different transcriptional complexes. The second burst of *CDC6* transcription appears to be redundant because the G1/M burst suffices for S-phase, even in cells which pause in G1 (Piatti, *et al.*, 1995). This is reminiscent of the case in *S. pombe,* in which *cdc18* appears to accumulate sometime in G1 on release from nitrogen starvation, and in mitosis in cycling cells. In both yeasts, these different timings may result from the two alternative ways of looking at gene expression; either in cells leaving mitosis or during re-growth from G1 arrest. In fact, two peaks of *CDC6/cdc18* expression are not observed in synchronous cultures in either organism. The low levels of *CDC6* mRNA in pheromone, may be the result of additional controls over *CDC6* transcription or mRNA stability, and in early G1 cells isolated by elutriation, cell physiology may be perturbed. The idea that the expression of certain endogenous targets of Mbp1p/Swi6p is inhibited by other controls in G1 is supported by the observation that expression from the synthetic triple MluI promoter (which recruits Swi6p/Mbp1p) is active in the presence of pheromone (and Figure 6.3a/b, and Lowndes, *et al.*, 1991). This may also explain the residual *CDC46* expression seen in a pheromone arrest, since the *CDC46* gene contains similar promoter elements to *CDC6*.

Finally, the current literature contains several complications concerning the early expression of "G1 transcripts" which suggest that the story is not yet complete. The expression of both *SIC1* and *CDC6* genes is thought to be mediated by Swi5p. My data suggest that *SIC1* may be transcribed in a metaphase arrest. *SIC1* expression has also been demonstrated in cells arrested in mitosis by the over-expression of a B-type cyclin (Toyn, *et al.*, 1997). However, the mitotic kinase has been shown to inhibit the action of Swi5p directly, by excluding the protein from the nucleus (Nasmyth, *et al.*, 1990). Also, although Mcm1p may be involved in the early expression of several genes, the protein also participates in the transcription of genes at

very different phases of the cell cycle: G2/M for *CLB2* and *SWI5* (Lydall *et al.*, 1991; Althoefer *et al.*, 1995) and M/G1 for pheromone induced transcripts (Hwang-Shum *et al.*, 1991). It is not clear what defines the difference between Mcm1p induced expression in G1, G2 and M-phase. Therefore, the mechanism and timing of the mitotic accumulation of several "G1" transcripts, remains unresolved. My observation, that the synthetic MluI promoter can drive metaphase gene expression, implies that the activity of Swi6p/Mbp1p may also be important in mitosis. It is conceivable that, either mitotic activation of this complex or alternatively relief from *SWI6/MBP1* mediated repression of transcription at G2/M, could generate this cell cycle profile of expression. (Although, expression from the MCB reporter presumably requires transcriptional activation by the Swi6p/Mbp1p complex).

In conclusion, it is clear that there are close similarities between the transcription of *CDC6* and *cdc18* transcription, in budding and fission yeast respectively. Firstly, in both yeasts, *CDC6/cdc18* transcription begins early in mitosis. For both cdc18p and Cdc6p, this is likely to be of functional significance (see Chapter 5). Also, it is possible that in both yeasts, expression in mitosis is mediated, in part, by homologous transcriptional complexes, acting at MCB promoter elements. If, alternatively, Mbp1p/Swi6p only activates *CDC6* expression late in G1, *S. cerevisiae* may utilise an additional layer of transcriptional control, *SWI5/MCM1*, to achieve expression prior to G1. If this is the case, the timing of *cdc18/CDC6* transcription may be similar in the two yeasts, but the machinery activating transcription different. Finally, it is conceivable that, in *S. pombe*, other unidentified transcription complexes contribute to the mitotic expression of S-phase transcripts.

a.



b.

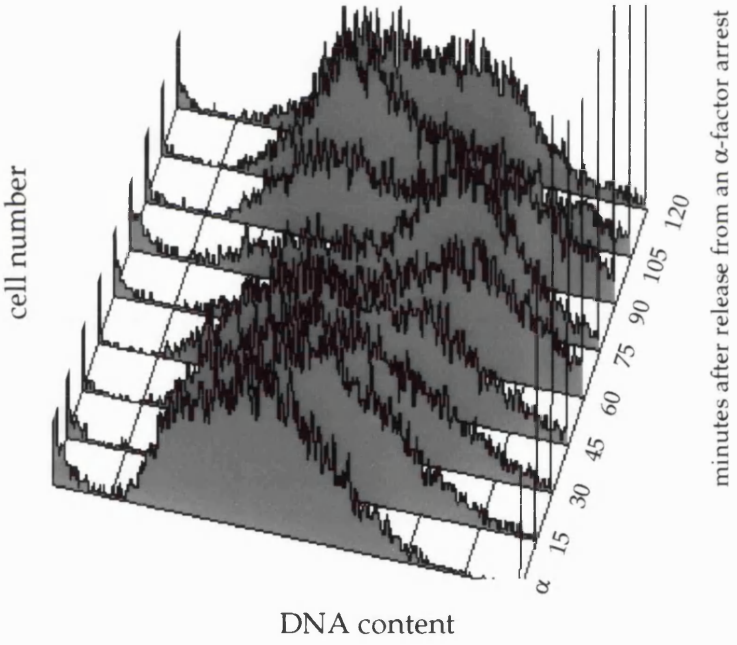
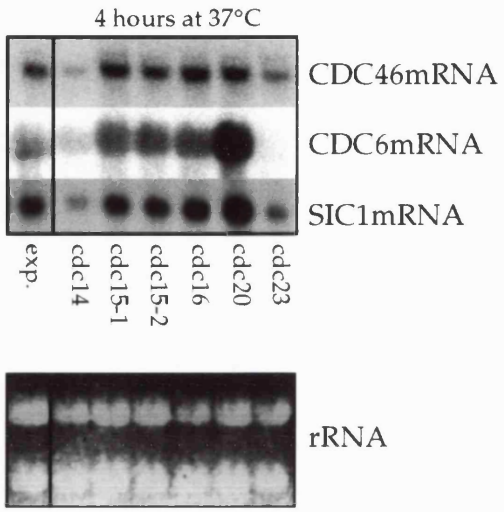


Figure 6.1

In a synchronous budding yeast cell culture, *CDC6* and *CDC46* mRNAs accumulate in mitosis.

Wildtype *S. cerevisiae* cells were arrested in G1 by the addition of α-factor and cells synchronously released into the subsequent cycle. **6.1a.** A Northern was blotted for *CDC6, CDC46, CLB5* and *HO* mRNAs. *ACT1* mRNA was used as a control. The percentage of cells in anaphase was measured by DAPI staining nuclei. **6.1b.** FACS analysis was used to assess the timing of S-phase and cell separation.

a.



b.

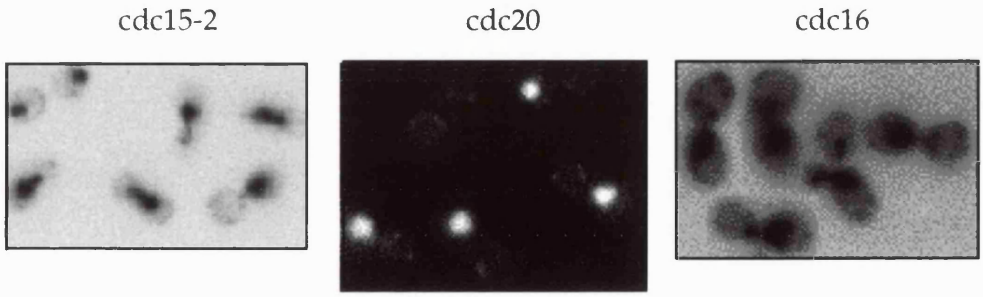


Figure 6.2

Genes expressed early in G1 are transcribed in a metaphase arrest.

ts-mutants which arrest in mitosis at the high temperature, *cdc14-1, cdc15-1, cdc15-2, cdc16-1, cdc20-1* and *cdc23-1* were shifted for 4 hours to 37°C. **6.2a.** RNA was isolated and analysed by Northern blotting to determine the levels of *CDC46, CDC6* and *SIC1* mRNAs. rRNA is shown as a loading control. **6.2a.** *cdc15-2, cdc16-1* and *cdc20-1* cells in the arrest were DAPI stained and photographed to show the mitotic arrest. To help visualise nuclei, the negative image is shown for *cdc15-2* and *cdc16* cells. Cell sizes are not directly comparable.

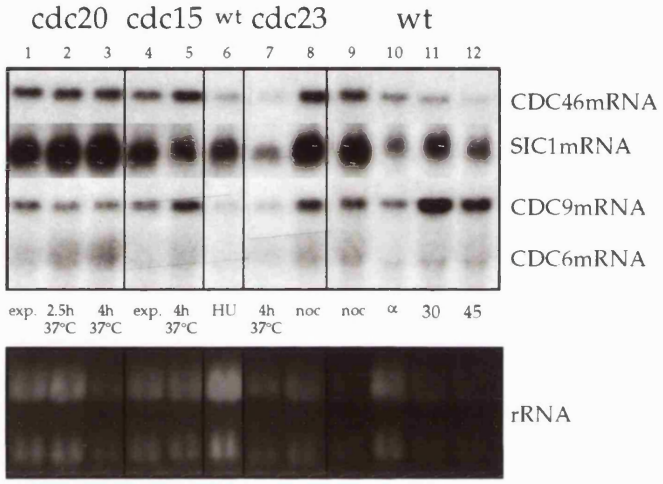


Figure 6.3

In *S. cerevisiae*, several "G1 genes" are expressed in a nocodazole induced metaphase arrest.

Wildtype and *cdc23 S. cerevisiae* cells were arrested in 20μg/ml nocodazole for 4 hours at 25°C (lanes 9 and 8 respectively). For comparison, *cdc20, cdc15* and *cdc23* budding yeast mutants were arrested in mitosis by a shift to 37°C; the arrest is complete after 4 hours. In addition, samples were included from the α-factor block and release experiment shown in Figure 6.1a, as an example of G1 /S levels of expression. The gel also contains a sample from wildtype cells arrested in 1M HU for 4 hours (lanes 6). rRNA was used as a loading control.

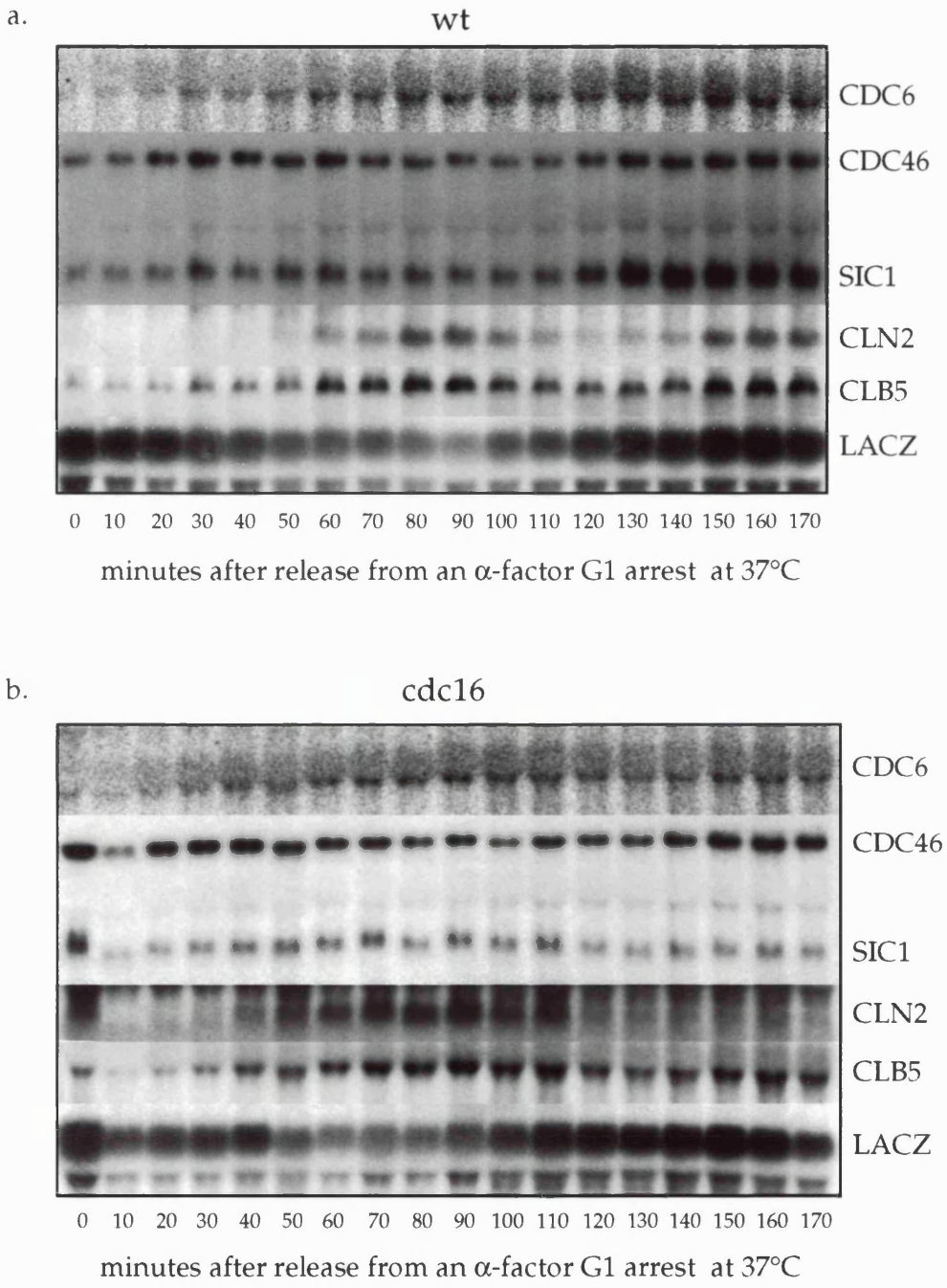
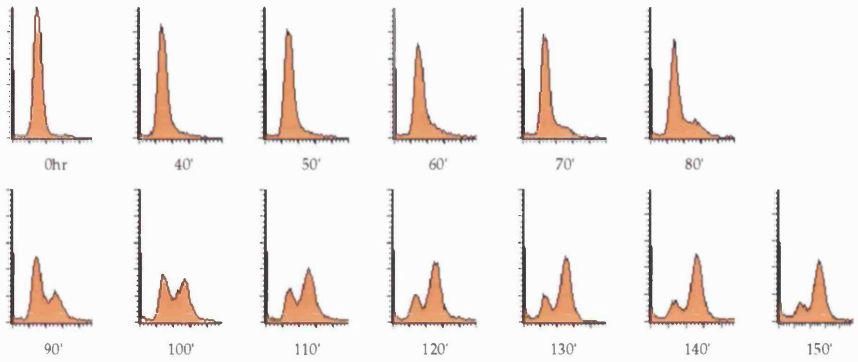


Figure 6.4

Expression from the triple MluI promoter is activated as cells enter mitosis.

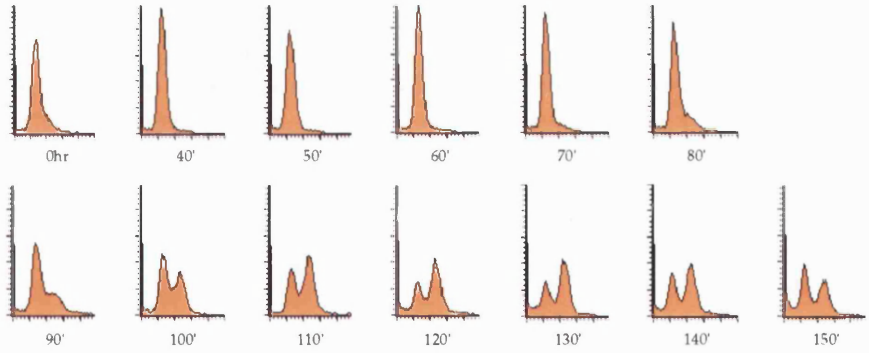
4a. Wildtype and **4b.** *cdc16* mutant *S. cerevisiae* cells, carrying a plasmid expressing *lacZ* from behind the synthetic triple MluI promoter, were arrested in G1 by treatment with α-factor for 3 hours at 26°C. Cells were then washed free of pheromone at 37°C synchronously releasing them into the subsequent cell cycle. mRNA levels were assessed by Northern blotting, probing for *CDC6, CDC46, SIC1, CLN2, CLB5* and *lacZ* transcripts. (FACS data is presented in Figure 6.4c).

6.4c



CDC16 α-factor block and release at 37°C

6.4d



WT α-factor block and release at 37°C

Figure 6.4 c & d

***cdc16* and wildtype *S. cerevisiae* cells synchronously entering mitosis at 37°C.**

FACS data is presented for **4c.** wildtype and **4d.** *cdc16* mutant *S. cerevisiae* cells, both carrying a plasmid expressing *lacZ* from behind the synthetic triple MluI promoter, after release from an α-factor induced G1 arrest at 37°C. S-phase began simultaneously in the two cultures at 80 minutes, and mitosis at about 110 minutes. As expected, *cdc16* cells became arrested in mitosis.

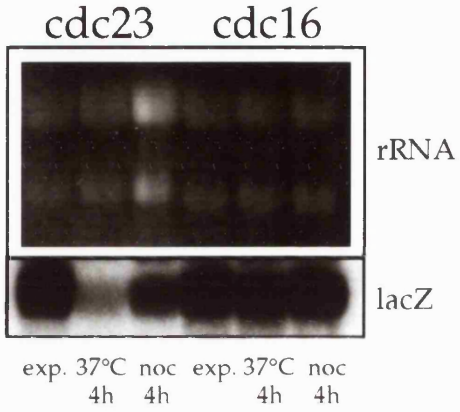


Figure 6.5

MCB elements can drive expression of a reporter gene in a mitotic arrest.

cdc16 and *cdc23* cells were transformed with the synthetic triple MluI-*lacZ* reporter plasmid, and arrested in metaphase in two ways. Firstly cells were shifted to the restrictive temperature, 37°C, for 4 hours, and secondly, cultures were grown in the presence of nocodazole for 4 hours at 25°C. A Northern blot was probed for *LacZ* mRNA. rRNA served as a loading control.

CHAPTER 7

General Discussion

Introduction

In this discussion I hope to frame some of my results in a wider context. I will discuss the problem of how transcription can be active in mitosis in condensed chromatin. I will then try to determine the significance of the mitotic activation of *cdc10* and link this with my findings about the regulated proteolysis of cdc18p. Next, I will extend my findings to *S. cerevisiae* and ask whether the controls governing *CDC6* function are directly analogous to those acting upon *cdc18*. Finally, I will investigate the mechanisms used by cells to generate a global sense of their position in the cell cycle, in order to assess how the periodicity of *cdc10* dependent transcription is likely to be generated.

The mitotic activation of S-phase transcription.

The most striking result from this analysis of S-phase transcription in fission yeast, is the finding that *cdc10* dependent transcription is activated as cells enter mitosis, in cells with condensed chromatin (Robinow, 1977). This is surprising because it is currently thought that a universal feature of mitosis in eukaryotes, is that it is incompatible with active transcription. The mitotic inhibition of transcription has been known for many years and has been most convincingly demonstrated in metazoan nuclei, where the degree of chromatin condensation is likely to exceed that in *S. pombe* by 10 fold. The inhibition of transcription in mitosis was originally thought to be a direct consequence of chromatin condensation (Johnston and Holland, 1965), however, more recently it has been shown, both *in vivo* and *in vitro* , that transcription is switched off directly by the mitotic kinase (Hartl *et al.*, 1993; Gottesfeld *et al.*, 1994; White *et al.*, 1995; Leresche *et al.*, 1996; Gebara *et al.*, 1997). Two wings of the transcriptional machinery have been shown to be independently inhibited by mitotic CDK activity. RNA polymerase function is inhibited by cdc2p/cyclinB mediated phosphorylation of core components of the holo-enzyme (e.g. of TFIIB and TFIID) (Hartl *et al.*, 1993; Gottesfeld, *et al.*, 1994; White, *et al.*, 1995; Leresche, *et al.*, 1996; Gebara, *et al.*, 1997; Segil *et al.*, 1996), and phosphorylation of transcription factors clears them from the mitotic chromatin (Roberts *et al.*, 1991; Segil *et al.*, 1991; Martinez-Balbas*et al.*, 1991). In view of this, mitotic inhibition of transcription may not be a direct consequence of condensation itself, but may be under the control of the mitotic kinase. This reverses the logic of the received wisdom and suggests the possibility that if transcription is not switched off directly, it will remain active in mitosis thereby interfering with the condensation process.

Having said this, specific mechanisms are likely to be required to enable *cdc10* to activate transcription in mitosis. The first potential problem is that of identifying DNA sequences in mitosis. In particular, it is difficult to imagine how a transcription factor can find its target site within condensed chromatin. This problem could however be circumvented if cdc10p were bound to the DNA in G2 cells in an inactive state. In this case, promoters of *cdc10* targets would already be marked for potential activation on entry into mitosis. An alternative hypothesis is that genetic elements in the vicinity

The mitotic activation of S-phase transcription.

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of mitotically active promoters prevent their incorporation into condensed chromatin, and keep active genes exposed at the surface of the chromatin. In this latter case, genes might be expected to be clustered in specific regions of the genome, (more *cdc10* targets must be mapped to assess this), and artificial promoter elements would only be able to activate transcription in mitosis, if integrated at particular chromosomal positions. Perhaps this could explain the inability of cells to express *cig2* in cells arrested in mitosis.

The next problem is how *cdc10* is able to recruit active RNA polII to promoters in mitotic chromatin. On activation, the *cdc10* complex may keep the local chromatin in a state of decondensation through mitosis. Alternatively, if the regions containing active promoters also become condensed, then there must be a mechanism, perhaps some local breathing, enabling the RNA polymerase to traverse the gene. Finally, it is also possible that the mutually exclusive nature of transcription and chromatin condensation is a feature of mitosis in "higher eukaryotes" where the nuclear envelope breaks down leading to the ~~the~~ mixing of cytoplasm and nucleoplasm. In this case prior inhibition of transcription may prevent incompletely processed RNAs from reaching the cytoplasm. In yeast, which experience a closed mitosis, it may not be necessary to shut off transcription. Interestingly, this conclusion is supported by experiments in which the rate of RNA synthesis has been assayed through the *S. pombe* cell cycle. In synchronous cultures, although some periodicity in the incorporation of radioactively labelled bases into RNA (both mRNA and rRNA) is observed, RNA synthesis and translation are not inhibited during nuclear division (Creanor and Mitchison, 1982; Elliott, 1983). In addition, RNA synthesis continues unabated in cells arrested in mitosis with condensed chromatin in the *cdc13-117* mutant (Novak and Mitchison, 1986; Novak and Mitchison, 1987). Since mitosis makes up as much as 10% of the life of a rapidly growing yeast cell (Nasmyth *et al.*, 1979), while growth limits its reproductive potential, it may be important for transcription to continue during this phase of the cycle.

Could CDK activity directly control the mitotic activation of *cdc10*?

How is the *cdc10* dependent transcription activated at the onset of mitosis? Presumably some signal generated during early mitosis induces the *cdc10* complex to activate transcription. Chromatin condensation itself is

unlikely to activate *cdc10* directly, since forced condensation of chromatin by *nimA* in G2 cells (O'Connell *et al.*, 1994) does not activate *cdc10* dependent transcription. At present however, there is little evidence in favour of one particular mechanism of activation of S-phase transcription in mitosis. One attractive possibility is that the mitotic kinase itself is responsible. In *S. cerevisiae*, Cln3p/Cdc28p is thought to activate the transcriptional complexes MBP1p/SWI6p and SWI4p/SWI6p, homologous to cdc10p/res1p/res2p, in late G1 (Peterson, et al., 1985; Breeden and Nasmyth, 1987a; Tyers *et al.*, 1993; Dirick *et al.*, 1995), possibly as the result of a direct interaction (Marini and Reed, 1992). Therefore, if *cdc10* dependent transcription is activated by the mitotic kinase, the mechanism of activation of S-phase transcription may have been conserved during evolution, but modified, making the S-phase transcriptional complex a better target for the mitotic CDK in *S. pombe* and a G1 CDK in *S. cerevisiae*. This would alter the timing of the activation of S-phase transcription in the two yeasts and could, in part, explain the lack of functional homologues of Cln3p/D-type cyclins in *S. pombe* (Forsburg and Nurse, 1994). If this was found to be the case, S-phase transcription in both *S. cerevisiae* and *S. pombe* would be activated at different times by essentially the same mechanism.

Recent work identified a possible *cdc2* consensus site in *cdc10*, which is phosphorylated *in vivo*, essential for *cdc10* activity and for its association with res1p (Connolly, *et al.*, 1997). Data was also presented suggesting that the association of cdc10p and res1p requires *cdc2* and *pat1* activity, and that puc1p (the *S. pombe* G1 cyclin homologue) and pat1p associate with cdc10p (Caligiuri *et al.*, 1997). It was concluded from these experiments that phosphorylation of cdc10p at serine 196, by either the pat1p or the cdc2p/puc1p kinase, would enable cdc10p to bind res1p to form an active transcription factor. However, this group and 2 other groups (Simon Whitehall and J. Ayte unpublished observations) have shown that cdc10p, res1p and res2p are associated in a complex throughout the cell cycle. This suggests that control over the association of res1p and cdc10p is unlikely to control the oscillation of S-phase transcription. Therefore, it is far from clear whether the phosphorylation of cdc10p and its binding to res1p are important for periodic transcription.

In work presented in this thesis I have amassed considerable evidence against the *cdc2-cdc10* hypothesis, conflicting with much of the data

presented above. I monitored *cdc10* activity directly and used ts-*cdc2* alleles and cyclin deletion strains to address whether *cdc10* is under the direct control of *cdc2*. However, in spite of the profound disruption of cell cycle progression caused by these perturbations of CDK activity, I was unable to identify a significant alteration in the activity of *cdc10* dependent transcription in G1. Even after 4 hours at the restrictive temperature in a $cdc2^{ts}$ mutant background, *cdc10* remains still active, whether cells are in G1, or in G2 in a *res2Δ* mutant background. I have also shown that *puc1*, the *CLN3* homologue in *S. pombe*, is not required for the oscillation in *cdc10* dependent transcription. These conclusions are not water-tight however, as ts-mutants can exhibit residual activity at the restrictive temperature, and in the cyclin deletion strains used, unidentified cyclin/CDK complexes could be responsible for activating S-phase transcription. Nevertheless, taken together, the data in this thesis supports the idea that the periodic activity of *cdc10* dependent transcription is independent of the CDK activity.

In order to rule out the interference of other cyclin/cdc2p complexes in the cyclin deletion experiments, it is possible to devise a method to limit the potential cdc2p partners to a single cyclin. This involves the expression of a fusion protein, in which cdc13p and cdc2p are connected through a disordered stretch of amino-acids. This would drive the association of cdc13p and cdc2p, by making it a first order reaction. This fusion protein would also probably be subject to APC mediated periodic destruction through the cyclin degradation box. If constitutive expression of this fusion protein could complement the *cdc2* deletion strain, a possibility suggested by the fact that cells deleted for *cig1*, *cig2* and *puc1* are viable, this would prove that an ordered cell cycle can be driven by a single cyclin/CDK complex. It would then also be possible to switch off the synthesis of this sole CDK and to test whether *cdc10* requires CDK activity at all for periodicity. In the future this may be an interesting approach to take.

The targets of *cdc10* dependent transcription.

Some of the confusion about the timing of *cdc10* dependent transcription in *S. pombe*, may stem from the idea that S-phase transcription is required primarily to express gene functions that replicate DNA (Johnston, 1992). Such transcription would only be required late in G1, after passage through

START. However, in *S. pombe,* S-phase transcription is initiated in mitosis. This being the case, what could its primary purpose be?

In *S. cerevisiae,* many genes of varying functions are important for DNA replication and are periodically transcribed prior to S-phase (Breeden, 1996). While several of the homologous genes in *S. pombe* have been shown to be subject to periodic *cdc10* dependent transcription prior to S-phase, many of the *S. pombe* homologues are transcribed aperiodically through the cell cycle (White *et al.*, 1986). In *S. pombe,* the genes whose expression is under control of the *cdc10* complex include: *cdc22, cdc18, cdt1* and *cig2* (Kelly *et al.*, 1993; Gordon and Fantes, 1986; Hofmann and Beach, 1994; Obara-Ishihara and Okayama, 1994). Although this is a small set of genes, they are likely to be involved in diverse functions, at different times within G1.

The phenotype of *cdc10* mutants serves as a first clue as to the function of S-phase transcription. Cells lacking *cdc10* function arrest in G1 and are unable to ~~to~~ pass through START and enter S-phase (Nurse and Bissett, 1981). Ectopic expresssion of *cdc18,* and *cdt1* to a lesser extent, can rescue the lethality of *cdc10* ts-mutations at the restrictive temperature (Kelly, *et al.*, 1993; Hofmann and Beach, 1994). The inability to transcribe *cdc18* and *cdt1* therefore explains, in part, why *cdc10* mutant cells are unable to enter S-phase. If these were the only critical targets of *cdc10, cdc10* mutants, like *cdc18Δ* and *cdt1Δ* cells, would be expected to enter mitosis directly from G1. However, in a *cdc10* block, B-type cyclins, which are necessary for S-phase and mitosis, cannot accumulate (Hayles *et al.*, 1994; Correa-Bordes and Nurse, 1997). This implies that one or more targets of *cdc10* are also required to inactivate the APC and/or degrade rum1p, both of which are important for targeting B-type cyclins for efficient proteolysis in G1 (Correa-Bordes and Nurse, 1997). This additional, hypothetical function of *cdc10* may also explain the recent observation that over-expression of the cyclin destruction box can also rescue the *cdc10* G1 arrest (Yamano *et al.*, 1996). This may effectively titrate out the proteolytic machinery required to destroy B-type cyclins enabling them to accumulate to a level sufficient to bring about S-phase in the presence of little cdc18p. In this thesis, it was also shown that, in cells leaving a G1 starvation arrest, *cdc10* targets other than *cdc18* are likely to be critical for the onset of S-phase. Therefore, the phenotype of a *cdc10* arrest, depends on experimental conditions, and is the result of the decrease in expression of a number of target genes.

Of the other known targets, *cdc22* encodes an enzyme (ribonucleotide reductase) necessary for ribonucleotide synthesis and therefore essential for the act of DNA synthesis itself (Fernandez *et al.*, 1993). In contrast, *cig2*, another cdc10p target, is a cyclin involved in the timing of the onset of S-phase (Fisher and Nurse, 1996; Mondesert *et al.*, 1996). Cig2p may also influence the decision to enter meiosis *vs* mitosis (Obara-Ishihara and Okayama, 1994), in part by affecting pheromone signalling. Another gene, *cut5*, encodes a protein involved in the DNA replication checkpoint (Saka and Yanagida, 1993; Saka *et al.*, 1994). The *cut5* promoter contains a single *cdc10* target site, and its expression is induced in mitotic blocks induced by mutations in *nda3* and *nuc2*. However, *cut5* appears to be transcribed constitutively through the cell cycle and the level of *cut5* transcripts is not affected by mutations in *cdc10* (Saka, *et al.*, 1994). Therefore, it is not clear whether *cut5* represents a true *cdc10* target. Other potential *cdc10* targets include enzymes required for meiotic DNA recombination (e.g. *rad51* (Jang *et al.*, 1996). Several of these genes have MluI boxes in their promoters and are expressed transiently as cells pass through meiotic S-phase. It is possible that the expression of these latter genes is mediated by an alternative meiotic *cdc10* complex lacking *res1* and *rep2*, because these genes are not highly expressed in the mitotic cycle (Miyamoto, *et al.*, 1994, Nakashima, *et al.*, 1995; Sugiyama, *et al.*, 1994).

Using one mechanism to drive differential gene expression.

The diverse nature of the functions downstream of *cdc10* may necessitate the use of different expression profiles. It may be important to express *cdc18* and *cdt1* at the boundary of mitosis and G1, but it may be sufficient to synthesize ribonucleotide reductase (*cdc22)* a little later in the cycle, as observed. However, a single transcription factor can be used to transcribe targets genes in subtly different patterns of expression. This can be accomplished using promoter elements with different affinities for the transcriptional complex, in various positions relative to each other and to other factors essential for transcription itself, and by differences in mRNA stability. In this way, a cell can generate coordinated, subtle and diverse control, at a low information cost (only several conserved base pairs are required). While such differences may appear trivial, they can be used to order cell cycle events. For example, a scenario can be imagined where

some genes are transcribed first after activation of a transcriptional activator, the transcription of the other low affinity targets then necessarily follows. This is directly analogous to the idea that S-phase and mitosis can be ordered simply by using a lower threshold of kinase activity to bring about S-phase than to initiate mitosis (Stern, 1996). This is also similar to the way in which spatial, rather than temporal, transcription factor gradients pattern *Drosophila* embryos, by activating the expression of different target genes at different concentration thresholds (Rushlow and Arora, 1990; Ip *et al.*, 1992). Such a mechanism could ensure that the accumulation of *cdc18* mRNA always precedes that of *cig2*, thereby enabling cdc18p to act before the peak in cig2p/cdc2p activity in late G1, which may cause its destruction. This is not a robust system however, because in a cell cycle arrest, all targets will accumulate simultaneously. Multiple transcriptional activators and repressors can therefore be used to achieve better temporal control over gene expression. Other transcription factors may in fact participate in the periodic expression of *cdc18, cdt1, cdc22* and *cig2*, to refine their expression profiles or to super-impose another pattern of transcription. The inability to accumulate *cig2* mRNA in mitosis, suggests that *cig2* expression may be subject to additional controls. Alternatively, transcription factors other than *cdc10* may contribute to the mitotic transcription of the *cdc18, cdt1* and *cdc22* genes. For this reason, it is very important in the future to dissect the promoters of these genes and to identify elements driving their expression. There may also be differences between promoters in meiotic and mitotic *cdc10* target genes.

What is the function of the mitotic expression of *cdc18?*

In the introduction I attempted to make a clear and important distinction between **i)** timing and **ii)** order in the cell cycle. In this section, I will discuss a possible role for the mitotic activation of *cdc10* dependent transcription in achieving these two separate functions of the cell cycle control machinery, by focusing on the expression of *cdc18* and controls over cdc18p stability.

i) Timing.

In all eukaryotes, entry into S-phase requires passage through a G1 decision point (Woollard and Nurse, 1995; Reed, 1992), called START in yeast, and the Restriction point in mammalian cells. This can be used to introduce a variable G1 delay into the cell cycle, in response to changes in the extracellular and intracellular environment. For example, in yeast, commitment to the mitotic cycle and entry into S-phase is inhibited by inter-cellular pheromone signalling (Davey and Nielsen, 1994). This is used to synchronise the cycles of two cells preparing to conjugate. In *S. pombe*, a G1 delay also acts as a size monitor to couple cell cycle progression to cellular growth (Nurse and Thuriaux, 1977; Nasmyth, *et al.*, 1979). In animal cells, the analogous G1 control determines whether cells will re-enter the cell cycle or arrest (e.g. for differentiation) (Reed, 1992). De-regulation of this control can therefore lead to unchecked cell proliferation and cancer (Elledge *et al.*, 1996). Consequently, the mechanics of this decision point in mammalian cells are of real medical importance, and have been the subject of intense analysis.

In *S. cerevisiae* and in "higher" eukaryotes, the activity of G1 CDKs (Cln3p/CDC28p in *S. cerevisiae* and CDK4 or CDK6 with D-type cyclins in mammalian cells) is the rate-limiting function controlling progression through G1 (Sherr, 1995). They are required to activate the S-phase transcription of B-type cyclins in late G1, which, in combination with a CDK subunit, catalyse the onset of S-phase (e.g. (Knoblich *et al.*, 1994). This G1/S transcription probably contributes to the cells decision to enter the mitotic cycle. At present, however, it is not understood how environmental stimuli and cell size, alter the activity of G1 CDK/cyclin complexes to

control progression through G1 in these organisms. In *S. pombe*, although *cdc10* is essential for committment to the mitotic cycle and entry into S-phase, it is already active in mitosis of the previous cell cycle. Therefore, the decision to activate *cdc10* dependent transcription is a prerequisite for, but not strictly part of, the decision to complete G1 and to enter a new round of the mitotic cycle. (Nevertheless, it is possible that cells could use the steady accumulation of *cdc10* target transcripts as a measure of cell cycle time since mitosis). If the activation of S-phase transcription is not equivalent to passsage through START, what is its purpose and what is the relevance of its activation in mitosis?

Under conditions of rapid growth, *S. pombe* cells have a short G1 phase and pass directly from mitosis into S-phase (for a recent review see MacNeill and Nurse, 1997). Therefore, the premature activation of *cdc10* could serve as an adaption to the *S. pombe* cell cycle, preparing cells in mitosis for the imminent onset of DNA synthesis. *S. pombe* also dispenses with the transcriptional control over the major B-type cyclin, *cdc13* (Fisher and Nurse, 1996; Correa-Bordes and Nurse, 1997). Therefore, the levels of cdc2p/cyclin B kinase activity are controlled primarily by the use of regulated cyclin degradation and CDK inhibitors (in particular rum1p) (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995; Correa-Bordes and Nurse, 1997; Fisher and Nurse, 1996). As a consequence, in exponentially growing *S. pombe* cells, the timing of S-phase may be determined primarily by the kinetics of inactivation of the APC, and the destruction of rum1p in G1. Such a strategy, combining the mitotic activation of S-phase transcription together with post-translational control over cyclin/CDK activity, effectively removes from the *S. pombe* cell the machinery which, in other eukaryotes (Sherr, 1995), introduces a delay in G1 progression.

It is possible that in certain circumstances other organisms use cell cycles analogous to that observed in rapidly cycling fission yeast. For instance, during the early embryonic cycles of many animals, mitosis and S-phase follow quick on each others heals without intervening gap phases. As in *S. pombe*, periodic transcription in these cells also takes a back seat in ordering the cell cycle (Edgar, 1995; Nothias *et al.*, 1995).

So far, we have discussed the possibility that the mitotic activation of *cdc10* is related to the time between mitosis and S-phase. However several observations demonstrate that, in *S. pombe*, the early activation of *cdc10* occurs irrespective of the length of G1. In particular, *cdc10* is active in wee cells throughout the extended G1 period, in small cells refed after nitrogen starvation and in cells arrested in G1 in response to pheromone. This data seems to argue that the timing of activation of *cdc10* dependent transcription is not related to the temporal proximity of mitosis and S-phase.

ii) Order.

Eukaryotic cells use a second type of control over S-phase, in addition to the G1 decision point, which ensures the dependency of DNA replication on the prior completion of mitosis. This may be a more primitive cell cycle control, because a system of this kind is an essential component of all cell cycles. The mitotic activation of *cdc10* dependent transcription could be one such mechanism coupling mitosis and S-phase.

In *S. cerevisiae*, there is good evidence that the transcription and translation of *CDC6* on exit from mitosis are important for the subsequent S-phase (Zwerschke *et al.*, 1994; Piatti *et al.*, 1995; Detweiler and Li, 1997). There is also evidence that Cdc6p acts at the M/G1 boundary in wildtype *S. cerevisiae* cells, because a genomic footprint, which absolutely requires Cdc6p (corresponding to the pre-replicative complex), is seen at origins in even the smallest elutriated cells (Cocker *et al.*, 1996). If the synthesis of *CDC6* is delayed until after the accumulation of CyclinB/Cdc28p at START, it is unable to function, S-phase is blocked and cells enter mitosis from G1 (Piatti *et al.*, 1996). These data argue that synthesis of Cdc6p at the exit from mitosis is likely to be critical for the efficient assembly of a pre-replicative complex before the resurrection of CDK activity (Nasmyth, 1996). Two observations presented in this thesis suggest that this may also be the case with *cdc18* in *S. pombe*. Firstly, in the absence of *cdc18*, cells enter mitosis from G1, and secondly, synthesis of cdc18p at the M/G1 boundary is probably sufficient for S-phase, because nitrogen starved G1 cells do not require additional cdc18p synthesis in order to re-enter S-phase. Therefore, in both *S. pombe* and *S. cerevisiae*, *CDC6/cdc18* may constitute the link between M and S-phase. It is not yet clear whether *cdc18/CDC6* homologues outside of yeast are expressed at the border between mitosis and G1. However, recent

work has shown that mammalian nuclei are competent or "licensed" to replicate their DNA soon after mitosis (when introduced into a *Xenopus* replication assay) (Wu and Gilbert, 1996). These studies also identified a point a few hours later, approximately 6 hours before the phosphorylation of Rb and the Restriction point, at which the control over origin use is determined. This suggests that some aspects of S-phase are complete very early in G1, even in "higher" eukaryotes, and*cdc18/CDC6* may turn out to be an essential element of all eukaryotic cell cycles.

Interactions between cdc18p and cdc2p may limit S-phase to one round per cell cycle.

In order to understand, mechanistically, how the link between the exit from mitosis and cdc18p function is established, we now turn to consider how cdc18p and cdc2p interact to bring about a single round of S-phase per cell cycle. A G2 nucleus can be induced to re-replicate, by manipulating cdc2p/cyclinB levels. CDK activity must first be inactivated entirely, and then restored to allow entry into S-phase (Broek *et al.*, 1991; Dahmann *et al.*, 1995). This implies that DNA replication is triggered by the sequential change from low to intermediate or high levels of CDK activity (Stern, 1996). In *S. cerevisiae,* under conditions of low CDK activity, the pre-replicative complex is set up (Dahmann, *et al.*, 1995). As a result of the subsequent rise in CDK/cyclin levels, components of the initiation complex are probably phosphorylated causing origin firing, the onset of DNA synthesis and the simultaneous destruction of the potential to re-initiate DNA replication. The observation that the ectopic production of cdc18p in G2 cells can also induce re-replication, first implied a direct interaction between *cdc2* and *cdc18* (Nishitani and Nurse, 1995). This, together with more recent work from Hideo Nishitani, Emma Greenwood and myself in the Nurse lab., suggests that the action of cdc2p kinase on cdc18p could constitute a molecular explanation for the control over re-replication.

The N-terminus of cdc18p contains 5 putative *cdc2* phosphorylation sites with the sequence S/T, P, X, K/R. Hideo Nishitani showed cdc18p is phosphorylated *in vivo*, and that cdc18p associates with cdc2p via its N-terminus. Other groups have established similar findings (Brown *et al.*, 1997). Data presented in this thesis also suggests that cdc2p mediates the periodic instability of cdc18p, by acting through the N-terminus of the

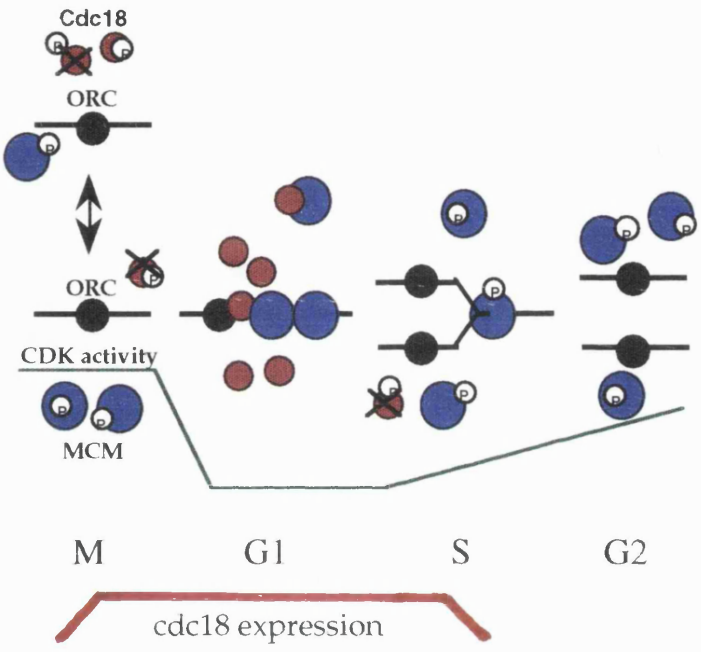
protein. Together, these results suggest that phosphorylation of the N-terminal portion of cdc18p, by the cdc2p/cdc13p complex, may mark the protein for recognition by the pop1p complex. Cdc18p is then ubiquitin conjugated, targeting it to the proteasome for destruction (Kominami and Toda, 1997). Further experiments are underway, using mutant versions of cdc18p in which the potential cdc2p phosphorylation sites are mutated to alanine, to prove this hypothesis definitively; by showing that the cdc2p consensus sites are required for the phosphorylation of cdc18p, its association with pop1p and its subsequent degradation. It should also be shown that the *in vivo* phosphorylation of cdc18p requires *cdc2* activity. Protein phosphatases could also play a role in the control of cdc18p turnover, by de-phosphorylating the protein. One such enzyme, phosphatase PP2C, was isolated as a supressor of a $cdc18^{ts}$ mutant (Hideo Nishitani unpublished data).

Interestingly, although *cdc2* is essential for S-phase (Nurse and Bissett, 1981), over-replication is induced by the ectopic expression of cdc18p lacking the cdc2p consensus sites (in preparation H. Nishitani). This over-replication is even more efficient than that induced by over-production of the wildtype protein. Although the cdc2p associated kinase levels remain high in these cells, the S-phase checkpoint is continously active thereby preventing them from entering mitosis (in preparation Hideo Nishitani and Emma Greenwood). These data suggest that *cdc18*'s potential cdc2p phosphorylation sites are dispensible for its S-phase function. It is still possible that cdc2p also acts through minimal SP or TP sites in cdc18p to activate the onset of replication directly. However, it appears that the primary role for the phosphorylation of cdc18p by cdc2p may be to limit its accumulation to the appropriate portion of the cycle. In wildtype cells this may be important for the prevention of endoreduplication from G2 of the cycle.

We can now use this information to construct a model for the control of S-phase by *cdc2* and *cdc18*.

Figure 7.1

A model for the control of S-phase by cdc2p kinase activity and cdc18p.



In this scheme, *cdc18* mRNA is present from early mitosis until S-phase. Therefore, the levels of cdc18p during this period are controlled primarily by the protein's instability in the presence of active cdc2p kinase. (CDK mediated phosphorylation of the cdc18p N-terminus may target the protein for ubiquitination by the pop1p complex and proteolysis). At the exit from mitosis, the sudden fall in the level of the mitotic kinase activity will lead to the simultaneous accumulation of cdc18p. On entry into G1, cdc18p, may then, by analogy with Cdc6p, set up pre-replicative complexes at the origins of replication, by recruitment of MCMs, in preparation for S-phase. At the transition from G1 into S-phase, B-type cyclins re-accumulate and, as an active complex together with cdc2p, they phosphorylate substrates at the origins of replication, initiating DNA synthesis. This determines the length of G1 and the timing of S-phase. Cdc18p is then phosphorylated, coincidentally with the initiation of S-phase, targetting it for destruction by

the ubiquitin mediated pathway, preventing origin re-firing. Thus, cdc2p and cdc18p serve as mutual antagonists (Kelly, *et al.*, 1993; Nishitani and Nurse, 1995; Bueno and Russell, 1992; Elsasser *et al.*, 1996): the pre-replicative state is defined by both the presence of cdc18p and the absence of cdc2p protein kinase activity, and the post-replicative state by high cdc2p kinase levels, leading to low levels of cdc18p. Previous models of the *S. pombe* cell cycle often contained a paradox: if the activation of *cdc10* was triggered in late G1 by CDK activity, then cdc18p would always be synthesized in the presence of CDK activity which inhibits its function. The observation that, *in S. pombe, cdc18* transcript and protein accumulate early in G1, independently of G1 cdc2p activity, explains how cells resolve this problem, by enabling cdc18p to act before the rise of CDK in late G1.

The mitotic instability of cdc18p may also function to prevent its accumulation in mitosis, since at high levels cdc18p can inhibit the mitotic kinase directly, via its N-terminus (Bueno and Russell, 1992; Brown, *et al.*, 1997 and Emma Greenwood personal communication), although this may not be of physiological significance. Finally, an additional control may exist to prevent expression of *cig2*, a *cdc10* target, in mitosis.

What is the function of the timing and periodicity of *cdc10* dependent transcription?

This discussion, focusing on the role of *cdc18*, has gone some way towards clarifying the possible roles of *cdc10* dependent transcription. In conclusion, it seems that the periodic activity of S-phase transcription could serve a number of discrete functions. The mitotic transcription of *cdc18* and *cdt1* may play key roles in ordering the cell cycle, perhaps by enabling cells to set up a pre-replicative complex early in G1. However, in contrast, early *cdc22* expression may simply be advantageous given the fact that *S. pombe* has a short G1 phase. Finally, additional controls may prevent the potentially detrimental accumulation of *cig2* mRNA in mitosis, since constitutive expression of *cig2* prevents cells from exiting mitosis (Patrick Zarzov personal communication), and in early G1, the presence of cig2p/cdc2p is likely to de-stabilise cdc18p.

Thus, there are reasons to think that the early onset of *cdc10* dependent transcription may be both, an adaption to the short G1 in rapidly growing *S.*

pombe cells, and a device to link completion of mitosis to the setting up of DNA replication, depending on which targets which consider. However, in both *S. cerevisiae* and *S. pombe,* periodic transcription of S-phase genes is not a prerequisite for an ordered cell cycle (Dirick *et al.*, 1992; Lowndes *et al.*, 1992a; Koch *et al.*, 1993). In *S. pombe,* both the *cdc10-C4* mutant and the *res2Δ* constitutively express *cdc10* targets (McInerny *et al.*, 1995). This is of little phenotypic consequence to cells in the mitotic cycle, in part, because of the redundant use of both periodic transcription and proteolysis to control levels of cdc18p, and presumably other key targets. It is also possible that expression of multiple *cdc10* targets in G2, e.g. *cig2* and *cdc18,* counter-balance each other's effect.

An alternative purpose of periodic transcription could be to increase the efficiency of cellular metabolism by producing functions only when required. This requires that the constraints on information storage are relaxed, to allow for the design of the machinery to generate periodic transcription. This is not always the case, and it may be, that for some organisms, it pays better to be genetically succinct than to use resources efficiency. Efficiency however, may be the primary purpose of the periodic expression of genes such as polymerases in *S. cerevisiae,* which are very stable proteins whose absence at discrete cell cycle times is not critical (Johnston and Lowndes, 1992). Periodic transcription may also have evolved as an adaptation to other situations. For example, in meiosis cells must carry out two rounds of nuclear division without an intervening S-phase. In this case, switching off *cdc10* dependent transcription at an appropriate time may be critical. Once the periodic transcriptional machinery has been invented, other processes can be brought under its control, to increase cellular efficiency.

Gene expression in mitosis and G1 in *S. cerevisiae.*

In the next section, I will look at gene expression in the period from mitosis until S-phase in *S. cerevisiae.* This will serve as an example of how multiple interacting transcription factors can potentially be used to define narrow windows of gene expression and to separate early and late G1 transcription. In addition, it will allow us to draw parallels between the two yeasts and to assess which transcription factors serve to transcribe the homologues of *cdc10* targets in *S. cerevisiae.*

The transcription factors involved in the expression of G1 genes in *S. cerevisiae.*

In *S. cerevisiae,* Swi5p, Ace2p, Swi4p/Swi6p, Mbp1p/Swi6p, Bry1p, Mcm1p and Ste12p all participate in the expression of different sets of genes from mitosis through until S-phase (reviewed in Lew *et al.*, 1997) (See Table 7.I). Ash1p confers promoters with mother/daughter specificity (Bobola *et al.*, 1996; Sil and Herskowitz, 1996). The use of different transcriptional complexes with overlapping patterns of activity obviously increases the potential for creating diverse patterns of gene expression. However, it also makes it very hard to unravel such controls. One of the problems with dissecting the various roles for these functions, is that promoters of target genes frequently contain multiple elements. For instance, *CDC6* is a target of Swi5p (Piatti, *et al.*, 1995), Swi6p/Mbp1p (Piatti, *et al.*, 1995) and possibly Mcm1p (McInerny *et al.*, 1997). A transcription factor homologous to the bacterial two component systems, Bry1p, has also been discovered which is thought to act at MCB promoter elements (Morgan *et al.*, 1995), and *CDC6* may also be subject to transcriptional inhibition in the presence of pheromone. An additional problem is that transcription factor consensus sequences are often ill defined. For instance, two groups have demonstrated the presence of a non-canonical upstream element in the *CLN2* promoter which confers Swi4p/Swi6p dependent gene expression (Stuart and Wittenberg, 1994; Cross *et al.*, 1994). Such complications cloud the ability to deduce transcriptional controls from DNA sequence information alone.

The different profiles of gene expression in *S. cerevisiae*, from mitosis until S-phase.

In this section, we will take a brief look at the types of genes expressed during the window of the *S. cerevisiae* cell cycle, from early to late G1. A summary of genes expressed in G1 and the transcription factors responsible for this expression, are shown below in Table 7.I. The signals thought to control the temporal profile of transcription are also included, where known.

Table 7.I

Periodic transcription in the G1 period of the *S. cerevisiae* cell cycle: transcripts, transcription factors responsible for their expression and the proposed mechanisms controlling the oscillation in transcriptional activity.

Target gene	Timing of expression	Transcription factors involved,	Activation signal	Inactivation signal
FUS1	M until late G1	Ste12p	α-factor	Cln/Cdc28
CLN3	M/G1	Mcm1p		
CDC6/SIC1	M/G1	Swi5p Mbp1p/Swi6p Mcm1p		Clb5/Cdc28?? CyclinB/Cdc28
CDC46/SWI4	M/G1	Mbp1p/Swi6p Mcm1p		
ASH1/EGT2/ CTS1	M/G1	Ace1p/Swi5p		CyclinB/Cdc28
CLB5/CLB6	G1/S	Mbp1p/Swi6p	Cln3/Cdc28	
RNR1/POL1/ POL9	G1/S	Mbp1p/Swi6p	Cln3/Cdc28	
CLN1/CLN2	G1/S	Swi4p/Swi6p	Cln3/Cdc28	CyclinB/Cdc28
HO	G1/S	Swi5p Ash1p Swi4p/Swi6p	Cln3/Cdc28 relieves Swi4p/Swi6p G1 repression.	CyclinB/Cdc28
Histone	S	Hir1p/Hir2p		CyclinB/Cdc28

i) Mcm1p

Recently an "early cell cycle box" (ECB) was identified in the promoters of several genes essential for the early phase of G1, including *CLN3, CDC46, CDC47, CDC6* and *SWI4* (McInerny, *et al.*, 1997). This ECB element is thought to bind Mcm1p, probably as a hetero-dimer. It is the only identified transcription factor binding site in the *CLN3* promoter, but it only imparts *CLN3* with a limited, three fold, oscillation in expression through the cycle, peaking at the end of mitosis (McInerny, *et al.*, 1997; Tyers *et al.*, 1993). Of the other potential targets, *CDC47, CDC46* and *CDC6*, are also transcribed in "late mitosis" (Dalton and Whitbread, 1995; Hennessy *et al.*, 1990; Zwerschke, *et al.*, 1994) and are all required to set up the pre-replicative complex (Nasmyth, 1996). However, these genes also possess other promoter elements (MCBs and/or *SWI5* target sites) important for their expression.

ii). Swi5p

Swi5p dependent gene expression is thought to be regulated by CDK mediated changes in nuclear localisation. Swi5p has been shown to enter the nucleus as the Clbp/Cdc28p kinase level falls at the exit from mitosis (Nasmyth, *et al.*, 1990; Dohrmann, *et al.*, 1992). This is thought to lead to the expression of target genes at the boundary of mitosis and G1. As predicted, constitutive nuclear localisation of the transcription factor (achieved by a truncation of Swi5p), can drive active transcription throughout the cell cycle (Nasmyth, *et al.*, 1990; Dohrmann, *et al.*, 1992). The action of Swi5p is then thought to be terminated by its rapid nuclear degradation (Nasmyth, *et al.*, 1990). *SWI5* target sites reside in promoters of a diverse collection of genes, from those expressed in late mitosis, e.g. *EGT2, SIC1* and *CDC6*, to those expressed in late G1, e.g. *HO* (Dohrmann *et al.*, 1992; Dohrmann *et al.*, 1996; Kovacech *et al.*, 1996; Piatti, *et al.*, 1995; Knapp *et al.*, 1996; Toyn *et al.*, 1997; Nasmyth *et al.*, 1990). This makes the temporal specificity of *SWI5* dependent transcription seem a little unclear.

iii) Mbp1p/Swi6p

In late G1, the activation of the Cln3p/Cdc28p kinase is thought to induce the expression of several genes which are important for the onset of S-phase, e.g. *CLB5/6*, together with transcripts coding for the enzymatic machinery necessary for DNA replication, e.g. *CDC9 and RNR1* (Johnston

and Lowndes, 1992). This S-phase transcription is mediated by MCB elements, the target sites for Swi6p/Mbp1p (see below).

iv) Swi4p/Swi6p

Swi4p/Swi6p dependent transcription is also thought to be activated by Cln3p/Cdc28p, late in G1. The targets of Swi4p/Swi6p appear to be primarily involved in morphological events coincident with S-phase e.g. budding (Wittenberg *et al.*, 1990; Lew and Reed, 1993; Igual *et al.*, 1996; Benton *et al.*, 1996). This transcriptional complex is switched off in G2 cells by the action of Clbp/Cdc28p complexes (Amon *et al.*, 1993). Also, the activity of Swi4p/Swi6p is subject to additional regulation by PKC induced activation of MAPK (Madden *et al.*, 1997).

These examples, also show how waves of CDK activity can be "read" and used to generate varying patterns of gene expression through the cell cycle, by both the activation and inactivation of different transcription factors (Lew, *et al.*, 1997). In this way, CDKs entrain gene expression to the cell cycle rhythm. (However, see page 2). These transcriptional systems can also be used in combination to refine the control over the expression of a particular gene. For instance, *HO* expression is thought to be confined to late G1 by a requirement for Swi5p (Nasmyth, *et al.*, 1990) and by Swi4p/Swi6p mediated inhibition of transcription in early G1 (Breeden and Nasmyth, 1987b). Ash1p ensures that it is only transcribed in the mother cell (Bobola, *et al.*, 1996; Sil and Herskowitz, 1996).

Mbp1p/Swi6p is likely to be the counterpart to *cdc10* in *S. cerevisiae*.

The targets of Mbp1p/Swi6p include functional homologues of the *S. pombe* genes: *cdc22 (RNR1;* Elledge and Davis, 1990), *cig2* (*CLB5?/CLB6?*, Epstein and Cross, 1992; Schwob *et al.*, 1994; Kuhne and Linder 1993) and *cdc18 (CDC6,* Lisziewicz *et al.*, 1988). The Mbp1p/Swi6p complex binds to MluI boxes in target genes (Verma *et al.*, 1991, Dirick, *et al.*, 1992, Lowndes, *et al.*, 1992a, Koch, *et al.*, 1993), similar to those found in promoters of *cdc22/cdc18* and *cdt1* in *S. pombe* (Lowndes *et al.*, 1992b), and Mbp1p/Swi6p, like cdc10/res1p, can drive the transcription of a reporter gene in a periodic fashion from the synthetic MluI promoter (Lowndes *et al.*, 1991; Lowndes, *et al.*, 1992b). *MBP1* and *SWI6* also share homology with *cdc10/res1* and *res2*

(Miyamoto *et al.*, 1994). In addition, deletion of *MBP1* or *SWI6* causes the aperiodic expression of several Mbp1p/Swi6p targets, as does deletion of *res2* (Dirick, *et al.*, 1992; Lowndes, *et al.*, 1992a; Koch, *et al.*, 1993). So, Mbp1p/Swi6p, like cdc10p, may be present in active and inactive forms. These data suggest that in *S. cerevisiae*, Mbp1p/Swi6p is likely to be the counterpart to cdc10p/res1p, (although there is no obvious rep2p homologue or functional equivalent, in *S. cerevisiae*). In *S. cerevisiae*, the expression of Mbp1p/Swi6p target genes has been shown to peak in late G1 (Lowndes, *et al.*, 1991; Breeden, 1996). It was primarily for this reason that the same was thought originally to be true for *cdc10* in *S. pombe*.

Like cdc10p/res1p, Mbp1p/Swi6p may be active in mitosis.

Having shown that *cdc10* dependent transcription, in contrast to its counterpart in *S. cerevisiae*, was not activated late in G1, but in metaphase cells, because of the superficial similarities between the two systems, I tested the inverse proposal, to see whether Swi6p/Mbp1p, like cdc10p/res1p, is active in a mitotic arrest.

Firstly, I investigated whether, by analogy with *cdc18*, *CDC6* and other "G1 genes" in *S. cerevisiae* were expressed in mitosis, in both synchronous cultures and in cell cycle arrests. *CDC6* and *CDC46* were found to be expressed to quite high levels in a metaphase arrest, achieved using nocodazole and ts-mutations in *cdc15-1*, *cdc16* and *cdc20* genes, and in a telophase arrest in the *cdc15-2* strain. The expression of both genes in mitosis is also observed in synchronous *S. cerevisiae* cell cultures. Although *CDC6* has already been shown to be expressed early in synchronous cultures and in a *cdc15-2* arrest (Zwerschke, *et al.*, 1994; Piatti, *et al.*, 1995), the data has been used to conclude that *CDC6* is first transcribed at the exit from mitosis (Piatti, *et al.*, 1995; McInerny, *et al.*, 1997). *CDC46* has been reported to be expressed some time in mitosis in synchronous cultures (Hennessy, *et al.*, 1990), although again this is thought to represent expression at the exit from mitosis (McInerny, *et al.*, 1997). The data presented in Chapter 6, however, suggest that, like *cdc18*, both *CDC6* and *CDC46* are expressed early in mitosis.

This observation, that *CDC6* is expressed in metaphase, potentially explains the ability of *S. cerevisiae* cells to over-replicate from a metaphase arrest.

Firstly, cells arrested using nocodazole can be made to set up a pre-replicative complex and to re-replicate after the ectopic expression of *SIC1* (Dahmann, *et al.*, 1995). Similarly, it has been reported that cells blocked in mitosis, by mutations in *CDC16* and *CDC20,* can undergo some measure of re-replication (Heichman and Roberts, 1996); although this observation remains contentious. In both cases, the process of endoreduplication presumably requires newly synthesized Cdc6p (Cocker *et al.*, 1996; Piatti *et al.*, 1995; Detweiler and Li, 1997). Previously the presence of *CDC6* mRNA was unexplained.

Both *CDC46* and *CDC6* genes contain MCB elements in their promoters, so it was conceivable that their mitotic expression is mediated by the Swi6p/Mbp1 complex. In addition, the periodic repression of *CDC6* transcription absolutely requires *SWI6* (Piatti, *et al.*, 1995). However, understanding the expression patterns of endogenous targets is complicated by their possession of multiple promoter elements. Alternatively, the expression of *CDC6* and *CDC46* in metaphase could be mediated through the recently described ECB element (McInerny, *et al.*, 1997), although Mcm1p is thought to mediate expression at the exit from mitosis. However, metaphase expression is unlikely to result from *SWI5* dependent transcription, since Cdc28p/cyclinB activity inhibits the nuclear localisation and therefore the activity of Swi5p.

I found that the synthetic triple MluI promoter, to which Mbp1/Swi6p binds (Lowndes *et al.*, 1991), can drive transcription of a reporter gene in cells arrested in metaphase by both nocodazole and ts-mutations in *cdc15-1, cdc16* and *cdc20* (all of which prevent the degradation of B-type cyclins). These observations suggest that Mbp1p/Swi6p is able to activate transcription in a metaphase arrest. This complex could therefore be responsible for the mitotic expression of *CDC6* and *CDC46.* If true, this could reconcile some of the differences, observed in this thesis, between S-phase transcription in budding and fission yeasts. It is possible that, just as the expression of *cdc18* from mitosis through until S-phase, appears to be mediated by cdc10p/res1p, the periodic activity of Mbp1p/Swi6p could drive both mitotic and late G1 peaks of *CDC6* mRNA, with altered timing under different experimental conditions. An additional piece of evidence potentially supporting the idea that Mbp1p/Swi6p is active in mitosis concerns *CLN3* activity. Cln3p/Cdc28p is thought to activate Mbp1p/Swi6p in late G1. Interestingly,

the levels of endogenous *CLN3* mRNA (McInerny, *et al.*, 1997), protein, and H1 kinase activity, all peak during mitosis in synchronous cultures (Tyers, *et al.*, 1993), and Cln3p associated H1 kinase is elevated in a nocodazole block (Tyers, *et al.*, 1993). Although, in each case, the extent of the oscillation is limited, it is possible that *CLN3* is able to bring about the activation (or de-repression) of transcription from MCB elements in mitosis.

Further data are required to validate the hypothesis that MCB elements drive the expression of endogenous genes in mitosis: **i).** Mitotic transcription from MCB elements should be shown to be dependent on *MBP1* and *SWI6* and possibly on *CLN3* activity. **ii).** This observation should be repeated using the endogenous MCB element from the *CDC6* gene. These experiments are underway.

Achieving periodicity of transcription in the cell cycle.

I will now turn to ask more generally whether we should consider the oscillation of one molecule or activity to be the seat of cell cycle control, or alternatively whether the cell cycle could be ordered by the use of multiple oscillators. I will use this analysis to explore the ways in which cell cycle periodicity can be imparted to a process such as *cdc10* dependent transcription.

The cell cycle is a series of events which follow a fixed sequence. Many cellular functions are under cell cycle control because passage through M and S-phase requires a dramatic reorganisation of the cell, in which many components must participate. For instance, cells reorganise their overall morphology through the cycle, and must distribute the cellular complement of each type of organelle evenly between the two daughter cells at each round of division. Even total cellular growth is coupled to the cell cycle (Fantes and Nurse, 1977; Nasmyth, *et al.*, 1979; Mitchison and Nurse, 1985). Therefore, many cellular components will take up an appropriate state with passage through the cycle.

CDKs are good clocks.

Just as control is invested in enzymes governing rate limiting steps in metabolic pathways e.g. glycolysis (Leninger, 1970), so it is in the cell's

interest to control the cell cycle using a few central players which can integrate various signals and then transmit "decisions" to the rest of the cell. Work in *S. pombe* has clearly demonstrated that manipulating the activity of a single CDK complex can reset a G2 cell to G1 of the cycle and can push a G1 cell into mitosis (Hayles, *et al.*, 1994; Fisher and Nurse, 1996). The logic of the cell cycle in *S. pombe* is therefore said to be governed by oscillations in the activity of *cdc2* (Stern, 1996). The key elements of the oscillation are: a steady increase in cyclin concentration through the cycle and its dramatic destruction at mitosis which resets the "clock". The CDK protein kinase activity may be a convenient choice of cell cycle regulator because it can modify many substrates in a reversible fashion, and thereby change the global order of the cell. The change in activity may also provide a precise measure of cell cycle time. For similar reasons, enzymes able to cause global changes in the cell architecture are likely to control other processes e.g. entry into a differentiated state such as meiosis.

However, even in *S. pombe*, other elements may contribute to control of the cell cycle. More particularly, during this analysis of periodic S-phase transcription, I found that, although *cdc10* may be activated by cdc2p/cdc13p in mitosis, the off switch, in late S-phase, occurs independently of the tyrosine phosphorylation of cdc2p and appears insensitive to changes in the moderate levels of CDK activity in G2 cells. Therefore, some aspects of the regulation of *cdc10* dependent transcription may occur independently of CDK activity. If this is the case, what aspects of the cell cycle could the *cdc10* complex read to bring about periodic transcription?

Other ways to measure passage through the cycle.

Firstly, molecules other than cdc2p/cyclin complexes may play a role in controlling certain cell cycle events. In *S. pombe*, ectopic expression of cdc18p can induce re-entry into S-phase, even in the presence of high levels of the mitotic kinase (Hideo Nishitani unpublished data). Therefore cdc18p, like cdc2p, should be regarded as the seat of much of the control over DNA replication. If this is true, it also follows that if *cdc10* was able to transcribe *cdc18* to a higher level, then the periodicity of *cdc10* dependent transcription could also become essential for ordering the cell cycle. It is possible that cdc18p could set up G1 and simultaneously inhibit mitosis. Then, during S-phase cdc18p would be cleared off the DNA leading to its proteolysis.

Finally, the degradation of cdc18p protein, and its dilution during cellular growth, could even serve to measure the ratio of DNA to cytoplasm, linking the cell cycle to growth. When all the cdc18p was destroyed, this would relieve the inhibition over mitosis, triggering cell division. The physical act of mitosis could then lead to the re-synthesis of cdc18p returning cells to G1. Similarly, plausible, but very cyclosome/APC centric versions of the cell cycle have been constructed (King *et al.*, 1996). These alternative models serve to illustrate that slight alterations in the circuitry can change the molecules perceived to be controlling the global order of the cell cycle.

It is also possible to build an ordered cell cycle using checkpoints alone to monitor the physical events of mitosis and S-phase and to ensure that one follows the other (Murray, 1992). In fact, in *S. pombe,* it is clear that the S-phase checkpoint measures some aspect of DNA replication, perhaps single stranded DNA, to inhibit tyrosine dephosphorylation of cdc2p delaying the onset of mitosis (Enoch and Nurse, 1991; D'Urso and Nurse, 1995). In contrast, in *S. cerevisiae* tyrosine phosphorylation of *CDC28* plays a minor role, probably in exit from mitosis (Minshull *et al.*, 1996). These examples demonstrate firstly, that the cell cycle is controlled in subtly different ways in different organisms but, more importantly, that aspects of a cells physical state, e.g. DNA replication itself (or the presence of a spindle (Murray, 1992)), can be monitored and used to contribute to the ordering of the cell cycle. In addition, these cell cycle events themselves could provide cues to control the periodic activity of other processes. The periodic transcription of histones during S-phase could be an example of transcription controlled in this way. Histone expression is subject to feedback control by the level of histone protein (Moran *et al.*, 1990). In theory at least, this feature alone could measure the histone/DNA ratio and drive its periodic transcription during S-phase, as DNA is synthesized, without measurement of CDK activity. Histone transcription would be induced, even in re-replicating cells, therefore, it might appear that the transcriptional machinery was controlled by CDK/cyclinB activity. Mechanisms of this type have the advantage that they are connected to actual cell cycle events themselves. However, this same feature removes flexibility of control from the system. Thus, there are several ways for a cellular component to measure cell cycle time.

Alternative systems to drive cell cycle oscillations.

Taking the argument further, it is also possible that cells contain components which exhibit periodic activity through the cell cycle independently of the CDK oscillation from mitosis to mitosis. A particularly interesting although complicated example of an oscillation independent of mitosis has been observed in *S. pombe* cells undergoing repeated rounds of S-phase without an intervening mitosis e.g. in *rum1* over-expression and *cdc13Δ* experiments (Moreno and Nurse, 1994; Hayles, *et al.*, 1994). These cells appear to re-enter S-phase periodically, and can maintain their mass to DNA ratio. This re-replication requires both *cdc10* activity and *cig2*, but is inhibited by constitutive *cig2* expression from a moderate promoter (unpublished work, Jacky Hayles). Because *cig2* is a target of *cdc10*, this implies that the activity of *cdc10* must oscillate if re-replication is to occur. Cig2p may also be periodically destroyed in these cells, even though they never pass through mitosis. This periodicity is subject to a size control, and therefore, the endo-reduplication cycle exhibits many features of a normal mitotic cell cycle. Although this system has not been studied in detail, it may be an example of a cryptic cell cycle clock, independent from the normal oscillation in CDK activity. In the normal cycle, this uncharacterised system could be used to order certain cellular events in time.

Can the periodicity of M/G1 transcription through the *S. cerevisiae* cell cycle be accounted for by oscillations in CDK activity alone?

In *S. cerevisiae*, several transcription complexes have been identified which are involved in the periodic expression of target genes through the cell cycle (Lew, *et al.*, 1997). In all cases, the oscillation in activity has been attributed to changes in CDK activity (see Table 7.I). Our understanding of the mechanics of the cell cycle control imposed on these systems, however, remains very limited. In fact, there is very little biochemical evidence for a link between changes in CDK activity and the activity of any cell cycle regulated transcription complexes (Sidorova *et al.*, 1995; Connolly, *et al.*, 1997). Perhaps the most comprehensive evidence for regulation of transcription by CDK mediated phosphorylation in yeast is the regulation by Pho85p/Pho80p of the expression of genes activated in response to a reduction in the levels of extracellular phosphate (Cross, 1995; Lenburg and

O'Shea, 1996). However, it is not clear whether this type of model is really applicable to cell cycle transcription.

I will look at two examples, already mentioned, of the way CDK oscillations in yeast are thought to control the periodic activity of cell cycle specific expression in *S. cerevisiae*, to clarify the limits of our knowledge.

i). In *S. cerevisiae*, the activity of both Swi6p/Mbp1p and Swi4p/Swi6p complexes is thought to be limited to late G1 by a requirement for Cln3p/Cdc28p activity (Tyers, *et al.*, 1993; Dirick, *et al.*, 1995). In the latter case, Cdc28p/cyclinB is also thought to inactivate the complex in G2 of the cycle, leading to its dissociation from the promoter (Siegmund and Nasmyth, 1996). However, the role of Cln3p/Cdc28p remains enigmatic. Firstly, levels of Cln3p and Cln3p associated H1 kinase activity display only a weak oscillation through the cycle, peaking in mitosis (Tyers, *et al.*, 1993). Also, constitutive high level expression of *CLN3* does not eliminate the periodicity of expression of target transcripts (Tyers, *et al.*, 1993). Therefore this accepted explanation for the expression of genes in late G1 is incomplete.

ii). A second and final example of periodic transcription concerns pheromone induced transcription in budding yeast. In *S. cerevisiae*, pheromone responsive genes (e.g. *FUS1*) are expressed from the onset of mitosis, (*FUS1* is transcribed in a nocodazole induced arrest) through G1 of the subsequent cycle, until passage through START, at which point *CLN1/CLN2* kinases inhibit their expression (Oehlen and Cross, 1994), possibly by inactivating components of the pheromone signalling cascade (Wassmann and Ammerer, 1997). This explains in part the nature of START: the inability of cells to pass into meiosis from late G1 and G2 of the mitotic cycle. However, it is clear that the periodic accumulation of CLNs is insufficient to explain the complete profile of expression. Interestingly, this is also a precedent for the activation of transcription in metaphase.

I have stressed the gaps in our knowledge to show that the oscillations in CDK activity may not be sufficient to explain all instances of periodic transcription through the cycle.

As yet, there is little evidence for oscillations in the activity of transcription factors, or of other cellular components, in response to other cell cycle changes e.g. the physical events accompanying passage through the cell cycle. Either such controls do not exist, or alternatively, it may simply be difficult prove that a hypothetical transcription factor of this type can sense a physical event, like passage of the replication fork, and use this information to alter gene expression. In *S. pombe*, one example of periodicity, independent of the CDK cycle, has been identified. In the wild type cell, oscillations in oxygen uptake and carbon dioxide production are entrained to the cell cycle rhythm. However, it has been shown that the oscillations can be maintained, in an altered state, even in a cell cycle arrest (Novak and Mitchison 1986; Novak and Mitchison 1987). This example shows that periodicity in cells is not limited to the CDK cycle.

The cdc10p transcriptional complex.

Far from explaining the periodicity of transcription, the physiological analysis of *cdc10* dependent transcription described in this thesis makes the control of *cdc10* activity seem even more mysterious. In an alternative approach the component parts of the cdc10p transcriptional complex were investigated, both *in vivo* and *in vitro*. Such an analysis has the potential to identify the elements in the complex receiving the periodic signal and translating this into cell cycle dependent gene expression.

Previous studies proposed that DSC1 was a good biochemical correlate of the active complex (Reymond *et al.*, 1993). Jérôme Wuarin and I compared the activity of DSC1, in gel-shift experiments, to the timing of *cdc10* dependent transcription. Although our data concerning DSC1 closely agree with those already published (Reymond, *et al.*, 1993), in light of the unexpected timing of *cdc10* dependent transcription, we came to the opposite conclusion: that the presence of the DSC1 band shift correlates in time with inactive transcription. This clarifies the DSC1 data and furthermore suggests the possibility that *cdc10* may bind promoters of target genes in G2 of the cell cycle. It is important to note however, that DSC1 is an *in vitro* bandshift complex and its activity may not reflect that of the cdc10p complex *in vivo*. For instance, although Swi4p/Swi6p binds DNA *in vitro* throughout the cycle (Taba *et al.*, 1991), genomic footprinting suggests that, *in vivo*, this complex only binds to active target promoters (Koch *et al.*, 1996). The observed periodicity of DSC1 complex formation could reflect the fact that the active cdc10p complex is tightly bound to DNA and difficult to extract. Alternatively, cdc10p may bind to promoter sites throughout the cell cycle in alternating forms, (as does MCM1 in *S. cerevisiae* (Althoefer *et al.*, 1995), and one of the two DNA binding complexes may simply be more labile when subjected to extraction. Recently Jérôme Wuarin also identified a new cdc10p containing complex of different mobility, using a low salt extraction buffer, in HU arrested cells and in G2 cells over-expressing *res1*. This novel complex may represent the active, *in vivo* complex. This emphasizes the point that the extraction procedure critically affects the gel-shift activity observed. For these reasons, much effort in the lab. has gone into developing genomic footprinting to

enable us to "see" the interaction of DNA with transcription factors as it occurs *in vivo* . So far however, this analysis has proved unsuccessful.

Jérôme Wuarin and I also showed that *cdc10*, *res1*, *res2* and *rep2* are all required to form the DSC1 complex. This hints at the possibility that there is a large inactive *cdc10* complex, similar to the hypothetical active complex, that binds *cdc10* target promoters *in vivo* in G2. DSC1 has since been shown to contain res2p, by using antibodies to super-shift the complex (Zhu, *et al.*, 1997).

In the next part of my analysis, I used a pertubation analysis (both deletion and overexpression) to look at the possible roles for the various components of cdc10 transcriptional complexes in the control of periodic S-phase transcription in the mitotic cycle. The results are summarised below.

Table 7.II

Table to show the deletion and over-expression phenotypes of genes simplicated in the S-phase transcriptional control.

Arrows indicate high or low level S-phase transcription.

	DELETION	OVEREXPRESSION *nmt promoter*
cdc10	aperiodic ↓	WT
res1	aperiodic ↓	aperiodic ↑
res2	aperiodic ↑	WT
rep2	periodic ↓	WT
res2Δrep2Δ	aperiodic ↑	
cdc10-C4	aperiodic ↑	
sct1-1 cdc10+	WT	
rep1Δ	WT	

cdc10, *res1* and *res2* functions are all required to maintain the periodicity of S-phase transcription. In particular, an unexpected role for res2p was identified in the periodic repression of S-phase transcription during the mitotic cell cycle. Previously, it was thought that res2p acted primarily during the meiotic cell cycle where it is essential (Miyamoto, *et al.*, 1994; Zhu *et al.*, 1994). In the *res2Δ*, the transcription of *cdc10* targets is likely to remain under the control of res1p, as the double mutant *res1Δ res2Δ* is lethal (Miyamoto, *et al.*, 1994). A truncated version of cdc10p, encoded by *cdc10-C4*, was also shown to be able to activate S-phase transcription in G2 cells (McInerny, *et al.*, 1995), implying a similar role for cdc10p in repressing transcription in G2 cells. It seems likely that there are two possible mechanisms for the inhibition of transcription in G2 cells: **1.** *res2* could inhibit the active complex by preventing it binding to DNA, or **2.** an inactive transcription factor complex or a repressor of transcription, may bind the promoters of target genes in G2 cells. (The difference between

inactivation of an active complex and the action of a repressor may often be a semantic one). Since res2p and cdc10p are both required to form the DSC1 band shift activity, it is possible that these proteins mediate the G2 repression of transcription through the action of the DSC1 complex. In budding yeast and in "higher" eukaryotes, there is also evidence which suggests that periodic S-phase transcription is brought about by both the activation and repression of transcription. In *S. cerevisiae*, the Swi4p/Swi6p complex inhibits transcription in early G1 cells (Koch, *et al.*, 1996), and Swi6p/Mbp1p complexes are required to prevent the expression of several target genes in G2 of the cell cycle (Dirick, *et al.*, 1992; Lowndes, *et al.*, 1992a; Koch, *et al.*, 1993), including *CDC6* (Piatti, *et al.*, 1995). In a similar fashion, in mammalian cells periodic S-phase transcription is controlled by the conversion of the an inactive E2Fp/DP1p/Rb complex, bound at the promoters of target genes, into an active form, by the release of Rb. (Zamanian and La, 1993; Adnane *et al.*, 1995; Bremner *et al.*, 1995). This role in transcriptional repression may explain why the deletion of *E2F* in mice is tumorigenic (Yamasaki *et al*, 1996; Field *et al*, 1996). Thus, this dual control over the periodic expression of S-phase genes (by both transcriptional activation and repression), may be conserved. This is not really surprising because, although gene expression is often assumed to be regulated by control over transcriptional activation, as Jacob and Monod first pointed out, in primitive organisms, constitutive gene expression probably suffices for most purposes. Then, with evolutionary time, the profile of expression of a gene is likely to be refined by the development of regulated transcriptional repression mechanisms.

I also analysed the function of the other components of the cdc10p complex. Res1p and cdc10p both appear to be required for active S-phase transcription and its oscillation through the cycle (including expression in mitosis). Significantly, high level, ectopic expression of res1p during G2 is sufficient to activate transcription to maximal levels (also see Ayte *et al.*, 1995). This is an important observation, which implies that res1p may play a key role in the periodic activation of S-phase transcription through the cell cycle. *Res1* can also rescue the *cdc10* deletion, suggesting that it is able to recognise MCB elements in the absence of *cdc10*, (although the genetic data imply that this probably requires res2p). Finally, rep2p does not appear to contribute to the periodicity of S-phase transcription but is required to elevate the absolute level of activity. However, in the absence of res2p, rep2p is not required for

active transcription. Together these data imply that *rep2* and *res2* (guilty by association) are in the active complex and that rep2p may enable active expression by counteracting the effect of the res2p repressor.

Now we have established possible roles for the individual components of the S-phase transcriptional machinery, it is possible to propose a model for the way in which they interact to promote periodic transcription (Figure 7.2). Cdc10p may provide a scaffold on which the regulation of transcription is imposed by the binding of res1p and res2p. Normally, res1p may inactivate the res2p inhibitor, activating transcription. When res1p is over-expressed during G2, inactivation of res2p would occur inappropriately, allowing the complex at the promoter to activate transcription. Ectopic expression of res2p cannot block active transcription, so its presence alone may not define the difference between the active and inactive complexes. This agrees with data from other groups which suggests that the stoichiometry of res1p and res2p in the complex is unaltered with passage through the cell cycle (personal communication Simon Whitehall and José Ayte; Connolly *et al.*, 1997). The role of the rep2p factor may be to increase the efficiency of the whole complex in promoting transcription in its active state, perhaps by masking the presence of res2p in the active complex.

Interestingly this model implies that 3 of the components in the complex are required for its measurement of cell cycle time, and that there is little difference between the active and inactive forms of the complex (although there is a difference *in vitro* in the level of observed DSC1 activity in cell extracts through the cycle). This leaves little clue as to the identity of the signal switching the complex between its active and inactive states forms. Therefore, although this analysis of the cdc10p complex has yielded surprising results, including roles for transcriptional repression and *res2*, it does not point clearly to an explanation for the periodicity of S-phase transcription.

Summary

In summary, I have investigated the controls governing the activity of S-phase transcription in the fission yeast mitotic cell cycle, and attempted to elucidate the function of the periodic expression of target genes. My physiological analysis lead me to re-address the timing of transcription. I found that *cdc10* dependent transcription is activated as cells enter mitosis and that *cdc10* target transcripts accumulate in cells arrested at metaphase with condensed chromatin and high levels of the mitotic kinase. This result implies that the oscillation in the activity of *cdc10* cannot be controlled as previously thought by G1 CDK activity. It also brings into question the significance of the *cdc10* bandshift complex, DSC1, which appears late in G1 and persists through G2. These data suggest that S-phase transcription is controlled very differently in *S. cerevisiae and S. pombe.*

I have tried to place the mitotic activation of S-phase transcription in a functional context and have shown that although *cdc10* is active in metaphase cells, it is unable to complete its function until some time later, in G1. The observation that cdc18p, its major target, is unstable in mitosis in part explains this phenomenon. Cdc18p is destabilised in mitosis, possibly through phosphorylation of its N-terminus by the mitotic kinase, and as a consequence, cdc18p only accumulates upon release from the mitotic block, as the kinase level falls. These results show that although I began my analysis by identifying differences between S-phase transcription in *S. cerevisiae* and *S. pombe,* both yeasts express *cdc18/CDC6* in mitosis. This conserved feature is probably important for ensuring that the protein products of these genes are synthesized before the accumulation of B-type cyclins, late in G1. In support of this conclusion, I present data suggesting that the synthesis of cdc18p at the boundary of M/G1 is likely to be sufficient to bring about the subsequent S-phase. Thus, in all eukaryotes it may be important to synthesize proteins that define the pre-replicative state of the DNA at the boundary of mitosis and G1, to couple exit from mitosis to the next S-phase.

I also conducted a limited analysis of the *cdc10* complex. This defined a new role for *res2,* in the G2 repression of *cdc10* dependent transcription in the mitotic cycle, and *res1* was shown to be a key activator of this transcription.

Finally, I used both synchronous culture and cell cycle arrests to show that, in *S. cerevisiae, CDC6* and other "G1" transcripts, are expressed in metaphase. In addition, a synthetic MluI promoter, which is designed to recruit the S-phase transcriptional complex (Swi6p/Mbp1p) can drive expression of a reporter gene in metaphase. This implies, unexpectedly, that in both *S. pombe and S. cerevisiae,* S phase transcription may be initiated in metaphase. Therefore, the differences initially identified between the mechanisms controlling *cdc10* and *SWI6/MBP1* activity may hide underlying similarities between the two systems.

In conclusion, this work has linked the disparate conceptions of S-phase transcription and the role of mitosis in resetting the state of origins of replication to a G1 state. What I have been unable to do, however, is to make much headway in explaining how the periodicity of *cdc10* dependent transcription is controlled. Therefore, many interesting questions remain for the future.

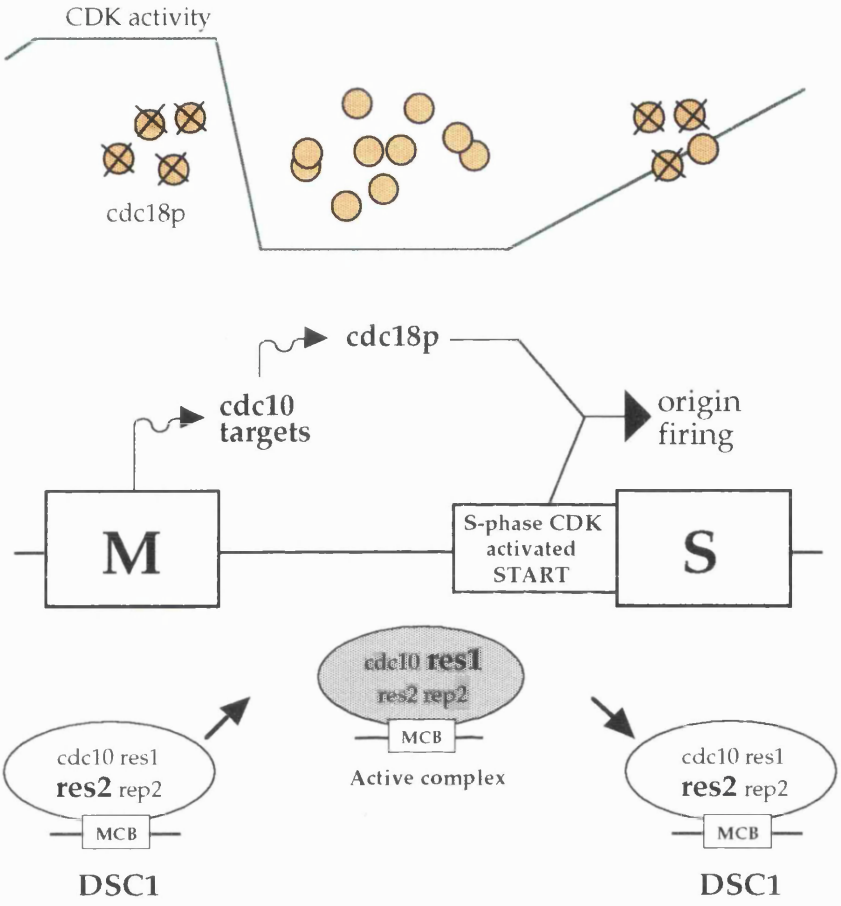


Figure 7.2.

Model for the control of *cdc10* dependent transcription and over the stability of cdc18p through the cell cycle.

cdc10 dependent transcription becomes active in metaphase. Although *cdc18* is expressed in metaphase, cdc18p cannot accumulate until cells leave mitosis. cdc18p may act, as soon as cells enter G1, to prepare cells for the subsequent S-phase. When cells in G1 have reached a sufficient size to pass START, B-type cyclins will re-accumulate. cdc2p/cyclin B complexes can then, in the presence of *cdc10* targets, initiate S-phase. This simultaneously destabilises cdc18p, preventing origin re-firing. During S-phase *cdc10* dependent transcription is switched off, possibly by passsage of the replication fork itself. It is possible that cdc10p, res1p, res2p and rep2p are present in large complexes bound to MCB elements in target genes throughout the cell cycle. The periodicity of transcription may be brought about by a switch between the active and inactive complexes. Res1p may be the critical activator component and res2p may inactivate the complex in G2 (bold print shows the dominant factor). These changes in activity may be brought about by alterations in the stoichiometry of res1p and res2p in the complex or by post-translational modification of components with passage through the cell cycle.

CHAPTER 8

Materials and Methods

Yeast physiology and genetics

Nomenclature

The genetic nomenclature of *Schizosaccharomyces pombe* is reviewed in (Kohli and Nurse, 1995). Fission yeast gene names consist of three italicized lower-case letters and a number, e.g. *cdc2*. Mutant alleles of a gene are denoted by a hyphen and a number, or a combination of letters and numbers, e.g. *cdc2-M26*. Alleles conferring temperature-sensitivity can be marked with a superscript, e.g. *cdc2-M26*^{ts}. The wild type allele can be designated with a superscript plus when the context requires specification of the wildtype allele. Phenotypes are denoted by a non-italicized 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. *res1Δ*. Integrated DNA is indicated with "::". A gene deletion marked with an integrated auxotrophic marker is indicated with "Δ::marker", e.g. *res1Δ::ura4*. Gene products are denoted by the lower-case, non-italicized gene name followed by "p" for protein, e.g. cdc2p. For truncated and mutated gene products the protein name is followed by a non-italicized tag, for example, cdc18p-d55. Budding yeast genes are designated with three italicized upper-case letters and a number, e.g. *CDC28*. Recessive mutant alleles are in lower case. Budding yeast gene products are denoted by the non-italicized gene name with the first letter in upper case and a "p" at the end, e.g. Cdc28p. The nomenclature used for mammalian genes and gene products are identical to those used for budding yeast.

Strain growth and maintenance.

Techniques used to grow and maintain fission yeast strains, to store and revive frozen cultures, to perform and analyse crosses by tetrad or random spore analysis, were performed exactly as previously described (MacNeill and Fantes, 1993; Moreno *et al.*, 1991) and will not be included.

Cell transformation.

All transformations were carried out by electroporation, using a Flowgen "Cellject" at 1500V, 40μF, 132Ω., as described previously (Prentice, 1992).

Induction of gene expression from the *nmt* promoter

The *nmt* promoter is derived from the *nmt1* gene and is repressed by the presence of exogenous thiamine (Maundrell, 1990). It exhibits a high basal level of expression in the presence of thiamine. Mutations in the TATA box were used to develop modified *nmt* promoters with intermediate or low levels of expression (Basi *et al.*, 1993; Forsburg, 1993). These mutations affect the activity of the promoter in both its repressed and its induced, active state. The Rep1 and Rep3 vectors contain the wildtype, strong *nmt1* promoter. The mutated promoters present in Rep41, 44 and 45 vectors, drive an intermediate level of expression, and in Rep81, low level expression. To achieve ectopic expression, cultures were grown in EMM containing thiamine (5μg/ml) to exponential phase ($O.D._{595}$ 0.1-0.5). Cells were washed three times in Eppendorf tubes with medium lacking thiamine and subsequently grown in the absence of thiamine for 4-5 generations (MacNeill and Fantes, 1993).

Physiological experiments with *S. pombe*: starvation, drugs and pheromone.

Strains were grown in complete media (YES) and minimal media (EMM) as previously described (Moreno *et al.*, 1991).

For nitrogen starvation experiments, cells were washed four times and resuspended in minimal-N_2 media and re-fed by the addition of 5g/l NH_4Cl.

P-factor was synthesized by a solid phase method using an automated synthesizer. P-factor was used at a concentration of 1.5μg/ml in liquid culture. Only *h⁻cyr1Δsxa2Δ* mutant cells will respond to P-factor.

Hydroxyurea (HU) was used at a concentration of 11mM to arrest cells at the onset of S-phase.
Cyclohexamide was used at 100μg/ml.

Thiabendazole (TBZ) was used, in DMSO, at a concentration of 150 µg/ml to depolymerise microtubules. It is not possible to arrest *S. pombe* in mitosis using agents which depolymerise microtubules alone.

Physiological experiments with *S. cerevisiae.*

Cells were grown in YPD medium (1% yeast extract, 2% bactopeptone, 2% glucose) accept in experiments in which strains carried the triple MluI plasmid. In this case cells were grown in synthetic media supplimented with uracil, adenine, leucine, tryptophan to a concentration of 20mg/ml to maintain selection for histidine.

Cells were stored in 15% v/v of glycerol at -70°C

To arrest cells in G1, α-factor (Sigma) was added to Bar+ a-type strains, for 150 minutes at 25°C, at a concentration of 5µg/ml. Cell cycle arrest was monitored by FACS analysis and by observating shmooing.

Nocodazole (noc.) was used at 15µg/ml, in DMSO.

0.1M HU was used to arrest cells early in S-phase.

Fission yeast strains.

Table 8.I. *Schizosaccharomyces pombe* strains were constructed for this study by random spore analysis unless stated otherwise.

h- sct1-1 cdc10^{+} ura4-D18 (constructed by tetrad analysis, from the sct1-1 cdc10Δ strain).
h- res2Δ::ura4^{+} / rep2Δ::ura4^{+} ura4-D18 (constructed by tetrad analysis).
h- cdc13Δ::ura4^{+} cig1Δ::ura4^{+} cig2Δ::ura4^{+} cdc10-V50 pREP41 cdc13::LEU2
h- cdc25-22 rep1Δ::ura4^{+}
h- nda3-km311 cdc10-129
h- nda3-km311 cdc10-V50
h- nda3-km311 cdc2-L7
h- nda3-km311 cdc2-33
h- cdc10-129 cdc13Δ90::sup3-5
h- res1Δ::ura4^{+} cdc13Δ90::sup3-5
h- res2Δ::ura4^{+} cdc13Δ90::sup3-5
h- cdc2-33 res2Δ::ura4^{+}

h- cdc25 res2Δ::ura4$^+$
h- cdc25 pRep41 nimA::ade6-704

Budding yeast strains.

Table 8.I. *Sacchtaromyces cerevisiae* strains used in this study:

These mutant strains were isolated in the original cdc screen conducted by Lee Hartwell.
cdc14-1
cdc15-1
cdc15-2
cdc16-1
cdc20-1
cdc23-1
wildtype 303

Flow cytometric analysis

10^{6}-10^{7} cells were fixed in 70% ethanol, washed in 3ml 50mM $Na_3Citrate$, resuspended in 1ml 50mM $Na_3Citrate$, 0.1mg RNAase, 2µg/ml propidium iodide, and incubated for 2h at 37°C. Cells were then sonicated for 60 seconds at setting 6 (Soniprep 150 sonicator, MSE), and the previously published protocol for flow cytometry followed (Sazer and Sherwood, 1990) using a Becton-Dickinson FACScan.

Cultures synchronised by elutriation

Elutriation in all cases was carried out using a Beckman J6 centrifuge and elutriator rotor. In the case of mutants grown at 30°C, the elutriator was pre-warmed at this temperature. *wee1-50* cells were grown at 25°C, loaded at this temperature and then eluted into media prewarmed at 36°C. Synchrony was evaluated by FACS analysis, cell number determination and the percentage of septated cells.

Cell number determination

Samples were fixed, in duplicate, by adding 400µl of culture to 1.6mls formal saline (0.9% saline, 3.7% formaldehyde) and sonicated as above. Cells were counted on a Sysmex Microcellcounter F-800 on the White Blood Cell channel.

Molecular biological techniques

General techniques

The following procedures were used as described in (Maniatis et al., 1982): preparation of competent bacteria, transformation of bacteria with DNA, restriction enzyme digests of DNA, gel electrophoresis of DNA, filling in of recessed 3' ends using Klenow, phosphatase treatment of vector DNA, ligation of DNA fragments, minipreps.

Western Blot analysis

Cells boiled for 6 minutes prior to storage at -70°C. Following glass bead lysis in HB buffer (Moreno, et al., 1991), protein concentration was determined and cell extracts were then re-boiled in 5x sample buffer. 50µg of protein from each sample was run on an 8% SDS-polyacrylamide gel (Laemmli, 1970). For Western Blots, the protein was blotted to Immobilon membrane (Millipore) and detected using ECL (Amersham).

Antibodies used	**Dilution for Western**
cdc18 polyclonal (H. Nishitani).	1:1500
α-tubulin monoclonal antibody (Sigma T5168).	1:50000
cdc2 polyclonal (C2, lab.).	1:1000
cdc13 polyclonal (SP4, lab.).	1:1000
cig2 (M. O'Connell and J. Correa-Bordes).	1:1000
rum1 polyclonal (J. Correa-Bordes).	1:1000
cdc10 polyclonal (J. Wuarin)	1:1000

H1 kinase assays

Extracts were prepared from 10^8 frozen cells using glass bead lysis in HB buffer (Moreno, et al., 1991) with the following protease inhibitors: Aprotinin 20µg/ml; Benzamidin 1mM; Pepstatin 10µg/ml; Leupeptin 20µg/ml; TLCK 50µg/ml; TPCK 50µg/ml; Pepstatin 1.8µg/ml; PMSF 1mM. 5µl of rabbit polyclonal cdc2p antisera, C2, (or preimmune serum), or 5µl of polyclonal cdc13p antisera, SP4, was added to 500µg of protein and incubated on ice for 45 minutes. Pre-equilibrated protein A Sepharose beads (Pharmacia Biotech.) were added and the mixture agitated for 30 minutes at

4°C. Beads were washed 3 times and then resuspended in 15µl of reaction buffer, containing 1µg/µl Calf Thymus Histone H1 (Sigma No. 382150), 200µM ATP and 40µCi/ml $\gamma(^{32}P)$ATP (Amersham). Extracts were then incubated at 30°C for 20 minutes, stopped by boiling for 5 minutes after the addition of 5X SDS sample buffer and run on a 12% SDS polyacrylamide gel (Laemmli, 1970). In experiments using *cdc2^{ts}* alleles, immunoprecipitates from control and *cdc2^{ts}* extracts were pre-incubated at 36.5°C for 5 minutes prior to the addition of reaction buffer (also at 36.5°C) and incubated for a further 20 minutes. Pre-immune sera gave no signal.

Bandshift analysis

PCR primers:
GCTCTAGACATTACCTGAAT
CGCGGAGCTCGTTTAAGAGCAGA
A dsDNA probe made from the *cdc18* promoter containing both MCB repeats was amplified by PCR (Zhu *et al.*, 1994), labelled by T4 polynucleotide kinase (BioLabs) with $\alpha(^{32}P)$dATP (Amersham) and gel purified on a 4% polyacrylamide gel. Glass bead lysis was carried out in 25mM Hepes pH 7.6, 0.1mM EDTA, 150mM KCl, 0.1% TritonX-100, 25% glycerol and 1M urea in the presence of 1mM DTT and protease inhibitors. 20µg or 40µg of soluble cell extract was pre-incubated for 10 minutes in gel shift buffer (25mM Hepes pH 7.6, 34mM KCl, 5mM $MgCl_2$) with 0.1µg/µl poly dI-dC and sonicated salmon sperm DNA, prior to the addition of excess radiolabelled probe. The gel shift reaction was incubated for a further 15 minutes at room temperature, then run on a native 4% acrylamide in 1xTBE for 3 hours. DSC1 disappears upon the addition of cold specific competitor DNA.

RNA preparation and Northern Blot analysis

Both *S. cerevisiae and S. pombe* cells were treated similarly.

Cultures were washed in STOP buffer (150mM NaCl, 50 mM NaF, 10 mM EDTA, 1mM NaN_3 pH 8), frozen on dry ice and then kept at -70°C. Subsequently, RNA was prepared using glass bead lysis, (Sigma No. G9268) in 0.1M EDTA, 0.1M NaCl, 0.05M Tris pH 8.0, in the presence of 1:1 v/v phenol:chloroform:Isoamyl alcohol (GIBCO-BRL) and 0.4% SDS. RNA was precipitated after 2 phenol:$CHCl_3$ extractions by the addition of NH_4OAc to 2.5M and 2.5 vol. EtOH. 10µg of sample RNA, as measured by $O.D._{260/280}$

was denatured in 1XMOPS, 8% formaldehyde and 67% de-ionized formamide, and run on a formaldehyde, 1.2% agarose gel in 1XMOPS. The RNA was transferred by Northern blotting in 10XSSC onto a GeneScreenPlus membrane (DuPont). Probes for blotting were prepared by random oligo priming with $\alpha(^{32}P)$dATP using a Prime-It Kit (Stratagene). The membrane was hybridised overnight in 1% SDS, 10% Dextran sulphate and 1M NaCl, and washed in 1%SDS, 2XSSC.

The following template DNAs were used to make probes for Northern analysis:

cdc18^{+} fragment from a REP1-*cdc18* cDNA plasmid (H. Nishitani).
cig2 fragment from a genomic *cig2* clone in pAL-SK (S. Moreno).
Histone *h2B*, fragment from pSJM211 (Matsumoto and Yanagida, 1985).
cdt1 fragment from PCR clone (H. Nishitani).
cdc22 a fragment was amplified by PCR from genomic DNA (primers from N. Lowndes).
res2 fragment from (N. Jones).
rep2 (Y. Watanabe).
rep1 (Y. Watanabe).
ura4 fragment from Rep4.
his3 fragment from a pKS-*his3* plasmid (lab.).
cdc2 from KS (J. Correa-Bordes).

Probes for *S. cerevisiae* genes were made by PCR amplification, from a preparation of genomic DNA, using standard PCR techniques.

The primers used were as follows:

CLB5	CCTTTAAGCCAGAACAAGAG
CLB5-	CAGAGCCATACCTTGGTTTC
ACT1	ATCCATTGGACCGTGTATC
ACT1-	GTTGCCCTCACGAGTGAAAG
HO	AACACGACTATTCTGATGGC
HO-	CACCTGCGTTGTTACCACAAC
SIC1	CGAAAATGACTCCTTCCACCC
SIC1-	CTTCAATGCTCTTGATCCC
CDC46	ACCGGAAATATACAGTGCTCC
CDC46-	CTTCGGGTTTCTACTTAGC

CDC9	TTGCCTTCTTTCATCTGCAC
CDC9-	CGCCATTTCTGGAATAAATCC
CLN2	ATGGCTAGTGCTGAACCAAG
CLN2-	TCTCGTCTACAGTGGCATCA
CDC6	ACCAATAACTCCAACTAAGCG
CDC6-	GCAGTGTATGGCTGAAAAACT

Nuclear Run-On

The procedure used is based on that previously described (Maundrell, 1990). $2x10^8$ cells were filtered and washed with 5mls of ice cold TMN buffer (10mM Tris/HCl; 5mM $MgCl_2$; 100mM NaCl). Cells were resuspended in TMN and allowed to equlibriate for 10' on ice. The TMN was then removed, cells were resuspended in 950µl cold H_2O, 50µl of 10% sarcosyl was added and cells were incubated on ice for an additional 20'. The detergent was removed and permeabilized cells were resuspended in 120ul of 'run-on' buffer (50mM TrisHCl pH 7.9; 80mM $MgCl_2$; 500mM KCl; 1mM DTT; 1mM rATP, 0.5mM rGTP and rCTP; 100 units of RNAase inhibitor (Sigma) and 100µCi rUTP-α^{32}P). The run on was carried out at 30°C for 10'. Cells were then washed once in TMN buffer and immediately broken to isolate labelled RNA as for a Northern. Isolated RNA was precipitated in the presence of 200µg tRNA and washed in 70% EtOH. The RNA was dissolved in 100µl 1xTE and denatured for 3' at 95°C, then added to the prehybridised membrane in 3mls of hybridisation buffer and incubated at 65°C for 2 days. The pre-hybridisation buffer and the hybridisation buffer identical and described in (Humphrey *et al.*, 1994).

The nylon membrane was soaked in 1xSSC and 5µg of ssDNA added in 400µl of 1xSSC and absorbed onto the membrane using a slot blot, and crosslinked using the STRATAGENE stratalinker.

Preparation of single stranded DNA

The procedure described in (Maniatis *et al.*, 1982) was followed. A gene of interest was inserted in both orientations into KS. This vector contains the M13 origin for the synthesis of ssDNA. XL1-Blue *E. coli* carrying the F' factor, maintained by the additon of tetracyclin, were transformed with KS. Cells were infected with M13KO7 bacteriophage, selected with Kanamycin, and single stranded DNA isolated and purified using the Qiagen M13 Midi-prep kit.

ssDNA probes used in nuclear run on experiments.
cdc18 KS+ and *cdc18* SK+
ura4 KS+ and *ura4* KS-
cdc2 KS+ *cdc2* KS-
KS+ and KS- were used to assess the background signal.

Visualisation of nuclei by DAPI staining

Both *S. cerevisiae* and *S. pombe* cells were treated similarly.
Cells fixed in 70% ethanol were rehydrated in water, heat fixed to a slide and mounted in 1µg/ml DAPI, 1mg/ml para-phenylenediamine, in 50% glycerol.

α–tubulin immuno-fluorescence.

The procedure used is adapted from (Moreno, et al., 1991). 10^8 cells were harvested by filtration and 20ml of methanol at -20°C was added to fix cells. Cells were resuspended in PEMS at a cell density of 5 x 10^7/ml. The cell wall was digested by the addition of Zymolyase, to a final concentration of equivalent to 0.5 mg ml^{-1} 100T. Digestion was followed by phase contrast microscopy. After cell wall digestion, cells were pelletted, resuspended in 1% Triton in PEMS for 30 seconds and then washed 3 times in PEM. Finally cells were resuspended in PEMBAL (Chappell and Warren, 1990) and incubated with TAT1 antibody at 1:10 dilution (donated by Keith Gull) overnight at room temperature. TAT1 is a mouse monoclonal antibody (raised against Trypanosome cytoskeletal preparations, (Woods *et al.*, 1989)). The primary antibody was washed out three times with PEMBAL before the secondary antibody was applied. If cells were resuspended in PBS + 0.2 µg/ml DAPI and washed one more time in PBS.

PEM	100mM Pipes, 1mM EGTA, 1mM $MgSO_4$, pH 6.9
PEMS	PEM + 1.2 M Sorbitol
PEMBAL	PEM + 1% BSA (essentially FA + globulin free, Sigma) 0.1% NaN_3 100 mM lysine hydrochloride.

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Control of S-phase periodic transcription in the fission yeast mitotic cycle

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In fission yeast, passage through START and into S-phase requires cyclin-dependent kinase (CDK) activity and the periodic transcription of genes essential for S-phase ('S-phase transcription'). Here we investigate the control of this transcription in the mitotic cell cycle. We demonstrate that the periodicity of S-phase transcription is likely to be controlled independently of CDK activity. This contrasts with the equivalent system in budding yeast. Furthermore, the CDK function required for S-phase acts after the onset of S-phase transcription and after the accumulation of cdc18p, a critical target of this transcriptional machinery. We investigate the role of individual components of the S-phase transcriptional machinery, cdc10p, res1p, res2p and rep2p, and define a new role for res2p, previously demonstrated to be important in the meiotic cycle, in switching off S-phase transcription during G2 of the mitotic cycle. We show that the presence of the *in vitro* bandshift activity DSC1, conventionally thought to represent the active complex, requires res2p and correlates with inactive transcription. We suggest that S-phase transcription is controlled by both activation and repression, and that res2p represses transcription in G2 of the cell cycle as a part of the DSC1 complex.
Keywords: cdc10/CDK/cell cycle/fission yeast/periodic transcription

Introduction

The periodic expression of genes required for S-phase is a common feature of cell cycle regulation in eukaryotes. It has been studied most thoroughly in the budding yeast *Saccharomyces cerevisiae* (Johnston, 1992; Koch and Nasmyth, 1994) and in mammalian cells (Martin *et al.*, 1995; Slansky and Farnham, 1996). In budding yeast, periodic transcription of S-phase genes is mediated by two transcriptional complexes, SWI4–SWI6 and SWI6–MBP1, which act through conserved promoter elements known as SCB and MCB sites respectively (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989; Lowndes *et al.*, 1991; Taba *et al.*, 1991; Dirick *et al.*, 1992; Koch *et al.*, 1993; for a recent review, see Breeden, 1996). A DNA-binding activity containing SWI6p and MBP1p, termed DSC1, that recognizes MCB elements, is thought to be involved in transcriptional activation (Verma *et al.*, 1991; Dirick *et al.*, 1992; Lowndes *et al.*, 1992a; Koch *et al.*, 1993). Periodic expression in budding yeast is controlled primarily by oscillations in cyclin-dependent kinase (CDK) activity through the cell cycle, although it is not fully understood how CDKs interact with the transcriptional complexes to regulate transcription. Transcription of genes containing SCB or MCB elements is activated in late G_1 by the CDK, CDC28–CLN3 (Tyers *et al.*, 1993; Dirick *et al.*, 1995). Transcription from SCB elements is then switched off in G_2 cells by the CDK activity of CDC28–CLB complexes (Koch *et al.*, 1996). An analogous control in mammalian cells involves the E2F–DP1 transcriptional complex which, although unrelated to SWI4/SWI6–MBP1, has a similar role. This is also regulated by CDKs, being activated by CDK4/6–cyclin D (reviewed in Martin *et al.*, 1995; Slansky and Farnham, 1996) and CDK2–cyclin E, and inactivated by CDK2–cyclin A activity in S-phase and G_2 (Xu *et al.*, 1994; Krek *et al.*, 1994).

In fission yeast, the S-phase transcriptional machinery is composed of cdc10p, res1p, res2p and rep2p proteins (Aves *et al.*, 1985; Lowndes *et al.*, 1992b; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Nakashima *et al.*, 1995) and operates through MCB promoter elements. Target genes for the machinery include *cdc18*, *cdc22*, *cdt1* and possibly *cig2* (Gordon and Fantes; 1986; Kelly *et al.*, 1993; Hofmann and Beach; 1994; Obara-Ishihara and Okayama, 1994). DSC1, an endogenous bandshift activity found in fission yeast cell extracts (Lowndes *et al.*, 1992b), binds MCB sites and contains cdc10p and res1p (Lowndes *et al.*, 1992b; Caligiuri and Beach, 1993). Res2p also interacts with cdc10p to form an alternative complex which can bind specifically to MCB elements *in vitro* (Zhu *et al.*, 1994). Genetic analysis suggests that cdc10p/res1p is the major transcriptional regulator during the mitotic cell cycle (Tanaka *et al.*, 1992), and cdc10p/res2p during the meiotic cell cycle (Miyamoto *et al.*, 1994). The mechanism controlling periodic transcription is not understood, but it has been suggested that DSC1 plays a role in transcriptional activation, and that the formation of DSC1 and the onset of *cdc10*-dependent transcription are triggered by G_1 cdc2p activity (Reymond *et al.*, 1993). In addition, a role has been proposed for cdc10p in repression of transcription because a truncation of cdc10p causes elevated levels of transcription throughout the cell cycle (McInerny *et al.*, 1995). The level of cdc10p throughout the cell cycle is constant, indicating that oscillations in cdc10p do not control the periodicity of transcription (Simanis and Nurse, 1989).

Here we further investigate the mechanism controlling periodic S-phase transcription during the mitotic cell cycle in the fission yeast *Schizosaccharomyces pombe*. We show that CDK activity does not appear to play a role in the

regulation of S-phase transcription, that cdc10p and res1p are required for transcriptional activation and res2p for the G_2 repression of this transcription, whilst rep2p is important for maintaining the level of transcription. Finally, we demonstrate that the presence of DSC1 correlates with repression of transcription during the G_2-phase of the cell cycle.

Results

cdc2p does not appear to activate cdc18 transcription prior to S-phase

We first investigated whether cdc2p is required in G_1 for the activation of *cdc10*-dependent S-phase transcription. *cdc10*-dependent transcription was monitored by assessing the transcript levels of a target gene, *cdc18*, in cells proceeding towards S-phase in the presence and absence of *cdc2* function. This was done using the temperature-sensitive alleles of *cdc2* most severely compromised for progression through G_1, *cdc2-M26* and *cdc2-33* (Broek *et al.*, 1991; MacNeill *et al.*, 1991). Similar results were obtained with both alleles and data is shown for *cdc2-M26*.

In the first experiment (Figure 1), temperature-sensitive *cdc2-M26* cells were synchronized in G_1 by nitrogen starvation at 25°C. On re-feeding with nitrogen, S-phase began within 3 h at 25°C but did not take place at the restrictive temperature of 36.5°C. The *cdc18* transcript level was low in nitrogen-starved cells, but began to increase 1.5 h after the addition of nitrogen at 25°C, that is ~1 h before the onset of S-phase (Figure 1A). This suggests that *cdc18* transcription is activated in small G_1 cells some time before they reach the critical size required for the onset of S-phase (Nurse, 1975; Nurse and Thuriaux, 1977). A similar increase in *cdc18* transcript level was observed at 36.5°C (Figure 1A), although cells were unable to enter S-phase at this temperature because they lacked *cdc2* function (see Figure 1C). Similar results were obtained using the other targets of *cdc10*, *cdc22* and *cdt1* (data not shown).

In order to assess whether residual *cdc2* kinase activity was likely to be present in these cells, the H1 histone kinase levels associated with cdc2p were determined in cdc2p immunoprecipitates from extracts of wild-type and *cdc2-M26* cells released from nitrogen starvation at 36.5°C. As demonstrated above, *cdc18* transcript accumulated in the *cdc2-M26* strain at 36.5°C, but cells failed to enter S-phase (Figure 1C and D). The H1 kinase assays were carried out at 36.5°C (Figure 1B) and quantified by phosphorimager analysis. In the *cdc2-M26* strain, H1 kinase activity at the time when *cdc18* transcript began to accumulate was only 0.13% of that seen in the wild-type strain. These data led us to conclude that cells released from nitrogen starvation in G_1 can activate *cdc10*-dependent transcription without significant cdc2p activity, although they require *cdc2* function for entry into S-phase.

In a second experiment, we studied the *cdc2* requirement for the activation of *cdc10*-dependent transcription in *cdc2-33* cells re-entering S-phase from G_2 (Figure 2). *cdc10* function is required for re-replicative DNA synthesis (Moreno and Nurse, 1994). Cells were arrested in G_2 at the restrictive temperature for *cdc2-33*, in the absence of nitrogen, and then subjected to a brief heat treatment at 49°C (Broek *et al.*, 1991). This procedure inactivates the

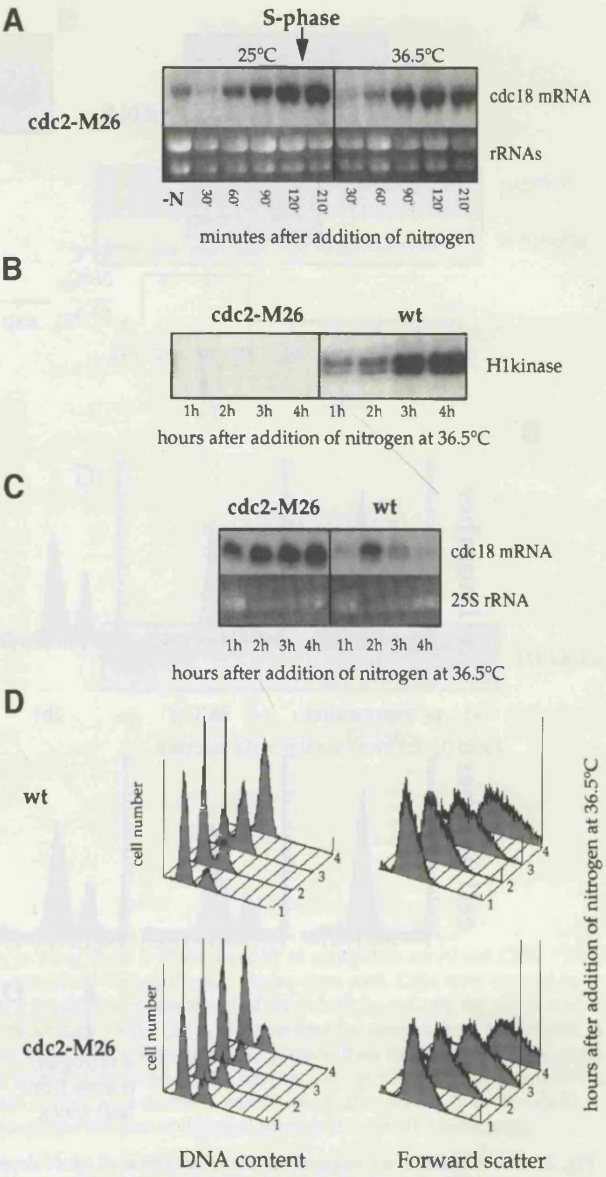

Fig. 1. *cdc2* function is not required for the activation of *cdc10*-dependent transcription following release from nitrogen starvation. *cdc2-M26* cells were arrested in G_1 by nitrogen starvation and re-fed at 25 or 36.5°C. (**A**) A Northern blot was probed for *cdc18*; rRNA was visualized in the gel using EtBr to control for sample loading. At 25°C, cells entered S-phase after 3 h (arrow), whereas at the restrictive temperature cells remained in G1. (**B**) Wild-type and *cdc2-M26* cells in G_1 were re-fed with nitrogen at 36.5°C. H1 kinase assays were carried out at 36.5°C using cdc2p immunoprecipitates from wild-type and *cdc2-M26* cells. (**C**) A Northern blot is shown of samples taken from the experiment in (B), the blot was probed for *cdc18*, and EtBr used to visualize rRNA. (**D**) Samples from (B) were taken for FACS analysis. Forward scatter (which represents cell mass) and DNA content are shown on separate histograms.

G_2 form of cdc2p and thereby allows cells to undergo an additional round of DNA synthesis. At the permissive temperature, 28.5°C, cells underwent an additional round of S-phase, 4–5 h after the re-addition of nitrogen (Figure 2B). However, when cells were incubated at 36.5°C, with functionally inactive *cdc2*, they failed to re-replicate their DNA. After the heat treatment, the level of *cdc18* mRNA was very low, but it increased to a peak level sufficient to bring about S-phase, 4 h after the addition of nitrogen at the permissive temperature. A similar increase in the

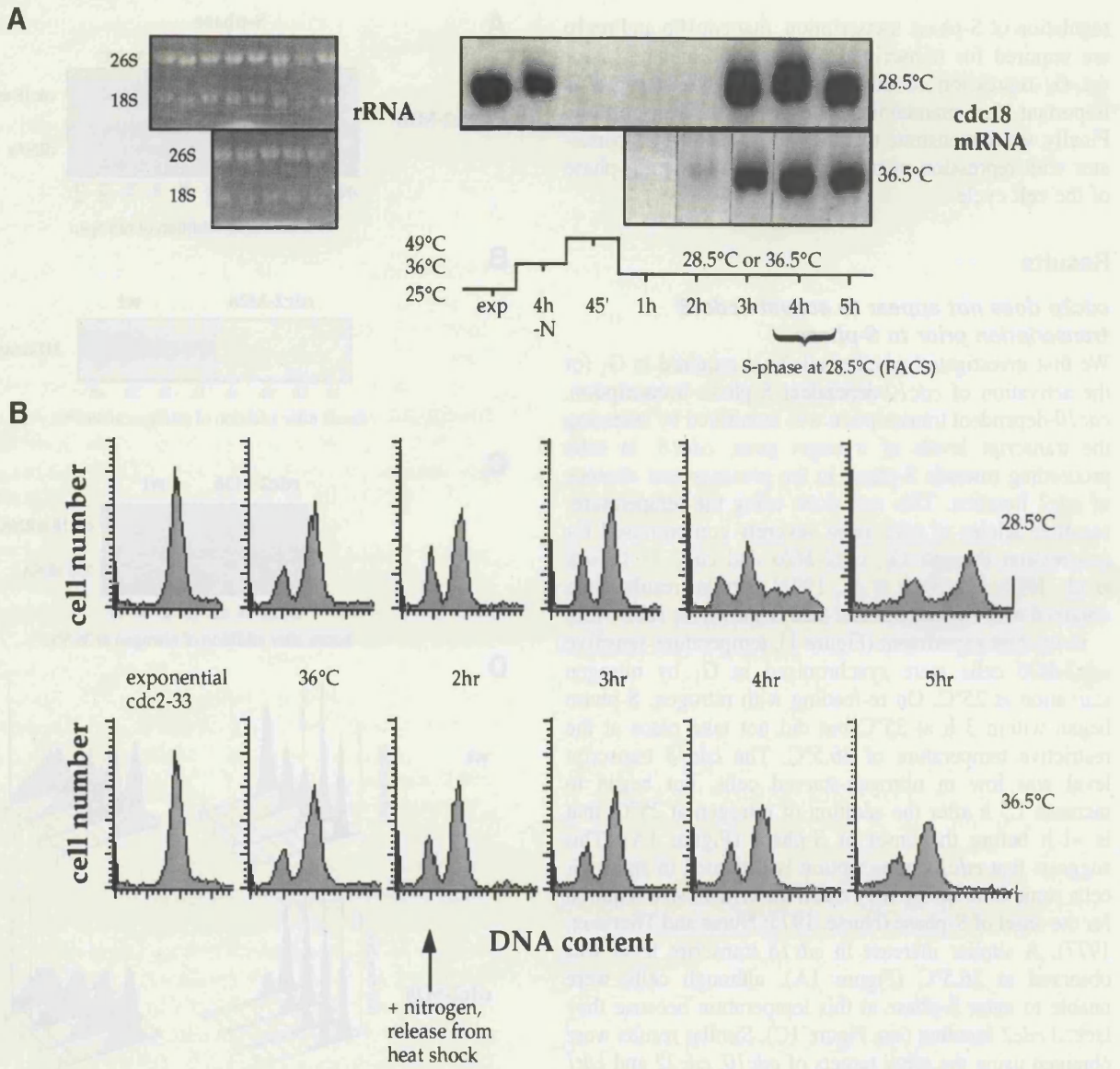

Fig. 2. *cdc2* function is not required for the re-activation of *cdc10*-dependent transcription following re-entry into S-phase from G_2. *cdc2-33* cells were arrested at G_2–M, subjected to a heat shock to induce them to re-enter S-phase and re-fed at the permissive or restrictive temperature for *cdc2-33* (28.5°C or 36.5°C). (**A**) The Northern blot was probed for *cdc18* mRNA; rRNA is shown as a loading control; exp denotes exponentially growing cells. (**B**) Fixed samples were analysed by FACS analysis to determine the timing of S-phase as cells with a 2C DNA content re-replicate their DNA, resulting in a 4C peak.

level of *cdc18* transcript was seen at 28.5 and 36.5°C, i.e. in both the presence and absence of *cdc2* function (Figure 2A). Similar results were obtained using the strain *cdc2-M26* (data not shown).

We conclude that *cdc10*-dependent transcription is activated in G_1 cells, and re-activated in cells re-entering S-phase from G_2, in the absence of significant *cdc2* function. We cannot rule out the possibility that although cells in these experiments were unable to enter S-phase at the restrictive temperature, sufficient residual cdc2p activity remained to activate *cdc10*-dependent transcription. However, this seems unlikely given the low levels of *in vitro* H1 kinase activity detectable in the $cdc2^{ts}$ strains at the restrictive temperature.

cig1, cig2 and cdc13 are required after cdc10 function to bring about the onset of S-phase

Three B-type cyclins act together with cdc2p in G_1 of the mitotic cycle to bring about S-phase (Fisher and Nurse, 1996). To determine whether these B-type cyclins are required downstream of *cdc10* function in G_1, we used a strain containing the temperature-sensitive *cdc10-V50* mutation in which two of the cdc2p cyclin partners, cig1p and cig2p, were deleted, with the remaining partner, cdc13p, placed under control of the thiamine-repressible promoter (Fisher and Nurse, 1996). This allows us to manipulate *cdc10*-dependent transcription and CDK activity independently. As a control strain, *cdc13::nmt cdc10-V50* $cig1^+cig2^+$ was used. Cells were shifted to the restrictive temperature for 3 h to arrest the majority of cells in G_1, and then *cdc13* was switched off by the addition of thiamine. After a further 1 h at 36°C, cells were shifted back to 25°C, re-activating *cdc10* function in the absence of the B-type cyclins needed to drive entry into S-phase (Figure 3C). Under these conditions, as a result of the instability of cdc13p in early G_1 cells (Hayles *et al.*, 1994), cdc13p levels were reduced to 2% of the level seen in the exponential population (data not shown).

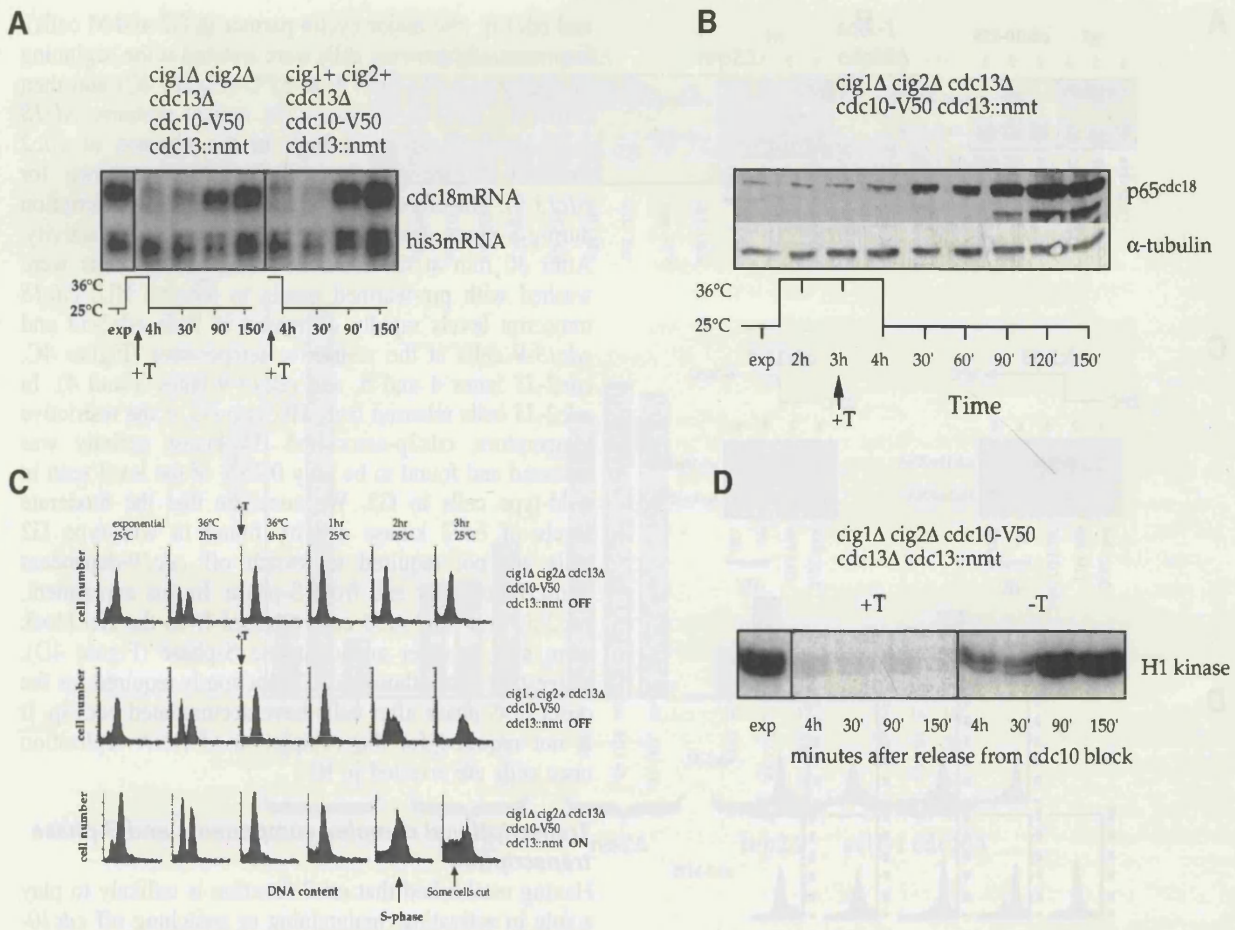

Fig. 3. *cig1*, *cig2* and *cdc13* functions are required after *cdc10* function in G_1 to bring about S-phase. In order to manipulate *cdc10* and CDK activity independently in G1, *cdc13::nmt cdc10-V50 cig1Δ cig2Δ* and *cdc13::nmt cdc10-V50 cig1*$^+$*cig2*$^+$ strains were used. Cells were arrested in G1 by incubation at 36°C, the non-permissive temperature for *cdc10-V50*, for 3 h. *cdc13* was then switched off in half the cells by the addition of thiamine. After an additional hour at 36°C, cells were shifted back to 25°C, reactivating *cdc10*. (**A**) A Northern blot for samples from both strains in the presence of thiamine was probed for *cdc18* mRNA; *his3* was probed as a control. (**B**) A Western blot of extracts from the strain lacking all three cyclins (*cdc13::nmt cdc10-V50 cig1Δ cig2Δ* in the presence of thiamine) was probed with antibodies for cdc18p, cdc13p (data not shown) and α-tubulin. (**C**) Fixed cells were taken for FACS analysis. Cells lacking all three cyclins were unable to enter S-phase after release from the *cdc10* block. (**D**) Extracts from *cdc13::nmt cdc10-V50 cig1Δ cig2Δ* cells in the presence and absence of thiamine were used in an H1 kinase assay.

The level of *cdc18* transcripts increased within 1.5 h of the release from the *cdc10* block, with similar kinetics and to a similar level in the presence or absence of G1 cyclins (Figure 3A). In this experiment, cdc18p levels were also monitored and found to be at a low in exponential cells (when most cells are in G2 of the cycle) and in G1 cells in the absence of the *cdc10* function. However, within 2 h of shift to 25°C (Figure 3B), cdc18p accumulated to a high level, exceeding that seen in *cig1*$^+$*cig2*$^+$ cells which enter S-phase (protein data not shown). These elevated levels of cdc18p were not sufficient to drive cells into S-phase in the absence of B-type cyclin partners for *cdc2* (Figure 3C).

Next we assessed the residual cdc2p kinase activity in the cyclin deletion strain upon release from the *cdc10* block, in the presence or absence of ectopic *cdc13* (Figure 3D). The level of cdc2p-associated H1 kinase activity in the absence of the three B-type cyclins was shown to be 1.5% of that in the control cells which express *cdc13* from the *nmt* promoter and enter S-phase (Figure 3C).

In conclusion, this experiment shows that the B-type cyclins, which are responsible for the vast majority of the measurable H1 kinase activity in G1 cells, act after the onset of *cdc10*-dependent transcription and after the accumulation of cdc18p, to bring about entry into S-phase. It is conceivable, nevertheless, that other cyclin partners for cdc2p are present in these cells which are unable to make a significant contribution to the overall H1 kinase activity, but can activate *cdc10*-dependent transcription.

cdc2 function is not required in S-phase or in G2 for the appropriate control of periodic cdc10-dependent transcription

The previous experiments suggest that *cdc2* function is not required for the onset of *cdc10*-dependent transcription. To investigate the control of *cdc10*-dependent transcription during S-phase and in G2 cells, we first asked whether *cdc10*-dependent transcription was active in cells arrested at the onset of S-phase by hydroxyurea (HU). Wild-type and *cdc10-129* cells were arrested with HU and then shifted to 36°C for 30 min to inactivate *cdc10* [HU prevented cells from entering S-phase, fluorescence-activated cell sorting (FACS) data not shown]. While both wild-type and *cdc10-129* cells that were arrested at the permissive temperature had elevated *cdc18* transcript levels (Figure 4A, lanes 2 and 5), cells at the restrictive

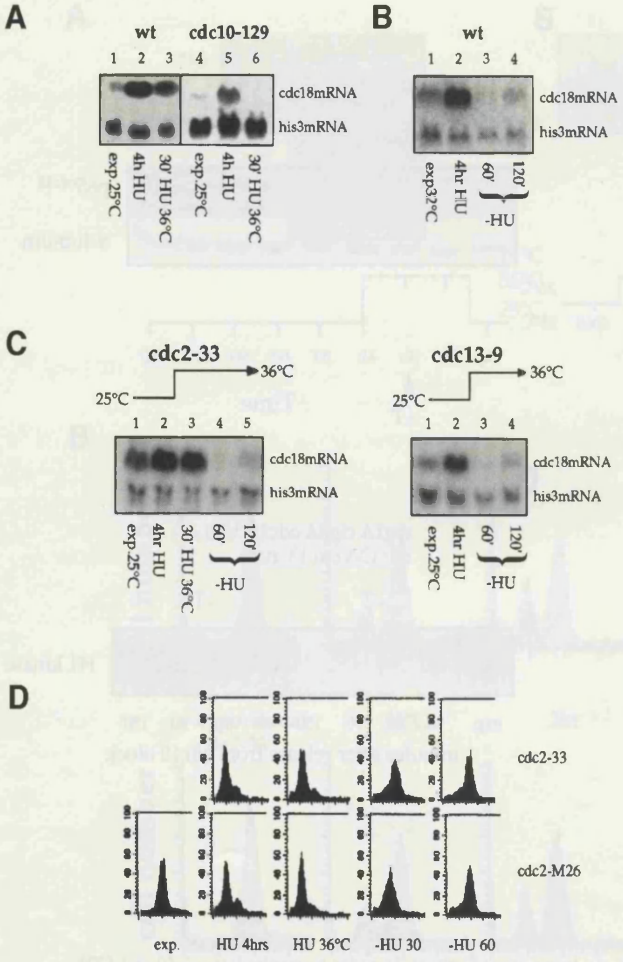

Fig. 4. *cdc2* function is not required for the maintenance of high levels of *cdc10*-dependent transcription in S-phase or for repression of this transcription in G2 cells. (**A**) Wild-type and *cdc10-129* cells were arrested at the onset of S-phase by the addition of 11 mM HU. Cells were then shifted to 36°C for 30 min. (**B**) Wild-type cells were arrested in HU for 4 h then washed, synchronously releasing cells into S-phase and G2. (**C**) *cdc2-33* and *cdc13-9* cells were arrested in HU for 4 h (lanes 1 and 2) and shifted to 36°C for 30 min to inactivate the temperature-sensitive functions (*cdc2-33*, lane 3; data not shown for *cdc13-9*). HU was then washed out at 36.5°C (final lanes) enabling cells to pass through S-phase and into G2 (confirmed by FACS analysis). Samples were taken for Northern analysis and probed for *cdc18* and *his3*. (**D**) *cdc2-33* and *cdc2-M26* strains were subjected to a HU block and release, like that carried out in (C), and samples taken for FACS analysis.

temperature for *cdc10-129* did not (Figure 4A, compare lanes 3 and 6). Thus, continued *cdc10* function is required to maintain *cdc18* transcript levels. In a second experiment (Figure 4B), *cdc18* mRNA levels were monitored in wild-type cells which were first arrested in HU and then washed free of HU, allowing them to pass through S-phase and into G2. As expected, *cdc18* mRNA levels rapidly decreased as cells passed through S-phase, remaining low in G2 cells.

To investigate whether cdc2p plays a role in maintaining *cdc10*-dependent transcription during S-phase, or in switching off this transcription at the end of S-phase, we monitored *cdc18* transcript levels, first in a HU block with and without *cdc2* function, and secondly after release from HU into mutant blocks lacking mitotic CDK activity. We utilized the CDK mutant strains *cdc2-33* and *cdc13-9*, which contain temperature-sensitive mutations in cdc2p and cdc13p (the major cyclin partner in G2 and M cells). Exponentially growing cells were arrested at the beginning of S-phase in HU for 4 h at 25°C (Figure 4C) and then shifted to 36°C to inactivate the mutant proteins. *cdc18* mRNA levels remained high in the absence of *cdc2* function (Figure 4C, lanes 1–3, data not shown for *cdc13-9*). Therefore, active *cdc10*-dependent transcription during S-phase does not require continued CDK activity. After 30 min at the restrictive temperature, cells were washed with pre-warmed media to remove HU; *cdc18* transcript levels rapidly decreased in both *cdc2-33* and *cdc13-9* cells at the restrictive temperature (Figure 4C, *cdc2-33* lanes 4 and 5, and *cdc13-9* lanes 3 and 4). In *cdc2-33* cells released from HU into G2 at the restrictive temperature, cdc2p-associated H1 kinase activity was assessed and found to be only 0.75% of the level seen in wild-type cells in G2. We conclude that the moderate levels of *cdc2* kinase activity found in wild-type G2 cells are not required to switch off *cdc10*-dependent transcription after exit from S-phase. In this experiment, *cdc2-33* and *cdc2-M26* cells released from the HU block were able to enter and complete S-phase (Figure 4D), suggesting that although *cdc2* function is required for the onset of S-phase after cells have accumulated cdc18p, it is not required for the completion of DNA replication once cells are arrested in HU.

Transcriptional complex components and S-phase transcription

Having established that *cdc2* function is unlikely to play a role in activating, maintaining or switching off *cdc10*-dependent transcription, we next assessed the role in periodic S-phase transcription of the components of the transcriptional complex itself. These studies used strains deleted for one of the genes, *cdc10*, *res1*, *res2* or *rep2*, that contribute to the function of the transcriptional complex (Aves *et al.*, 1985; Marks, 1992; Tanaka *et al.*, 1992; Caligiuri and Beach, 1993; Miyamoto *et al.*, 1994; Zhu *et al.*, 1994; Nakashima *et al.*, 1995). The *cdc10* deletion is inviable, so the double mutant *cdc10Δ sct1-1* (Marks *et al.*, 1992; Caligiuri and Beach, 1993) was used in which a point mutation in *res1* (*sct1-1*) suppresses the lethality of the *cdc10Δ*. *sct1-1 cdc10*$^{+}$, *res2Δrep2Δ* strains were also studied (Figure 5C).

Cells harbouring deletions of components of the transcriptional machinery were grown at 30°C, where they are viable, subjected to a HU-induced arrest followed by release, and analysed by Northern blotting to assess *cdc18* message levels. FACS analysis (data not shown) confirmed that, in all cases, at least 90% of cells were arrested with a G1 DNA content 4 h after the addition of HU to cultures, and that >95% of cells were in G2 an hour after the removal of HU.

During log-phase growth of *res1Δ* cells, levels of *cdc18* transcript were similar to the low levels seen in wild-type cells, exponentially growing *rep2Δ* and *cdc10Δ sct1-1* cells exhibited even lower *cdc18* transcript levels (Figure 5B, columns 1, 4, 10 and 13), whereas in the *res2Δ* strain, *cdc18* transcript levels were elevated (Figure 5B, column 7). After treatment with HU for 4 h, cells arrested in early S-phase. In the wild-type, *cdc18* transcript levels were elevated during S-phase. However, no significant elevation was seen in *res1Δ* or *cdc10Δ sct1-1* cells and only a small

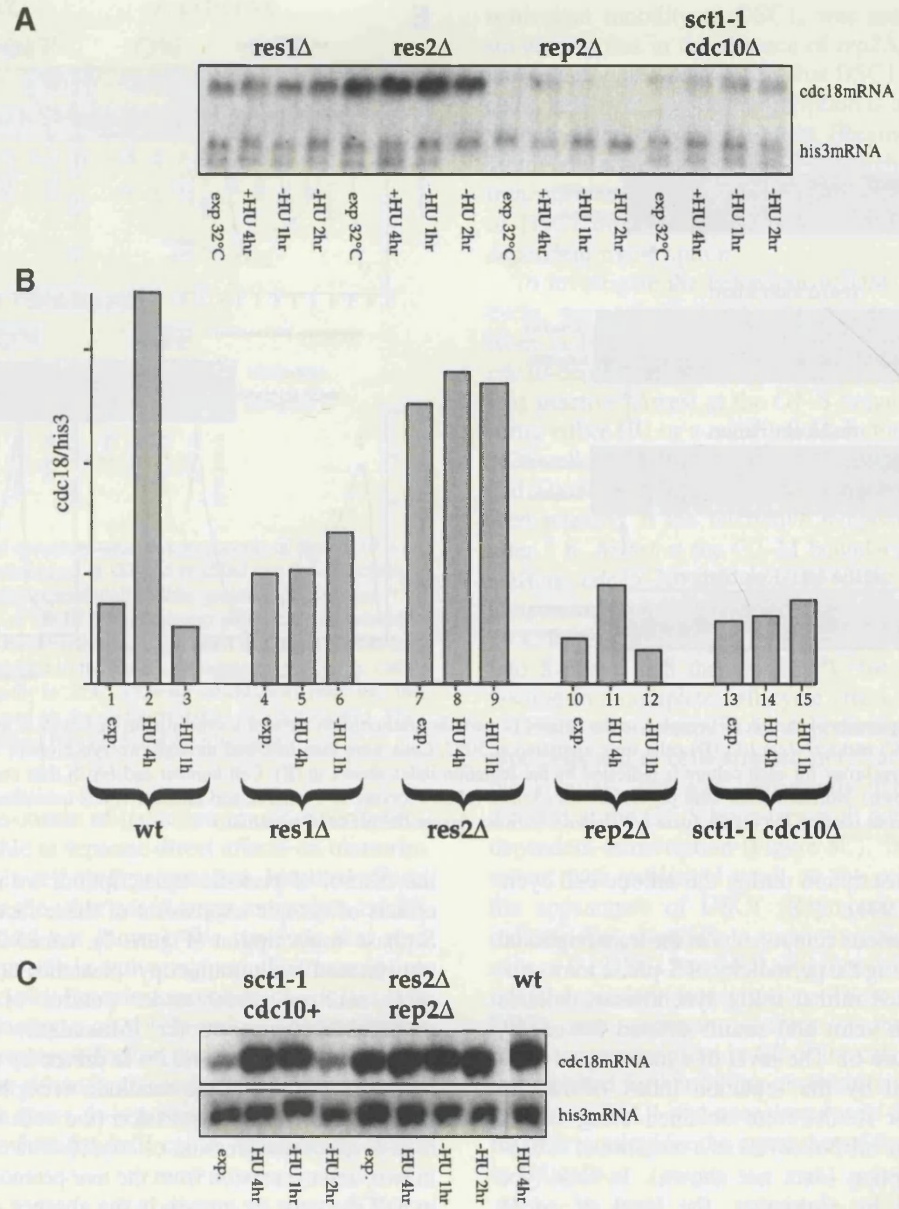

Fig. 5. The role of components of the *cdc10* complex in the control of periodic transcription in mitotic cells. *res1*Δ, *res2*Δ, *rep2*Δ and *sct1-1 cdc10*Δ (**A** and **B**) and the double mutant *res2*Δ *rep2*Δ and *sct1-1 cdc10*$^{+}$ (**C**) strains were grown at 30°C, where they are all viable, and then arrested at S-phase by the addition of 11 mM HU for 4 h. HU was then washed out, enabling cells to pass through S-phase and into G2 (confirmed by FACS analysis, see text). Samples were taken for Northern analysis (A and C) and probed for *cdc18* mRNA, *his3* serving as a loading control. (B) The Northern blot containing samples from experiments shown in (A) and in Figure 4B was quantified by phosphorimager analysis (using an arbitrary scale).

elevation was seen in *rep2*Δ cells, suggesting that these gene products are required for the elevated levels of *cdc18* transcription (Figure 5B, columns 2, 5, 11 and 14). In the *res2*Δ strain, *cdc18* transcript levels were high, both during and after release from the HU block (Figure 5B, columns 8 and 9), and were close to the peak level seen in S-phase wild-type cells. In wild-type cells, within 1 h of removing HU, as S-phase was completed, *cdc18* transcript levels fell dramatically (Figure 5B, column 3). *res1*Δ and *cdc10*Δ *sct1-1* did not exhibit a reduced *cdc18* transcript level after HU was removed, whereas a reduction was observed in *rep2*Δ cells (Figure 5B, columns 6, 12 and 15). As a control for *cdc10*Δ *sct1-1*, the single mutant *sct1-1* was also monitored. Periodic transcription of *cdc18* was retained in the *sct1-1* strain (Figure 5C), although there was some delay in the down-regulation of *cdc18* message as cells left the HU block, suggesting that the *sct1-1* mutation may cause a partial deregulation of *res1* function. However, the experiment confirms that the *sct1-1* mutation is not responsible for the aperiodic behaviour of *cdc18* transcription in the *cdc10*Δ *sct1-1* strain.

These results show that *cdc18* transcript levels are reduced and constant during the HU block and release in *res1*Δ and *cdc10*Δ*sct1-1* strains. *cdc18* transcription is elevated and constant in *res2*Δ cells, and is reduced but periodic in *rep2*Δ cells. In the double mutant *rep2*Δ*res2*Δ, transcript levels are high (Figure 5C), confirming that rep2p has no role in the absence of res2p (Nakashima *et al.*, 1995). We also confirmed previous work (data not shown) suggesting that *rep1*, a *rep2* homologue, has no

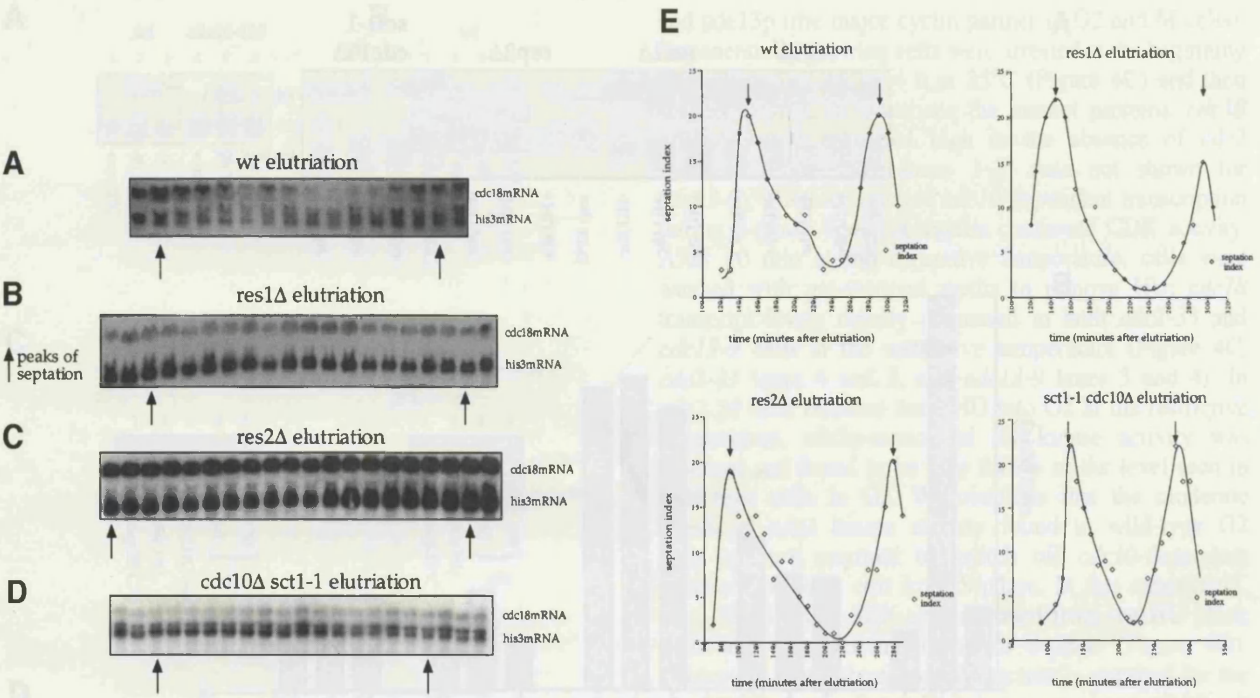

Fig. 6. The role of components of the *cdc10* complex in the control of periodic transcription through a synchronous cell cycle. Cultures of wild-type (**A**), *res1*Δ (**B**), *res2*Δ (**C**) and *sct1-1cdc10*Δ (**D**) cells were elutriated at 30°C. Cells were then followed through two synchronous rounds of cell division. The level of synchrony for each culture is indicated by the septation index shown in (**E**). Cell number and FACS data confirmed the cell synchrony (data not shown). Northern blots were probed for *cdc18*, *his3* mRNA serving as a control, and message levels quantified by phosphorimager analysis to confirm the results (data not shown). (Arrows denote the peaks of septation.)

role in S-phase transcription during the mitotic cell cycle (Sugiyama *et al.*, 1994).

The role of the various components of the transcriptional complex in controlling the periodicity of S-phase transcription was investigated further using synchronous cultures of wild-type fission yeast and strains deleted for *cdc10*, *res1* and *res2* (Figure 6). The level of synchrony of each culture is indicated by the septation index (shown in Figure 6E). Similar results were obtained using *cdc18*, *cdc22*, *cdt1* and *cig2* mRNA levels as a measure of *cdc10*-dependent transcription (data not shown). In wild-type cells synchronized by elutriation, the level of *cdc18* transcript is periodic during the cell cycle, being maximal around the peak in septation (Kelly *et al.*, 1993) (Figure 6A). In the *res1*Δ strain (Figure 6B), *cdc18* transcripts were present at low levels throughout the cell cycle. Thus, res1p is required to activate *cdc10*-dependent transcription during the cell cycle and, in its absence, no periodicity is observed and the absolute level of transcription is reduced. In the *res2*Δ strain, no significant periodicity in the level of *cdc18* message was observed in the synchronous culture (Figure 6C). We conclude that res2p is required to repress transcription periodically during the cell cycle; in its absence, no periodicity is observed and absolute levels are increased. In the *cdc10*Δ *sct1-1* strain (Figure 6D), *cdc18* transcript levels were constant but very low throughout the cell cycle. We conclude that although the *cdc10*Δ *sct1-1* strain can activate transcription of *cdc10* targets to a low level, cdc10p is absolutely required for the periodicity of this transcription. Finally, we confirmed our previous observation that in a *rep2*Δ strain, *cdc10*-dependent transcription was still periodic, although at a reduced absolute level during the cell cycle (data not shown).

To explore further the role of *resl*, *res2* and *cdc10* in the control of periodic transcription, we investigated the effects of ectopic expression of these factors on periodic S-phase transcription (Figure 7). *cdc25-22* strains were transformed with multicopy plasmids containing either *res1*, *res2* or *cdc10* under control of the thiamine-repressible *nmt* promoter (Maundrell, 1993). Ectopic expression of *res1* and *res2* was driven by the full strength *nmt* promoter while the medium strength promoter was used to drive *cdc10* expression (the cells became sick on high-level overexpression of *cdc10*). The culture was split in two, and expression from the *nmt* promoter was induced in half the cells by growth in the absence of thiamine, for 20 h at 25°C. Both induced and uninduced cultures subsequently were incubated at 36°C for 4 h to inactivate the $cdc25^{ts}$ function, thereby arresting cells in G2, where *cdc18* transcript levels are normally low. Cells were then released at 25°C into mitosis and a subsequent cell cycle.

In the presence of thiamine, which prevents ectopic expression of *res1*, *res2* or *cdc10* (marked OFF in Figure 7), *cdc18* transcript levels were low in G2, increased to peak levels on release into mitosis and decreased after entry into S-phase. However, in G2-arrested cells expressing *res1* from the *nmt* promoter (marked ON in Figure 7), *cdc18* transcription was activated to maximal levels (other *cdc10* targets, *cdc22*, *cdt1* and *cig2*, were similarly affected, data not shown). Following release of the *res1*-expressing cells into mitosis and the subsequent cell cycle, *cdc18* transcription was maintained at high levels. These results are in agreement with previously published data showing that overexpression of res1p can drive expression of *cdc10* targets (Ayte *et al.*, 1995). However, our results differ from those of Ayte *et al.*, in that we observed no significant G1 arrest after release from the *cdc25* block. This difference may be explained

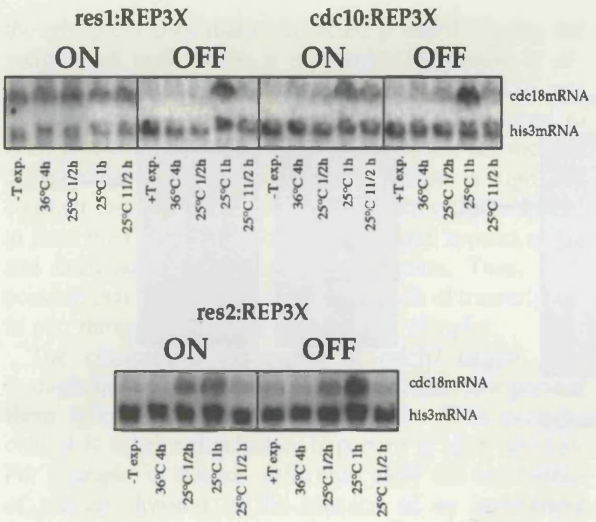

Fig. 7. The effects of overexpression of components of the *cdc10* complex in cells synchronized in G2 and released into the cell cycle. *cdc25-22* cells growing exponentially in the presence of thiamine, containing *res1*, *res2* or *cdc10* behind the *nmt* promoter, were washed four times to induce *nmt*-driven gene expression and, after growth for 20 h at 25°C, were shifted to the restrictive temperature for 4 h. Cells were then cooled rapidly to 25°C, allowing synchronous entry into the mitotic cycle (confirmed by the septation index, data not shown). The Northern blot was probed for *cdc18* and *his3* message.

by the short time-course of induction in our experiments. Thus, we were able to separate direct effects on transcription from blocks in cell cycle progression. Identical effects were observed on the other *cdc10* target transcripts, *cdc22*, *cdt1* and *cig2* (data not shown). We conclude that *res1* plays an important role in activating periodic transcription during the cell cycle. Ectopic expression of *cdc10* (Ayte *et al.*, 1995; McInerny *et al.*, 1995) and *res2* had no strong activating or repressing effect on the periodic transcription of *cdc10* targets, although transcription may be slightly repressed in both cases. It is possible that if expressed at higher levels, *res2* and *cdc10* could significantly affect transcription.

Analysis of the composition and cell cycle behaviour of DSC1

To provide a biochemical correlate for our analysis of *cdc10*-dependent transcription, we investigated the behaviour of DSC1, the bandshift activity which binds to MCB-containing promoters and contains cdc10p and res1p (Lowndes *et al.*, 1992b; Ayte *et al.*, 1995). The DSC1 bandshift was obtained by incubating cell extracts with a radiolabelled fragment of the *cdc18* promoter containing both putative palindromic MCB repeats. The bandshift was shown to contain cdc10p (Figure 8A) and to be sensitive to cold competitor DNA (data not shown). It is therefore likely to represent the same complex previously identified as DSC1.

To determine which gene functions are required to generate the DSC1 bandshift activity, its presence was monitored in extracts made from wild-type, *res1*Δ, *res2*Δ, *cdc10*Δ*sct1-1* and *rep2*Δ cells (Figure 8b). Previous work has shown the presence of *cdc10*, *res1* and *res2* in DSC1 (Zhu *et al.*, 1997; Lowndes *et al.*, 1992b; Ayte *et al.*, 1995). All four genes were required to generate the DSC1 bandshift activity, although a very faint bandshift, of equivalent mobility to DSC1, was seen in *rep2*Δ cells, suggesting that in the absence of *rep2*Δ, DSC1 may form inefficiently. It is noteworthy that DSC1 is absent in *res2*Δ cells, in which S-phase transcription is high. DSC1 is also absent in the *cdc10-C4* mutant (Reymond and Simanis, 1993; McInerny *et al.*, 1995), although the effect of this truncation on *cdc10* function is unclear. Thus, the presence of DSC1 does not correlate with the activity of *cdc10*-dependent transcription.

To investigate the behaviour of DSC1 through the cell cycle, we performed gel-shift experiments with extracts from cells arrested in the mitotic cycle; in G1 where *cdc10*-dependent transcription is active, and in G2 where it is inactive. Arrest at the G1–S boundary was achieved using either HU or a $cdc2^{ts}$ allele. In the latter case, *cdc2-M26* cells (from the experiment shown in Figure 1B, C and D) were arrested in G1 by nitrogen starvation and then released at the restrictive temperature and sampled after 3 h. Arrest at the G2–M boundary was achieved by shifting *cdc25-22* and *cdc2-33* cells to the restrictive temperature for 4 h (*cdc2-33* cells were first shifted to 34°C for 2 h, enabling cells arrested in G1 to leak through into S-phase, and then to 36.5°C for an additional 2 h leading to a complete cell cycle arrest in G2).

DSC1 was detectable in exponentially growing wild-type cells and in cells arrested in G2 at the *cdc25* or *cdc2* block points, but was consistently undetectable in cells arrested at the G1–S boundary with high levels of *cdc10*-dependent transcription (Figure 8C). These data are consistent with published work on the cell cycle timing of the appearance of DSC1 (Reymond *et al.*, 1993) but indicate that, contrary to previous conclusions, the presence of the DSC1 bandshift activity correlates with inactive *cdc10*-dependent transcription. These data suggest that DSC1 may represent a form of the complex which represses transcription in G2 of the cell cycle. This notion is supported further by the observations that res2p is both present in DSC1 and necessary for its formation, and that res2p is required for the repression of S-phase transcription in G2 cells.

Discussion

We have investigated the role of cdc2p and of various components of the S-phase transcriptional machinery in regulating the periodic expression of genes required for S-phase during the fission yeast cell cycle. Cdc2p protein kinase activity does not appear to be required for the activation of *cdc10*-dependent transcription in G1, for the maintenance of this transcription during S-phase or for its repression after S-phase. This behaviour contrasts with the situation in budding yeast, in which *CDC28* has been implicated in both activating and repressing S-phase transcription (Tyers *et al.*, 1993; Dirick *et al.*, 1995; Koch *et al.*, 1996) and in which temperature-sensitive mutations in *CDC28* dramatically reduce the activity of S-phase transcription mediated by *SW14/SW16–MBP1* (Peterson, 1985; Breeden and Nasmyth, 1987). In the parallel experiment in *S.pombe*, where temperature-sensitive alleles of *cdc2* had no effect on *cdc10*-dependent transcription, we cannot rule out the possibility that some residual *cdc2* activity, insufficient for entry into S-phase and undetectable in an *in vitro* protein kinase assay, was still present. In

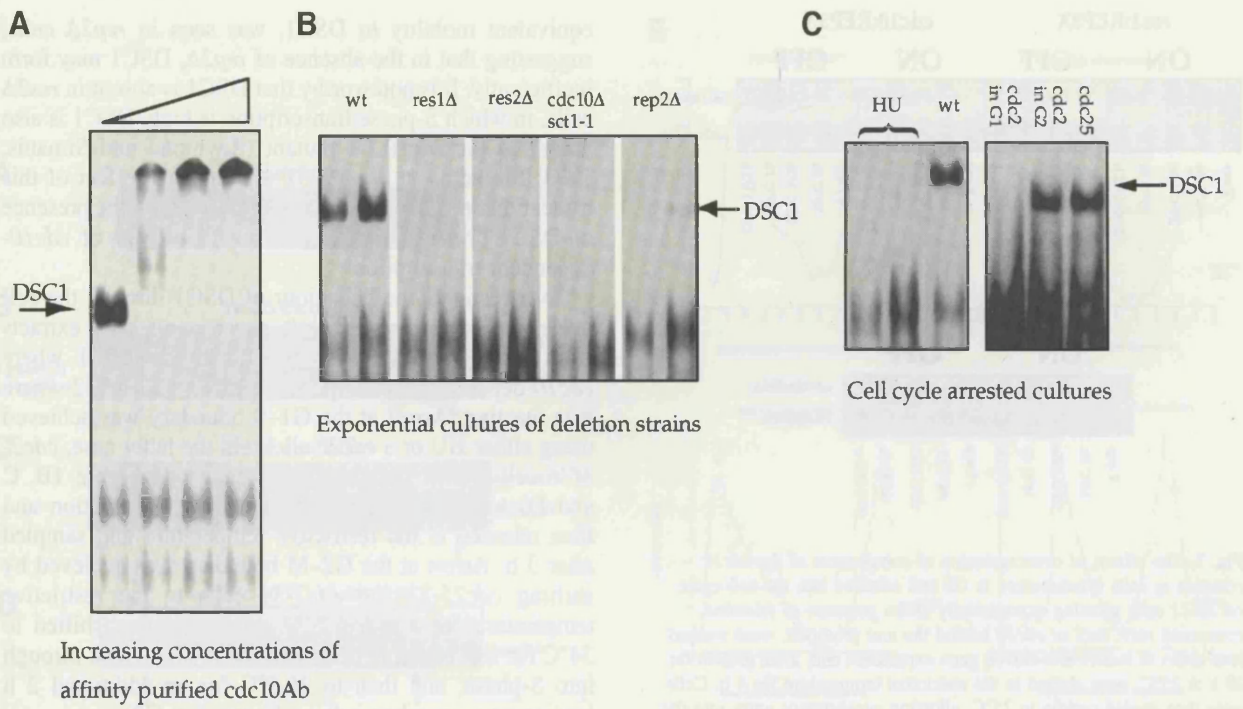

Fig. 8. Components of the *cdc10* complex required for the formation of DSC1 and the periodic appearance of DSC1 throughout the cell cycle. Bandshifts were carried out on cell extracts using the radiolabelled MCB-containing element from the *cdc18* promoter as a probe. In (**A**), wild-type cell extracts were incubated with increasing concentrations of affinity-purified, polyclonal rabbit cdc10Ab. (**B**) Gel-shift assays were performed with cell extracts from exponential cultures, grown at 30°C from wild-type cells and *res1*Δ, *res2*Δ, *cdc10*Δ in a *sct1-1* background and *rep2*Δ mutants. Samples were prepared in duplicate at two different concentrations of cell extract (20 and 40 μg) and loaded in adjacent lanes. (**C**) Gel-shift assays were carried out for cells arrested at various points in the cell cycle. Extracts were taken from exponentially growing wild-type cells and cells arrested at the onset of S-phase by the addition of 11 mM HU for 4 h and loaded on the same gel. Two bandshift assays were conducted using 20 and 40 μg of the HU extract. On a second gel, bandshifts were carried out using 40 μg of cell extracts from *cdc2-M26* cells arrested in G1, 3 h after release from nitrogen starvation (from the experiment shown in Figure 1) and from cells arrested in G2 using mutations in *cdc2* and *cdc25* in which *cdc10*-dependent transcription is inactive (RNA data not shown).

support of our conclusion, similar results were obtained in G1 cells deleted for the three B-type cyclins cig1p, cig2p and cdc13p, in which the H1 kinase activity was reduced to 1.5% of that seen in control cells expressing cdc13p. However, although this latter experiment is not subject to the concern about residual kinase activity of $cdc2^{ts}$, other cyclins may complex with cdc2p and activate *cdc10*-dependent transcription in this strain. In fact, a CLN type cyclin, known as puc1p, has been isolated from *S.pombe*. By analogy with CLNs in *S.cerevisiae*, puc1p could play a role in promoting the passage of cells through G1 into the mitotic cycle, in part by activating S-phase transcription. In contrast to the CLNs in *S.cerevisiae*, however, no role has been established for puc1p in the mitotic cell cycle, in which puc1p is barely detectable by Western blotting. *puc1* expression is induced on cell cycle exit, and its major function may be in controlling entry into meiosis (Forsburg and Nurse, 1994). In additional experiments, we have found that *cdc18* transcripts oscillate normally in *puc1*Δ and *puc1*Δ*cig2*Δ strains subjected to a HU block and release (data not shown), thus ruling out a situation analogous to that in *S.cerevisiae* in which *CLN3* deletions have a profound effect on the activity of *SWI4/SWI6–MBP1* (Tyers *et al.*, 1993; Dirick *et al.*, 1995). In further support of our interpretation, we observed high levels of *cdc18* mRNA in *res2*Δ*cdc2-33* cells after 4 h at the restrictive temperature (data not shown), suggesting that *cdc2* is not required for the elevated transcription seen in *res2*Δ cells. Thus, we conclude that CDKs are not universally responsible for regulating the periodic expression of genes required for S-phase.

In several situations, we observed active *cdc10*-dependent transcription in cells that were effectively pre-START (Hartwell, 1974; Nurse, 1975; Nurse and Bissett, 1981). Firstly, in small G1 cells re-fed after nitrogen starvation, *cdc10*-dependent transcription was activated at least an hour before the onset of S-phase and the observed increase in *cdc2*-associated H1 kinase. Secondly, *cdc10*-dependent transcription was activated in cells arrested pre-START at the G1 $cdc2^{ts}$ block and is active in cells arrested by pheromone (Stern and Nurse, 1997). These observations suggest that the activation of *cdc10*-dependent transcription and the accumulation of its major target, cdc18p, are not rate-limiting for the onset of S-phase. We also demonstrated that cdc2p protein kinase activity is required at a late stage in G1 and peaks close to the onset of S-phase. Therefore, the passage of cells beyond START may be driven by the action of G1 CDK activity rather than by the activation of *cdc10*-dependent transcription, and *cdc10*-dependent transcription in early G1 may provide the necessary gene products for a mitotic or meiotic S-phase, depending on the subsequent decision of cells to pass START (see also Miyamoto *et al.*, 1994).

Our studies have uncovered the roles of some of the components of the transcriptional complex in conferring the periodicity of S-phase transcription. We identified an unexpected role for res2p in the periodic repression of S-phase transcription during the mitotic cell cycle. It was

thought previously that res2p acted primarily during the meiotic cell cycle where it is essential (Miyamoto *et al.*, 1994; Zhu *et al.*, 1994). We have shown that res2p acts to inactivate transcription at the end of S-phase and to keep it low throughout the G2 phase of the mitotic cell cycle. When res2p is absent, transcription is high and constant throughout the cell cycle. Res2p is also required to form the DSC1 bandshift activity, which appears in G2 and correlates with inactive transcription. Thus, it is possible that res2p brings about repression of transcription in part through the action of the DSC1 complex.

The constitutive expression of *cdc10* targets seen throughout the cell cycle in *res2*Δ cells does not prevent them from dividing relatively normally. Thus periodic control is either redundant or important in other settings. For example, in meiosis, cells must carry out two rounds of nuclear division in the absence of an intervening S-phase. In this case, switching off *cdc10*-dependent transcription at an appropriate time may be critical. Alternatively, res2p may serve a different function in mitotic and meiotic cells; its role in periodic transcriptional control in mitotic cells may be of selective advantage simply because it ensures the efficient use of resources.

In contrast to res2p, res1p has an activating role in regulating periodic transcription. Res1p is required to maintain transcription in S-phase; in its absence, transcription is low and constant throughout the cell cycle. Importantly, high level ectopic expression of res1p during G2 is sufficient to activate transcription to a high level (see also Ayte *et al.*, 1995). This suggests that res1p is a key component in activating transcription periodically during the cell cycle. The cdc10p component is also critical for active transcription and is therefore essential for entry into S-phase (Nurse and Bissett, 1981). However, a mutation in the *res1* gene known as *sct1-1* (Marks, *et al.*, 1992; Caligiuri and Beach, 1993) enables the *cdc10*Δ strain to survive. Transcription in *sct1-1 cdc10*Δ cells is low and constant throughout the cell cycle, whereas it is more or less wild-type in the *sct1-1 cdc10*$^{+}$ strain, suggesting that cdc10p is also essential for the periodicity of S-phase transcription. Ectopically overexpressed cdc10p does not affect the periodicity or level of transcription, an observation supported by previous work (Ayte *et al.*, 1995). However, a truncated version of cdc10p encoded by *cdc10-C4* has been shown to activate transcription in G2 cells in the absence of wild-type cdc10p (McInerny *et al.*, 1995), suggesting an additional role for cdc10p in repressing transcription in G2 cells. Thus, it seems likely that res1p and cdc10p act together to promote activation of periodic S-phase transcription and that an altered complex containing cdc10p and res2p represses this transcription in G2 cells.

The rep2p component does not appear to contribute to the periodicity of S-phase transcription but is required to elevate the absolute level of activity. In the absence of rep2p, transcription is still periodic, but is much reduced in magnitude. Such a general activating role is consistent with its having a strong transcription activation domain (Nakashima *et al.*, 1995; P.Stacey, personal communication). Rep2p is not required to activate transcription when the res2p repressor is absent, suggesting that the role of rep2p may be to counteract the res2p repressor. These results support previous genetic data showing that *rep2* probably acts through *res2* (Nakashima *et al.*, 1995). Finally, we confirmed earlier reports that rep1p does not play a role in the mitotic cell cycle in the S-phase transcriptional control (Sugiyama *et al.*, 1994).

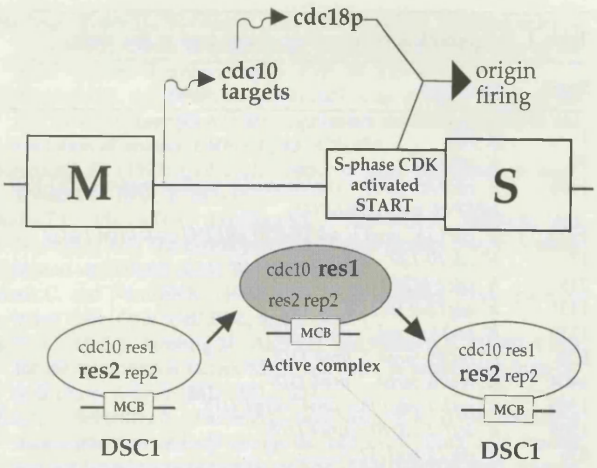

Fig. 9. Model for the control of *cdc10*-dependent transcription. We present a model for the way in which *cdc10*-dependent transcription may be controlled. We propose that *cdc10*-dependent transcription becomes active early in G1, independently of cdc2p activity, and that transcription persists until some time in S-phase. Cdc2p acts late in G1, after the accumulation of *cdc10* targets, to bring about the onset of S-phase. We suggest that cdc10p, res1p, res2p and rep2p are present in large complexes bound to MCB elements in target genes throughout the cell cycle. The periodicity of transcription may be brought about by a switch between an active and an inactive complex. Res1p may be the critical activator component and res2p may inactivate the complex in G2 (bold print shows dominant factor). These changes in activity may be brought about by alterations in the stoichiometry of res1p and res2p in the complex, or by post-translational modification of components with passage through the cell cycle.

The above experiments allow us to identify the roles of the S-phase transcriptional components in cell cycle regulation and to propose a model for the way in which they interact to promote periodic transcription (Figure 9). Cdc10p may provide a scaffold on which the regulation of transcription is imposed by the binding of res1p and res2p. Deleting either *res2* or *res1* eliminates cell cycle periodicity, indicating that both are necessary for the switch between active and inactive transcription. Normally, res1p may inactivate the res2p inhibitor, activating transcription. When res1p is overexpressed during G2, inactivation of res2p would occur inappropriately, allowing the complex at the promoter to activate transcription. Ectopic expression of res2p cannot block active transcription, so its presence alone may not define the difference between the active and inactive complexes. However, the stoichiometry of res1p and res2p in the complex may be altered with passage through the cell cycle and define its activity as a repressor or activator of transcription. The role of the *rep2* factor may be to increase the efficiency of the whole complex in promoting transcription in its active state, perhaps by masking the presence of res2p in the active complex.

We have shown that *cdc10*, *res1*, *res2* and *rep2* are all required to form the DSC1 bandshift activity and that the presence of the bandshift correlates with repressed transcription. These observations suggest that all of these components are part of an *in vivo* complex related to DSC1, which binds to MCB sites in G2 cells, but which

Table I. *Schizosaccharomyces pombe* strains used in this study

Strain	Genotype
1	*h⁻ 972*
19	*h⁻ cdc2-33*
1416	*h⁻ cdc13Δ::ura4+ cig1Δ::ura4+ cig2Δ::ura4+ cdc10-V50 pREP41::cdc13int LEU2*
1320	*h⁻ cdc13Δ::ura4+ cdc10-V50 pREP41::cdc13int LEU2*
17	*h⁻ cdc10-129*
275	*h⁻ cdc2-M26*
1435	*h⁻ cdc13-9 leu1-32*
1359	*h⁻ res1Δ::ura4 ⁺ ura4-D18*
B50	*h⁻ res2Δ::ura4 ⁺ ura4-D18*
1404	*h⁻ rep2Δ::ura4 ⁺ ura4 D18*
1365	*h⁻ sct1-1 cdc10Δ:: ura4⁺ ura4-D18*
1366	*h⁻ sct1-1 cdc10+ ura4-D18*
836	*h⁻ cdc25 leu1-32*
1405	*h⁻ res2Δ::ura4/ rep2Δ::ura4⁺ ura4-D18*
B51	*h⁻ cdc25-22 rep1Δ::ura4⁺*

is unable to activate transcription. In contrast, previous studies proposed DSC1 as a good biochemical correlate of the active complex (Reymond *et al.*, 1993). In budding yeast, there is also evidence for a role for *SWI4/SWI6–MBP1* in both the activation and repression of target gene expression (Dirick *et al.*, 1992; Lowndes *et al.*, 1992a; Koch *et al.*, 1996). In mammalian cells, the periodicity of transcription required for S-phase is also controlled in part by the conversion of the active E2F–DP1 transcription factor into a repressor by the binding of Rb early in G1 (Zamanian and La, 1993; Adnane *et al.*, 1995; Bremner *et al.*, 1995). Thus control of periodic expression of S-phase genes by both transcriptional activation and repression may be conserved, although our observation that transcriptional periodicity in fission yeast occurs independently of CDK activity indicates that similar transcriptional mechanisms may be regulated in different ways in different organisms.

Materials and methods

Fission yeast strains and methods

All strains used were constructed using standard procedures and are shown in Table I. Strains were grown in complete media (YES) and minimal media (EMM) as previously described (Moreno *et al.*, 1991). For nitrogen starvation experiments, cells were washed four times and resuspended in minimal-N_2 media and re-fed by the addition of 5 g/l NH_4Cl. In the experiment in Figure 1, cells were nitrogen starved for 15 h and then re-fed at 25 or 36°C. Upon starvation, a small fraction of *cdc2*^{ts} mutant cells always remain in G2. In the *cdc2-33* re-replication experiment (Figure 2), cells were arrested at G2–M at 36°C for 4 h in the absence of nitrogen (a small population of cells arrest at the *cdc2*^{ts} block in G1) and induced to re-enter S-phase by heat shock (Broek *et al.*, 1991). On re-feeding with nitrogen at 28.5°C, all the cells re-entered S-phase (at 25°C the recovery takes longer), but at 36.5°C cells were unable to duplicate their DNA. To enable *cdc10* and CDK activity to be manipulated independently in G1 cells, *cdc13::nmt cdc10-V50 cig1Δ cig2Δ* and *cdc13::nmt cdc10-V50 cig1⁺cig2⁺* strains were constructed (Figure 3). They were arrested in G1 by shifting to 36°C, which inactivates the *cdc10* gene function and, after 3 h at 36°C, *cdc13* was switched off in half the cells by the addition of 5 μg/μl thiamine (Maundrell, 1993). After an additional 1 h at 36°C, cells were shifted back to 25°C, reactivating *cdc10*. In the strain expressing cdc13p but lacking cig1p and cig2p, some cells underwent premature mitosis after entering S-phase, probably as a result of the elevated levels of *cdc13* expressed from the Rep41 promoter. For the HU block and release experiments (Figures 4 and 5), 11 mM HU was added for 4 h to block cells at the G1–S boundary, and removed by two washes with pre-warmed media. To ectopically express *cdc10* components in the *cdc25* block and release experiment (Figure 7), *cdc25-22 leu1-32* cells were transformed with a multicopy plasmid (Maundrell, 1993) containing *res1* or *res2* behind the full strength *nmt* promoter in Rep3X, or with *cdc10* behind the medium strength *nmt* promoter in Rep41X, in the presence of 5 μg/μl thiamine. Cells were then washed to remove thiamine, grown for 20 h at 25°C to induce *nmt*-driven expression and shifted to the restrictive temperature for *cdc25-22* for 4 h. Cooling to 25°C then allowed cells to enter the mitotic cycle in synchrony. In the bandshift experiment shown in Figure 8C, *cdc2-33* cells were shifted to 34°C for 2 h to enable cells arrested in G1 to leak into G2 and then to 36.5°C for another 2 h to complete the cell cycle arrest. The *cdc25-22* cells were blocked at G2–M for 4 h at 36°C.

Flow cytometric analysis

A total of 2×10^6 cells were fixed in 70% ethanol, washed in 3 ml of 50 mM sodium citrate, resuspended in 1 ml of 50 mM sodium citrate, 0.1 mg of RNase, 2 μg/ml of propidium iodide, and incubated for 2 h at 37°C. We followed the previously published protocol for flow cytometry (Sazer and Sherwood, 1990), using a Becton-Dickinson FACScan.

Cultures synchronized by elutriation

Elutriation in all cases was carried out using a Beckman J6 centrifuge and elutriator rotor. The septation index was counted and plotted. Synchronicity was evaluated further by FACS analysis and cell number determination (using a Sysmex Microcellcounter F-800, on the white cell channel) of fixed cells. In the case of mutants grown at 30°C, the elutriator was pre-warmed at this temperature.

RNA preparation and Northern blot analysis

Cultures were washed in STOP buffer, frozen on dry ice and then kept at –70°C. Subsequently, RNA was prepared using glass bead lysis (Sigma No. G9268) in 0.1 M EDTA, 0.1 M NaCl, 0.05 M Tris pH 8.0, in the presence of phenol:chloroform:isoamyl alcohol (Gibco-BRL) and 0.4% SDS. RNA was precipitated after two phenol extractions by the addition of NH_4OAc to 2.5 M and 2.5 vols of EtOH. Ten μg of sample RNA was denatured in 1× MOPS, 8% formaldehyde and 67% formamide, and run on a formaldehyde, 1.2% agarose gel in 1× MOPS. The RNA was transferred by Northern blotting in 10× SSC onto a GeneScreenPlus membrane (DuPont). Probes for blotting were prepared by random oligo priming with [α-^{32}P]dATP using a Prime-It Kit (Stratagene). The template DNA for the probes were: an *Nde*I–*Bam*H1 *cdc18*$^+$ fragment from REP1-*cdc18* cDNA; an *Nde*I–*Eco*RV *cig2* fragment from a genomic *cig2* clone in pAL-SK (Sergio Moreno); a *Sal*I–*Kpn*I *his3* fragment from a pKS *his3* plasmid; a *Hin*dIII *ura4* fragment from Rep4; a *cdt1* fragment from a PCR-derived cDNA clone (H.Nishitani) and a *cdc22* PCR fragment (primers from N.Lowndes). The membrane was hybridized overnight in 1% SDS, 10% dextran sulfate and 1 M NaCl, and washed in 1% SDS, 2× SSC.

Antibodies

The following antibodies were used: cdc18p polyclonal rabbit antiserum (H.Nishitani); cdc13p polyclonal rabbit antiserum (lab); cdc10p polyclonal rabbit antiserum raised against His_6TAG-cdc10 purified from *Escherichia coli* (J.Wuarin); an α-tubulin monoclonal antibody (Sigma); and rabbit polyclonal cdc2p antibody, C2 (lab).

Western blot analysis

Cells were boiled for 6 min prior to storage. Following glass bead lysis in HB buffer (Moreno *et al.*, 1991), protein concentration was determined and cell extracts were then re-boiled in 5× sample buffer. Then 50 μg of protein from each sample were run on an 8% SDS–polyacrylamide gel (Laemmli, 1970). For Western blots, the protein was blotted to Immobilon™-P membrane (Millipore) and detected using ECL (Amersham). Dilutions of the antibodies were 1:1000 for all polyclonal antibodies and 1:50 000 for the anti-α-tubulin monoclonal antibody (Sigma T5168).

H1 kinase assays

Extracts were prepared from frozen cells using glass bead lysis in HB buffer (Moreno *et al.*, 1991). Then 5 μl of rabbit polyclonal cdc2 antibody C2 (or pre-immune serum) was added to 500 μg of protein and incubated on ice for 45 min. Pre-equilibrated protein A–Sepharose beads (Pharmacia Biotech.) were added and the mixture agitated for 30 min at 4°C. Beads were washed three times and then resuspended in 15 μl of reaction buffer, containing 1 μg/μl calf thymus histone H1 (Sigma No. 382150), 200 μM ATP and 40 μCi/ml [γ-^{32}P]ATP

(Amersham). Extracts were then incubated at 30°C for 20 min, stopped by boiling for 5 min after the addition of 5× SDS sample buffer and run on a 12% SDS–polyacrylamide gel (Laemmli, 1970). In experiments using temperature-sensitive alleles, immunoprecipitates from control and *cdc2*^{ts} extracts were pre-incubated at 36.5°C for 5 min prior to the addition of reaction buffer (also at 36.5°C) and incubated for a further 20 min. Pre-immune sera gave no detectable signal.

Bandshift analysis

A double-stranded DNA probe made from the *cdc18* promoter containing both MCB repeats was amplified by PCR (Zhu *et al.*, 1994) and labelled by T4 polynucleotide kinase (BioLabs) with [γ-^{32}P]dATP (Amersham) and gel purified on a 4% polyacrylamide gel. Glass bead lysis was carried out in 25 mM HEPES pH 7.6, 0.1 mM EDTA, 150 mM KCl, 0.1% Triton X-100, 25% glycerol and 1 M urea in the presence of 1 mM dithiothreitol and protease inhibitors. Forty µg of soluble cell extract was pre-incubated for 10 min in gel-shift buffer: 25 mM HEPES pH 7.6, 34 mM KCl, 5 mM $MgCl_2$ with 0.1 µg/µl poly(dI–dC) and sonicated salmon sperm DNA, prior to the addition of excess radiolabelled probe. The gel-shift reaction was incubated for a further 15 min at room temperature then run on a native 4% acrylamide gel in 1× TBE for 3 h. DSC1 disappears upon the addition of cold-specific competitor DNA.

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