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The Role of MCB Elements in Transcriptional Activation of Cell-Cycle Regulated Genes in Fission Yeast

By

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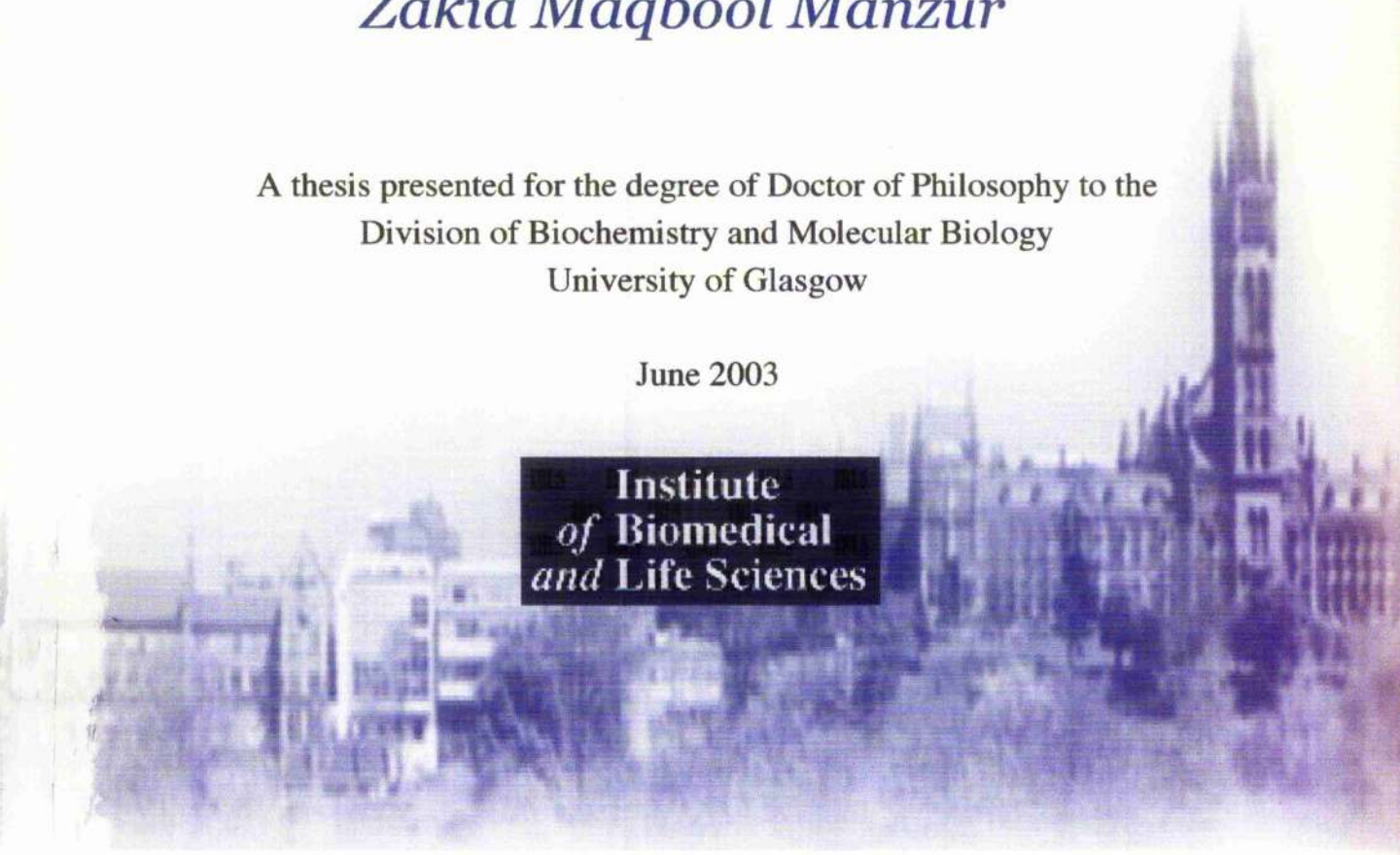

In the name of Allah, Most Gracious, Most Merciful.

SUMMARY

To understand cancer and the uncontrolled divisions that malignant cells undergo to form tumours, it is essential to elucidate normal cell cycle control mechanisms. The use of mammalian cell systems in this area of research is difficult, and thus alternative simpler model systems, such as yeast, have been exploited. The application of yeast genetics has proved to be a particularly powerful tool, permitting the identification of a number of genes required for cell cycle progression, that have subsequently been shown to have human homologues. For example, transcriptional mechanisms involving passage through START in G1 and commitment to S phase are conserved from yeast to mammals.

In the work presented in this thesis, I have investigated the transcriptional regulation of *cdc22*$^{+}$, a fission yeast, *Schizosaccharomyces pombe*, G1/S expressed gene, to understand cell cycle regulated mechanisms at START. We hope that such information will contribute towards a better understanding of transcriptional mechanisms of G1/S regulated genes in other eukaryotes, including mammalian systems.

In fission yeast progression through the cell cycle is dependent on passage through START, which is regulated in part by the transcription factor complex DSC1. DSC1 controls the expression of genes at the G1/S transition essential for DNA synthesis. Each of these genes contains an upstream activation sequence (UAS) that possess a conserved core element of one or more hexameric sequences, coinciding with the *Mlu*I restriction site (ACGCGT), called a *Mlu*I cell cycle box, or MCB motif. These MCB motifs bind specifically to DSC1 to form the transcription control system.

cdc22^{+} was the first fission yeast G1/S transcribed gene to be discovered, and found to contain an interesting array of MCB motifs in its promoter. The promoter contains two clusters of MCB motifs, named in this thesis as "MCB1" and "MCB2", within which there are three *Mlu*I motifs that are identical to the *Mlu*I recognition sequence, and five MCB motifs containing the central CGCG core thought to be

essential for function. There is also a single core *Mlu*I site in between the two MCB clusters, named here "MCBX". The mapping of transcriptional start of *cdc22*$^+$, using primer extension and Northern blot analysis, revealed that the start point of the gene lies between the two MCB clusters. Furthermore, *in vitro* DNAseI footprint analysis showed there are protected regions over MCB2, implying that either one or both of the MCB clusters is essential for the binding of DSC1, and activating transcription of *cdc22*$^+$.

My initial experiments showed that both of MCB clusters, MCB1 and MCB2, can confer G1/S transcription. This was achieved by separately cloning the two clusters into a UAS-*lacZ* reporter construct containing the *CYC1* minimal promoter and examining their ability to confer G1/S transcription to *lacZ*. Both MCB clusters could confer cell cycle regulation to the heterologous gene, but only when present in the correct orientation. This latter observation was shown to be a generic property of MCB motifs in fission yeast, as MCBs in other endogenous genomic locations were also found to only activate cell cycle transcription in one orientation.

In another experiment, using an integration vector containing the entire promoter region of *cdc22*$^+$ linked to *lacZ*, we were also able to show that intact MCB motifs in both MCB clusters are necessary for the correct cell cycle regulation. It was observed that MCB1 was required for stimulating cell cycle transcription, and that MCB2 is essential for basal transcription of the gene. The finding that MCB2 participates in the transcriptional activation of *cdc22*$^+$ is particularly interesting, because this is the first evidence in fission yeast of a transcriptional control sequence downstream to the transcriptional start for a RNA polymerase II regulated gene. We therefore name this type of new sequence a DAS (downstream activating sequence).

In vitro gel retardation analysis showed that DSC1 specifically binds to MCB2 and MCB1, confirming the importance of both clusters. Significantly, MCB2 had a higher "on-off" rate that MCB1, suggesting a mechanism by which this cluster could activate *cdc22*$^+$ transcription, while allowing RNA polymerase II to pass. Bringing these observations together, a model is proposed to explain DSC1-MCB regulation of *cdc22*$^+$ transcription, which we believe will be applicable to most MCB-regulated genes in fission yeast.

Dedication

I would like to dedicate this work to my Beloved Mother, Safia Maqbool.

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All praises to Allah, He is the best of providers.

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Abbreviations

Ax	absorbance (turbidity) at x nm wavelength
APC	anaphase promoting complex
ARS	autonomous replication sequences
ATP	adenosine triphosphate
BSA	bovine serum albumin
CAK	CDK-activating kinase
cdc, CDC	cell division cycle
CDK	cyclin dependent kinase
cDNA	complementary DNA
CKI	CDK-inhibitory
CTD	carboxy-terminal domain
c-term	C-terminus
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DAS	downstream activation sequence
DEPC	diethyl pyrocarbonate
DNA-PK	DNA dependent protein kinase
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide
dNTPs	all four deoxy triphosphates
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
DSC1	DNA synthesis complex 1
EDTA	ethylenediaminetetraacetic acid
EMM	Edinburgh minimal medium
GB	Glasgow collection number for bacteria
GG	Glasgow collection number for fission yeast
GO	Glasgow collection number for oligonucleotide primer
HU	Hydroxyurea
IR	ionising radiation
IPTG	isopropylthio-β-D-galactoside
kb	kilobase
kDa	kilodalton
MBF	*Mlu*I cell- cycle box binding factor
MCB	*Mlu*I cell cycle box
MCM	minichromosome maintenance
min	minutes
MOPS	3-(N-morpholino)propane-sulphonic acid
MPF	maturatin promoting factor
mRNA	messenger RNA
MW	molecular weight
ORC	origin-recognition complex

ORI	origin of replication initiation
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulphonylfluoride
PIK	phosphoinositide-3-kinase
PNK	polynucleotide kinase
RCE's	retinoblastoma control elements
RNA	ribonucleic acid
RNR	ribonucleotide reductase
rpm	revolutions per minute
RB	retinoblastoma
RNAP I	RNA polymerase I
RNAP II	RNA polymerase II
RNAP III	RNA polymerase III
RNR	ribonucleotide reductase
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
SBF	Swi4/Swi6 cell cycle box binding factor
TBP	TATA box binding protein
TFII	transcription factor RNA polymerase II
ts	temperature sensitive
UAS	upstream activating sequence
v/v	volume/volume
w/v	weight/volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YE	yeast extract medium

Chapter 1: Introduction

1A Introduction

Of the estimated nine million new cancer cases every year more than half occur in developing countries (Neal and Hoskin 1997). In the UK alone during 1993, 160,000 people died from different forms of the malignant disease, which represented about 1 in 4 of all deaths (WHO 1996). One of the major causes of cancer is uncontrollable cell division, when cells divide at incorrect times.

Replication of a cell is governed by the cell division cycle, which follows a pre-determined genetic programme. This genetic programme results in the progression of cells through growth, DNA replication and mitosis, followed by cytokinesis, to allow the segregation of the hereditary material into two daughter cells. The precise co-ordination of the progression through cell cycle phases is critical, not only for normal cell division, but also for effective arrest under conditions of stress or after DNA damage. As a consequence, a derangement in the cell cycle machinery may contribute to uncontrolled cell division, which is a principal feature of many forms of cancer (Malumbres and Barbacid 2001).

Thus, an understanding of the normal eukaryotic cell cycle is an essential requirement to understanding cancer and the uncontrolled divisions that malignant cells undergo to form tumours. The application of yeast genetics to this field of work has proved to be a powerful tool, as it has allowed the identification of a number of genes that are critical for controlling cell division in all eukaryotes (Qin and Li 2003).

In this introduction, a review of the current knowledge regarding the cell cycle in mammalian systems is outlined, followed by the present understanding of cell cycle controls in yeasts. Finally, the importance of yeast species, and particularly the fission yeast *Schizosaccharomyces pombe*, as model organisms for elucidating eukaryotic cell cycle control mechanisms is described.

1B Cell cycle

I *Mitotic cell cycle*

The cell cycle in eukaryotic cells occurs in a particular defined order, which is commonly divided into four phases (Nurse 2000) (Figure 1). The first gap, called G1, is the longest phase in mammalian cells, during which the cells prepare for DNA replication. This is followed by S phase (DNA synthesis), during which the DNA is replicated and a complete copy of each of the chromosomes is made. The second gap phase, G2, occurs after S phase and before the next stage, M phase (mitosis). Together, G1, S and G2 comprise interphase. M phase is when the duplicated chromosomes are segregated between the two daughter cells as the cell divides.

M phase consists of the various stages of nuclear division, which can be sub-divided into five parts. Prophase, during which the chromosomes condense. Prometaphase, when the chromosomes interact with the centrosome to form the spindle. Metaphase, during which the sister chromatids attach at the centromere and become aligned in the centre of the cell. Anaphase, where the sister chromatids separate and move to opposite poles or spindles and segregate into the daughter cells. And finally, telophase, when cytokinesis pinches the cell into two daughter cells and a nuclear envelope re-forms around the two groups of chromosomes (Mazia 1961). After mitosis, proliferating cells enter G1 of the next cell cycle (Nurse 2000).

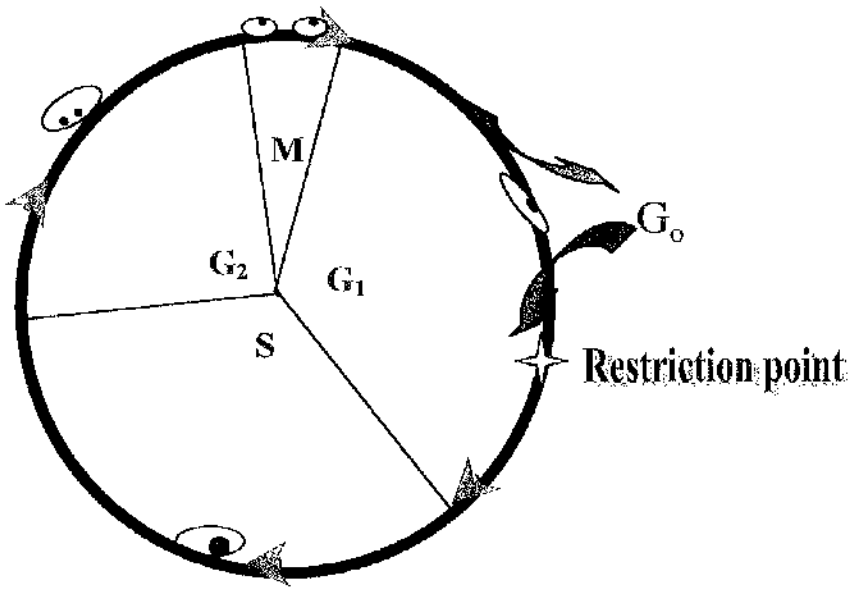


Figure 1. A schematic representation of the mitotic cell cycle of mammalian cells.
The cell cycle is classically divided into four phases: G1 before DNA synthesis; S when DNA synthesis occurs; G2 after DNA synthesis and before M, mitosis, when nuclear division occurs. Under certain circumstances cells exit the cell cycle during G1 and remain in G_0 state as non-growing non-dividing (quiescent) cells. Appropriate stimulation of such cells induces them to return to G1 and resume growth and division. The restriction point is the stage during G1 when cells chose to either enter S phase or G_0.

Cells normally enter S phase only if they are committed to mitosis. Under certain circumstances non-dividing cells may exit the cell cycle after mitosis and remain in a non-proliferative resting phase, called G_0, or quiescence (Baserga 1976; Prescott 1976a; Pardee et al. 1978).

The initiation of the cell division cycle is dependent on the presence of extra-cellular growth factors known as mitogens. In the absence of mitogens, cells withdraw from G1 and enter G_0. The point in G1 at which information regarding the environment of the cell is assessed, and when the cell decides whether to enter another division cycle, is called the restriction point ("R" point) (Pardee 1989). The R point is of crucial significance in understanding the commitment of mammalian cells to undergo a cell division cycle, as deregulation of the R point may lead to cancerous growth of cells *in vivo* (Sherr 1996; Blagosklonny and Pardee 2002). In most cell types the R point will occur a few hours after mitosis. Cells that are starved of mitogens before reaching the R point will enter G_0 and fail to undergo cell division. In contrast, cells that are starved of mitogens after reaching the R point are committed

to enter S phase and must complete cell division before entering G0 from the next cell cycle (Blagosklonny and Pardee 2002).

II *Meiosis*

In all multi-cellular organisms, and in single-celled organisms that are diploid at some phase of their life cycle, another important type of cell division occurs that fundamentally differs from mitosis. This alternative life cycle is called meiosis (Prescott 1976b). Meiosis is a reductive form of cell division, which results in the production of haploid cells, sperm and egg cells in higher plants and animals (Cavalier-Smith 2002).

1C Checkpoint controls

Catastrophic genetic damage can occur to cells if they progress to the next phase of the cell cycle before the previous phase is properly completed. Thus, a crucial regulation of eukaryotic cell cycle is exerted through *checkpoint controls* that operate to ensure that each stage of the cell cycle is fully completed before the following stage is initiated (Rhind and Russell 2000a). There are different stages at which checkpoint controls can arrest passage through the cell cycle if cell conditions are unsuitable. For example, DNA damage due to irradiation or chemical modification prevents G1 cells from entering S phase and G2 cells from entering mitosis. Un-replicated DNA can also cause S phase arrest and prevent entry into mitosis, and improper spindle formation results in M phase arrest preventing the cell from entering anaphase (Qin and Li 2003). Passage through checkpoints in the cell cycle is controlled by the activity of cyclin-dependent protein kinases (Nurse 2002).

I *Cyclins and CDKs - activities*

The cyclin-dependent protein kinase complex is made up of a regulatory sub-unit called a cyclin, and a catalytic sub-unit, initially discovered in yeast, called a cyclin-dependent kinase (CDK) (Walworth 2000). The CDKs have no catalytic activity unless they are associated with a cyclin, and each can associate with more than one type of cyclin (Fisher 1997). The cyclin family of proteins share homology in a 100 amino acid region called the cyclin box, through which they bind specifically to the CDKs (Pines 1996). In addition to controlling CDK activation, the cyclin sub-unit may also contribute to substrate specificity and determine which target proteins are phosphorylated (Peeper et al. 1993).

In mammalian cells a range of cyclin sub-units activate CDKs to trigger their transition from one phase to the next during the cell cycle. There are three different classes of cyclin-CDK complexes associated with either G1, S or M phases. The G1 CDK complexes CDK4, CDK6 and CDK3 activate transcription factors that cause expression of enzymes needed for DNA synthesis and the genes encoding S phase CDK complexes, and so prepare the cell for S phase (Reed 1997). The S phase CDK complex, CDK2, stimulates the onset of DNA synthesis and helps to ensure that each chromosome is replicated only once (Morgan 1997). It also induces chromosome condensation and orders chromosome separation into the two daughter cells (King et al. 1994). Entry into mitosis employs a highly conserved mechanism dependent on CDK1 (Riabowol et al. 1989; Hamaguchi et al. 1992). CDK1 was the first human cyclin-dependent kinase to be identified and was found by complementing fission yeast *cdc2* mutants (Lee and Nurse 1987) (Introduction, pages 28 and 32).

During G2 CDK1 joins newly synthesised cyclin B to form a complex called maturation promoting factor or MPF, that controls the G2/M transition (Doree and Hunt 2002). Full activation of CDK1 is essential to stabilise its association with cyclin A. This activation is stimulated by the phosphorylation of CDK1 at its stimulatory threonine-161 (Thr-161) site (Artherton-Fessler et al. 1993); and an inhibitory tyrosine-15 (Tyr-15) site, that is de-phosphorylated by a protein phosphatase Cdc25, after DNA synthesis has been completed (Izumi et al. 1992). In human cells, three CDK phosphatases have been identified named Cdc25A, Cdc25B

and Cdc25C (Galaktionov and Beach 1991). Once activated, the CDK1 can then phosphorylate many target proteins, initiate mitosis, and also switch off the complex by triggering enzymes that degrades the cyclin polypeptide.

II *Cyclins and CDKs – regulation and control*

Both cyclins, and some CDK-inhibitory proteins (CKIs), are regulated by synthesis and ubiquitin-mediated proteolysis (Ciechanover et al. 2000). This ubiquitin-proteosome system involves a large number of enzymes mediating ubiquitin activation (E1), ubiquitin conjugation (E2) or ubiquitin ligation (E2), which modulate turnover of cell cycle regulatory proteins (King et al. 1997). Ubiquitin-dependent degradation performs a key regulatory function during G2/M and completion of mitosis is regulated by the anaphase promoting complex (APC) - another high molecular weight ubiquitin ligase complex essential for chromosome segregation. Apart from degradation of cyclins at specific stages during the cell cycle, ubiquitination-dependent proteolysis may also be important for regulating the activities of oncoproteins (protein products encoded by oncogenes) with the potential to transform cells to malignancy (Appella and Anderson 2000).

Late in the cell cycle ubiquitination and subsequent degradation of B type cyclins is initiated by the APC, which functions by catalysing the binding of ubiquitin to cyclin B (Peters 2002). Activation of MPF and the mitotic cyclin degradation system occur sequentially, as the time lag between these two events allows MPF to remain active to induce mitotic events before APC activation ends the mitotic process. The APC is itself cell cycle regulated, as ubiquitination and proteolysis of B type cyclins only occurs in mitosis and G1 (Irniger and Nasmyth 1997). The regulation of the APC in mammalian cells is poorly understood and is better studied in simpler model systems, such as fission yeast (Blanco et al. 2000).

CDKs can also be regulated by binding CKIs and other proteins such as Suc1, that modify their specificity or accessibility to regulators (Patra and Dunphy 1996). CKIs inhibit CDK activity after their function has been exerted and provide an

efficient mechanism to regulate the sequential activation of specific cyclin/CDK complexes throughout cell cycle progression (Serrano et al. 1995).

CDKs are essential for regulating the function of transcription factors, and recent studies implicate CDK complexes in regulating transcription by functional and physical interaction with components of the basal transcription apparatus (Dynlacht 1997; Walworth 2000). To date, the three known CDKs which appear to have a role in basal transcription, have been reported to be capable of utilising the carboxy-terminal domain (CTD) of the large sub-unit of RNA polymerase II as a substrate. This leads to the hyper-phosphorylation of the CTD, which is an important process in transcription (Svejstrup et al. 1997). CDK-activating kinase (CAK) is another cyclin/CDK complex composed of cyclin H, CDK7 and a third protein called MAT1 (Devault et al. 1995; Fisher and Nurse 1995; Tassan et al. 1995). CAK may provide a link between transcription and cell cycle control, as CDK7 is a component of TFIIH, a protein belonging to the basal transcription machinery. CDK7 can phosphorylate the C-terminus (C-term) of RNA polymerase II, and is possibly participating in the induction of RNA transcription more than in activation of cyclin/CDK (Feaver et al. 1994; Serizawa et al. 1995). Significantly, perturbation of CDK activities can result in tumourogenesis (Senderowicz 2002), and their regulation, by both positive and negative factors, plays an important part in cell cycle progression.

III *Mammalian DNA damage checkpoints*

Mammalian cells display a cell cycle response to DNA damage similar to that of yeasts, but in addition they can also activate the cell death pathway (Qin and Li 2003). In mammalian cells the G1 DNA damage checkpoint is controlled by the two related kinases, ATM (ataxia telangiactasia mutated) and ATR (ataxia-and Rad related protein) (Abraham 2001), p53 (a protein encoded by a tumour suppressor gene) (Ko and Prives 1996) and a CKI, p21 (Brugarolas et al. 1995; Deng et al. 1995). The signals sensed by ATM and ATR are transmitted through two kinases, Cds1 and Chk1, which phosphorylate and thus modify the function of the targets of the checkpoint response (McGowan 2002). These two kinases are conserved at the level of primary protein sequence in eukaryotes, although current evidence suggests that

they might not have equivalent function in all species, for example yeast (Rhind and Russell 1998). More recently, the precise functions of Chk1 and Cds1 in mammalian cells are beginning to emerge (Figure 2). Cds1 is activated in response to ionising radiation (IR) (Brown et al. 1999a; Chaturvedi et al. 1999), and is needed for IR-induced stabilisation of p53, but not to initiate the G2 damage checkpoint (Hiraro et al. 2000). Chk1 is found to be an essential gene that is required both for the S/M checkpoint and the G2 DNA damage checkpoint (Liu et al. 2000a).

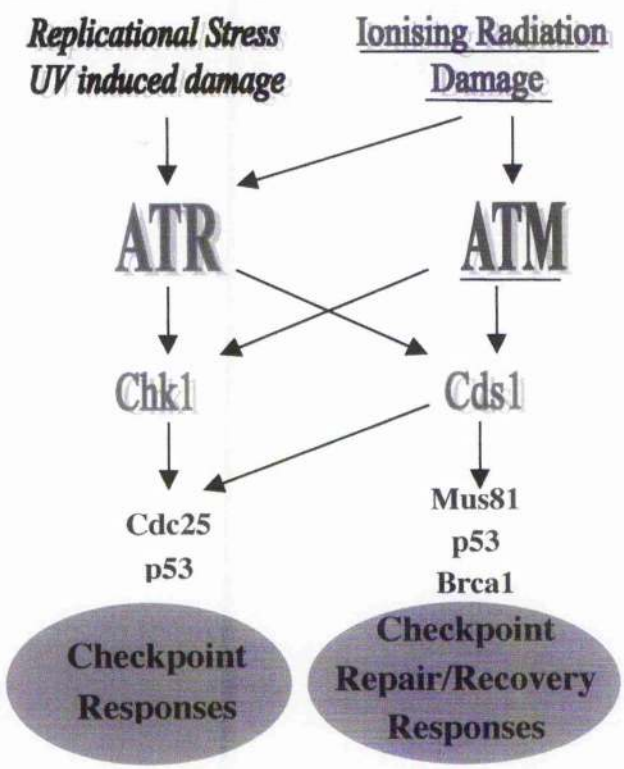


Figure 2. Schematic representation of the DNA damage and replication checkpoint in mammalian cells.

Arrows represent either biochemical or genetic evidence for a connection. Regulators of the DNA damage response underlined. Regulators that act only in the replication/intra-S phase checkpoint are in italics. Kinases that are used by both branches of the pathway are in green. For full explanation see text, pages 7 and 8.

IV The spindle assembly checkpoint

In most eukaryotic cells the presence of unattached chromosomes or defects in spindle assembly activates the intracellular signalling pathway known as the spindle assembly checkpoint, that blocks anaphase onset and stabilises the APC substrate during mitosis (Elledge 1996). In normal mitosis the checkpoint intervenes only when the fidelity of the segregation system needs to be enhanced (Clarke and Gimenez-Abian 2000). In addition, loss of this checkpoint may contribute to aneuploidy in cancerous cells (Wells 1996).

1D DNA replication in mammalian cells

I *Origins of replication*

In mammalian cells the initiation of DNA replication occurs mostly at intergenic regions in chromosomes, in large DNA elements ranging in size from 0.5 to 55 Kb. These elements are called autonomously replicating sequences (ARS) (Clyne and Kelly 1997). A purified multi-protein complex called the ORC (origin recognition complex) binds the ARSs, and the interaction of ORC with so-called minichromosome maintenance (MCM) proteins play an important role in the initiation of replication. ORC sub-units were initially identified in budding yeast, and homologues were identified in higher eukaryotes, suggesting similar mechanisms occur in mammalian cells. So far, most knowledge about ARSs and ORCs has come from yeasts (Fujita 1999; Wegrzyn and Wegrzyn 2001) (Introduction, pages 29-31).

II *Ribonucleotide reductase*

Ribonucleotide reductases (RNRs) are a family of enzymes that have an essential role in all living organisms, as they provide the only mechanism by which ribonucleotides can be converted into deoxy-ribonucleotides (Eklund et al. 2001). These enzymes are therefore vital for both DNA replication and repair.

RNR classes

RNRs are divided into three classes based on the nature of the cofactor providing the free radical for the ribonucleotide reduction reaction (Reichard 1993) (Stubbe and van Der Donk 1998). Class I RNRs, present in eukaryotes and micro-organisms, use an iron centre to produce a stable tyrosyl radical, that is stored in one of the sub-units of the enzyme. Class II RNRs, present only in micro-organisms, alternatively use adenosylcobalamin (AdoCbl), a precursor to a 5'-deoxyadenosyl radical and Cob(II)alamin. Class III RNRs, only found in anaerobic micro-organisms, utilise an iron-sulphur protein and S-adenosyl-methionine to generate a stable glycyl radical (Mulliex et al. 1999).

Although RNRs from different organisms are a diverse set of enzymes, they contain certain homologous structural features. The best understood of these enzymes is the RNR class I from *E. coli*, which are thought to be representative of other RNR classes (Figure 3). *E. coli* class I RNR consists of two homodimeric proteins, a small sub-unit called NrdA (α_2) and a large sub-unit called NrdB (β_2) arranged as a heterotetramer ($\alpha_2\beta_2$). Based on sequence identity and allosteric properties, class I RNRs are sub-divided into class Ia and Ib, encoded respectively by the *nrdABs* and *nrdEFs* genes. Class II RNRs are mainly α_2 homodimers encoded by the *nrdJ* genes, and class III RNRs are structurally α_2-β_2 with sub-units encoded by the *nrdDG* genes (Torrents et al. 2002).

The sequences of the class I RNRs from different species show weak but significant sequence similarities distributed along most of the polypeptide chain, except from the first 145 residues where there are large deviations. The similarities strongly suggest that the small sub-units from all species have similar overall three-dimensional structure to the *E.coli* small sub-unit, but with variations in the N-terminus (Torrents et al. 2002).

The class I RNR non-identical homo-dimeric sub-units are often referred to as R1 and R2 (Figure 3). *E. coli* R1 has a molecular weight of 172,0000 daltons, where each sub-unit contains 761 amino acid residues (Carlson et al. 1984; Nilsson et al.

1988). R1 is S-shaped and is composed of three domains: one β/α-barrel of about 480 residues, an N-terminal portion, and another small domain attached to the β/α-barrel. The R1 dimer is the enzymic portion of the molecule where nucleotide reduction occurs. It contains both the active sites and binding sites for allosteric effectors, and the essential cysteines that serve as the immediate electron donor in the reduction of the ribose unit.

The R2 dimer has an essential role in the catalysis to generate a free radical in each of its two chains. It contains one di-nuclear iron centre and one stable tyrosyl radical per monomer, which are both essential for enzymatic activity. Amino acid sequence alignments of R2 proteins from different species demonstrate high similarities within the eukaryotic proteins (Nordlund and Eklund 1993). The *E. coli* R2 sub-unit has a molecular weight of 87,000 daltons, where each sub-unit contains 375 amino acids (Carlson et al. 1984). The R2 dimer is heart shaped, consisting of two β-hairpins and α-helices. Each R2 sub-unit is a single domain consisting of 13 helices and two β-sheet strands. Within the interior of the protein is Tyr122, the tyrosyl free radical, which is generated by a nearby iron centre consisting of two ferric ions bridged by an oxide ion (Eklund et al. 2001) (Figure 3).

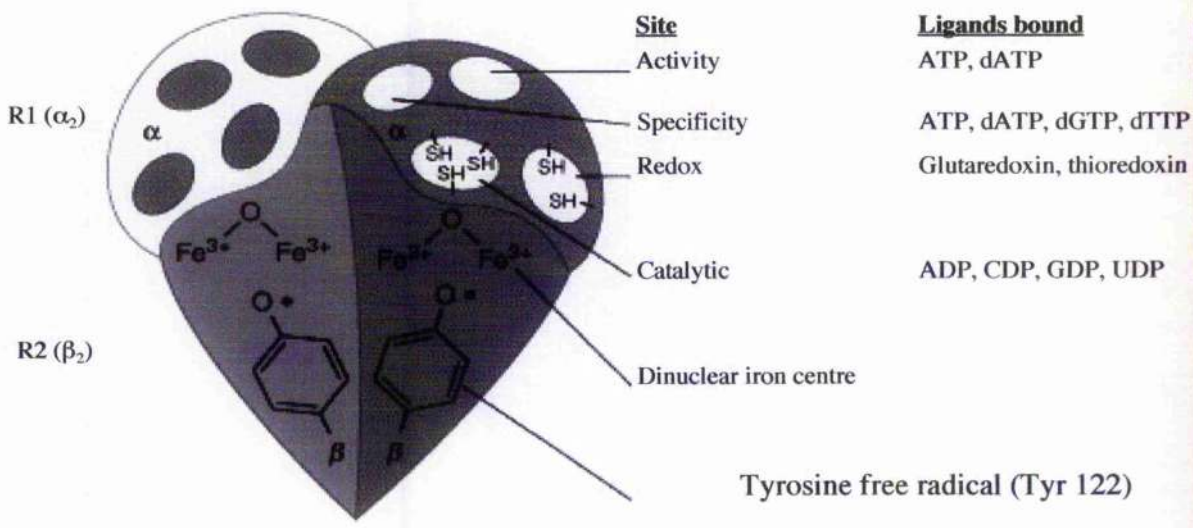


Figure 3. R1-R2 model of ribonucleotide reductase class I from *E. coli*.
The R1 dimer is positioned on top of the R2 dimer and the different sites necessary for its function are labelled accordingly (Uhlin and Eklund 1994).

Pathways

DNA is built from deoxy-ribonucleotides, which are synthesised from the corresponding ribonucleotides. In deoxy-ribonucleotide synthesis, a deoxyribose sugar is generated by the reduction of the ribose within a fully formed ribonucleotide. The methyl group that distinguishes the thymine of DNA from the uracil of RNA is added at the last step in the pathway. RNR is responsible for the reduction reaction of the ribonucleotides, where the 2'hydroxyl group bonded to the C-2' on the ribose moiety is stereo-specifically replaced by a hydrogen atom, with retention of the configuration at the C-2' carbon atom (Figure 4). Thus, the essence of the catalytic reaction is a transient transfer of radical properties from the enzyme to the substrate. Mechanistic studies suggest that the chemistry catalysed by all classes of RNRs, with the exception of the reductant used, is very similar (Stubbe and van Der Donk 1998) (Eklund et al. 2001).

The deoxy-ribonucleotide synthesis pathway occurs in a number of steps, shown schematically in Figure 4. The pathway begins with step 1, where an electron transfer from a cysteine (Cys439) on R1 to a tyrosine radical (Tyr122) on R2 generates a highly active cysteine thiyl radical within the active site of R1. In step 2, this thiyl radical then abstracts a hydrogen atom from C-3' of the ribose unit, generating a radical at the carbon atom. The presence of the radical at C-3 promotes step 3: the ejection of OH- from C-2, that when protonated by a second Cys225 residue, the departing OH- leaves as a water molecule. In step 4 a hydride (proton on two electrons) is then transferred from a third Cys462 to complete the reduction of C-2 position from a disulphide bond. The hydrogen atom abstracted by the radical is concomitantly returned to C-3. Thus, the C-3 radical recaptures the same hydrogen atom originally abstracted by the first Cys439 residue and the dNTP is free to leave the enzyme. After substrate reduction by RNR, a disulphide bridge between Cys225 and Cys462 is formed and has to be reduced (step 5) before the enzyme can be active again. The oxidised enzyme can be reduced by at least two systems, the thioredoxin and glutaredoxin system (Jordan and Reichard 1999).

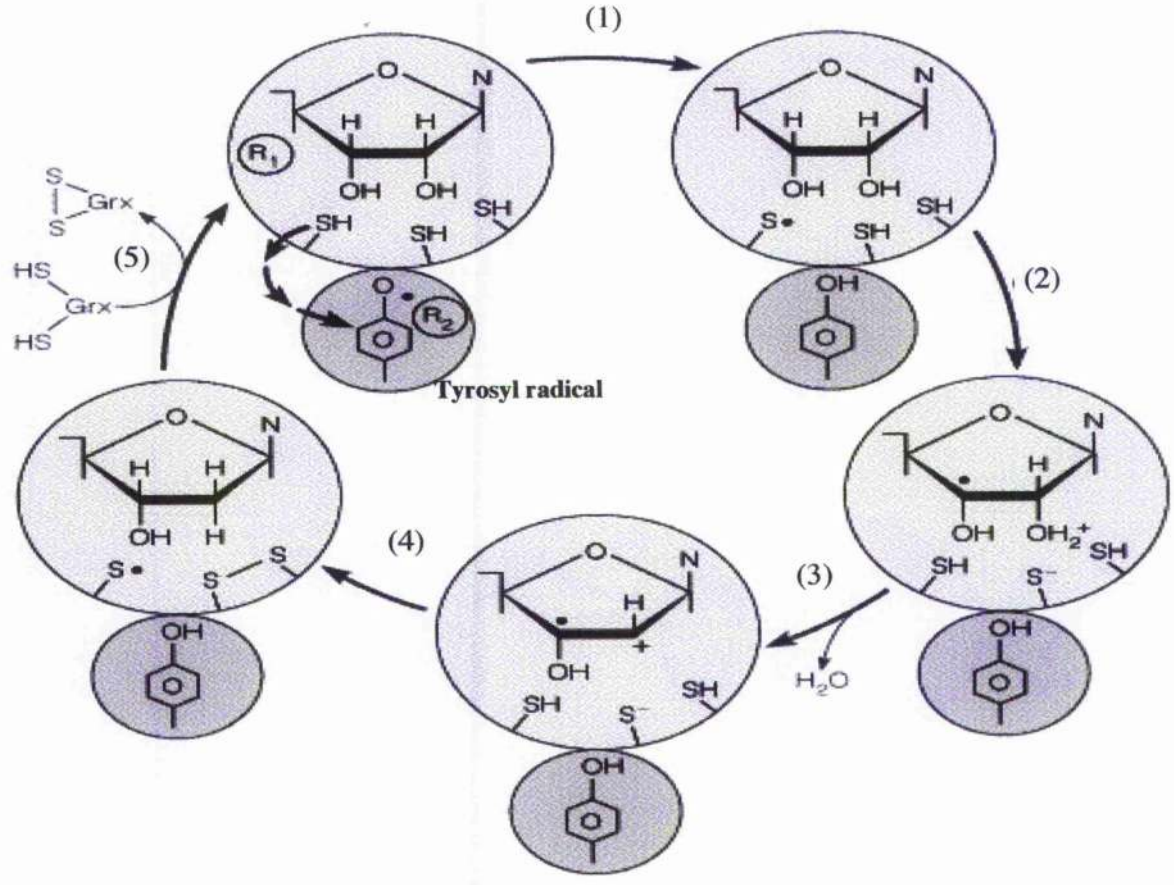


Figure 4. Biochemical pathway showing the reduction of ribonucleotides to deoxy-ribonucleotidesby ribonucleotide reductase.
For details of the 5 steps see text page 12 (Reichard and Ehrenberg 1983).

In both cases exposed cysteine residues are oxidized to a disulfide in the reaction catalysed by RNR. In turn, the reduced thioredoxin or glutaredoxin is regenerated by electron flow from NADPH, the ultimate reductant. This reaction is catalysed by flavin enzymes thioredoxin reductase or glutathione reductase, respectively (Figure 5).

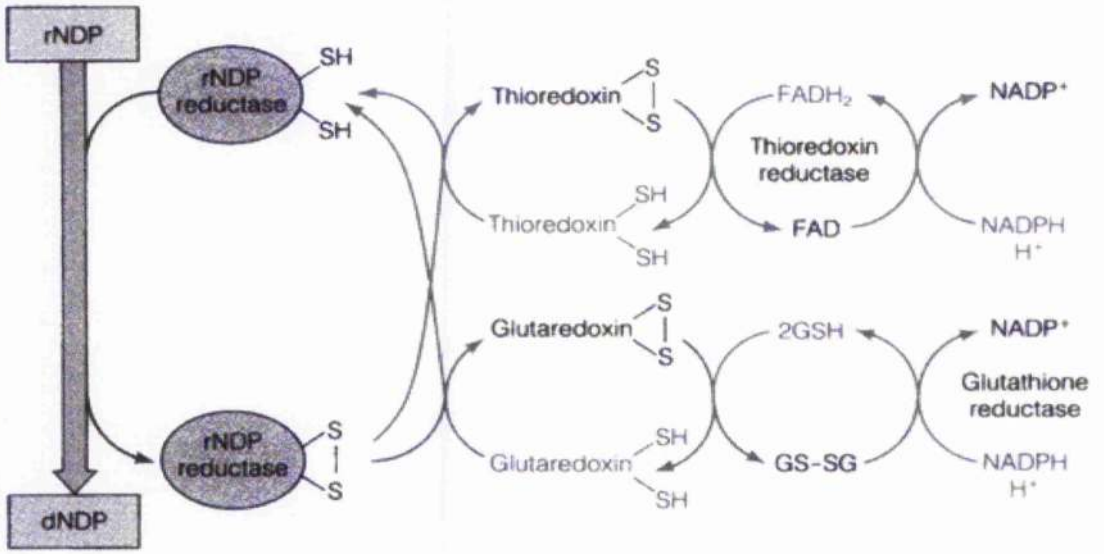


Figure 5. Reductive electron transport sequences in the action of ribonucleotide reductase.

Electrons flow from NADPH to bound FAD (oxidized form of flavin adenine dinucleotide) of the reductase and then to the disulfide of the oxidised thioredoxin/glutaredoxin (Mathews and van Holde 1996).

Regulation

RNR is regulated in mammalian cells by three mechanisms:

i) Allosteric control of the activity and specificity of RNR by NTP effectors (Kashlan et al. 2002).

ii) S-phase dependent transcription of RNR genes (Bjorklund et al. 1990).

iii) Rapid, proteosome-dependent proteolysis of R2 (RNR small sub-unit) in late mitosis (Chabes and Thelander 2000).

i) Balanced pools of dNTPs are essential for the fidelity of DNA replication. Allosteric regulation of NDP (CDP, ADP, GDP, UDP) reduction by ATP and dNTPs is one of the major regulatory mechanisms for deoxy-ribonucleotide synthesis (Kashlan et al. 2002) (Table 1). Errors in allosteric control can lead to unbalanced dNTP pools, mis-incorporation of deoxy-ribonucleotides in DNA, genetic abnormalities or cell death (Jordan and Reichard 1999).

Nucleotide Bound to			
Activity Site	Specificity site	Activates Reduction of	Inhibits Reduction of
ATP	ATP or dATP	CDP, UDP	
ATP	dTTP	GDP	CDP, UDP
ATP	DGTP	ADP	CDP, UDP*
dATP	Any effector		ADP, GDP, CDP, UDP

*dGTP binding inhibits reduction of pyrimidine nucleotides by the mammalian enzyme but not by the *E. coli* enzyme (Stubbe and van Der Donk 1998)

Table 1. Allosteric control of the activity and specificity of RNR by NTP effectors.

Each polypeptide of the R1 sub-unit contains two allosteric sites, one of which controls the overall activity of the enzyme, whereas the other regulates substrate specificity. Overall catalytic activity of RNR is diminished by the binding of dATP to the allosteric site, which signals the abundance of dNTPs; this feedback inhibition is reversed by the binding of ATP. Substrate specificity is controlled by binding ATP, dATP, and dTTP/dGTP to the allosteric site, all of which can compete for the same allosteric specificity site: dATP and ATP stimulate CDP reduction, dTTP stimulates GDP reduction and dGTP stimulates ADP reduction (Elledge et al. 1992). In addition, class Ia, II, and some class III RNRs contain an extra allosteric site, referred to as the active site. This active site activates or inhibits the overall activity of the enzyme, with ATP and dATP acting as enhancer and inhibitor, respectively (Jordan and Reichard 1999). dATP, which can bind to both sites, binds more strongly to the substrate specificity site, which has also been called the high affinity site (Stubbe and van Der Donk 1998).

Alignment of the available sequences of the R1 sub-unit shows that the residues involved in the binding of the allosteric effector nucleotide are conserved to a high degree. An example of this is Asp232 and Arg262 in loop 2, that are both essential for binding of the sugar and phosphate of ribonucleotides, and are both perfectly conserved. Other residues interacting with the base maintain the same chemical character in different species. The residues allowing for the high flexibility, required to accommodate allosteric regulation, are also conserved (Torrents et al. 2002).

ii) Mammalian RNR is located in the cytoplasm and is transcriptionally regulated during the mitotic cell cycle. Levels of R1 are constant and in excess during the cell cycle because of a long half-life (more than 20 hours). The R2 protein, in contrast, is made in the late G1 phase slightly before DNA replication and is completely stable during S and G2. It has a half-life of 3 hours in exponentially growing cell cultures and is limiting for activity (Bjorklund et al. 1990; Eriksson et al. 1984; Mann et al. 1988).

iii) RNR small sub-unit R2 is specifically degraded by a proteosome-dependent process, both during the mammalian cell cycle and indirectly after DNA damage or a replication block (Chabes and Thelander 2000). In combination with the dATP feedback control, the controlled degradation of the R2 directly regulates ribonucleotide reduction in proliferating mammalian cells. The specific degradation of R2 in late mitosis, inactivating ribonucleotide reduction in G1, may contribute to preventing reduplication of DNA before the next S phase. This enables the S phase dNTP pools to be regulated at a level that is optimal for replication, but which does not increase even when the limiting R2-protein is overproduced.

A recently discovered R2-like gene product, p53R2, is induced in response to DNA damage by the p53 protein, unlike R2 (Tanaka and Okayama 2000; Lozano and Elledge 2000). Cells in S and G2 phases of the cell cycle, which do not normally make R2, induce DNA-repair in a p53-dependent manner. R1 can function as the normal partner of p53R2 protein and the R1-p53R2 complex may explain how resting cells can supply deoxy-ribonucleotides for DNA repair. This observation strongly suggests that p53R2 may be most important for repair in phases of the cell cycle outside S and G2 (Guittet et al. 2001).

1E G1 regulators – E2F and RB

In mammalian systems, progression through G1 and into S phase is in part regulated by activation (and in some cases inhibition) of gene transcription, whereas progression through the later cell cycle phases appears to be regulated primarily by post-transcriptional mechanisms. These G1 transcriptional control mechanisms are conserved from yeast to mammals (La Thangue 2001).

In mammalian cells passage through the R point during G1 critically depends on the activation of the transcription factor E2F (Ginsberg 2002). There are six known members of the E2F family, E2F1 to E2F6, and two hetero-dimerization partners of E2F, Dp-1 and Dp-2, encoded by the DP gene family (La Thangue 1996; Cartwright et al. 1998). A majority of E2F-regulated genes encode proteins that are essential in DNA replication and in cell cycle progression, for example DHFR, *CDC6* and cyclin E (Geng et al. 1996; Wells et al. 1997; Hateboer et al. 1998).

E2F itself is a key downstream target for the retinoblastoma tumour suppressor protein RB, the product of one of the most common tumour suppressor genes in human cancer (La Thangue 2001). Also, two RB-related proteins, p107 and p130, which show related structures and similar biochemical properties to RB, are involved in these processes (Classon and Dyson 2001). Collectively, these three proteins are known as pocket proteins.

RB is an active repressor of gene transcription when bound to E2F-regulated promoters during G1, which play an important role in cell-cycle control. Thus, RB regulates progression through R point in G1, and the expression of proteins essential for the initiation of DNA replication, including the three classes of RNA polymerases I, II and III (Lipinski and Jacks 1999).

The RB pocket proteins interact directly with E2F complexes with different specificity, and associate with the E2F complexes in a temporally modulated schedule during the cell cycle (Dyson 1998). RB binding to E2F1 in G1 stabilises a RB/E2F1/DP1 complex that allows RB itself to express its control on the G1/S phase progression, and so protects E2F1 from degradation by the ubiquitin-proteosome

pathway. Like RB, p107 and p130 interact with and inhibit the transcriptional activity of E2F/DP heterodimers, and they are also substrates of G1 CDK/cyclin complexes (Baldi et al. 1995). However, p107 and p130 appear to function in growth-signalling pathways distinct from those involving RB (Vairo et al. 1995). p107 and p130 interact with E2F4, E2F5 and RB to specifically bind E2F1-3, although an E2F4/RB complex is detectable during G1/S phase transition (Pagi et al. 1996). This suggests that RB might play the role of universal regulator of E2F/DP member function (Malumbres and Barbacid 2001).

Phosphorylation of RB proteins by CDKs during middle and late G1 phases releases RB from E2F, leading to de-repression and/or activation of E2F–dependent genes, and subsequent entry into S phase (Figure 6) (Lipinski and Jacks 1999).

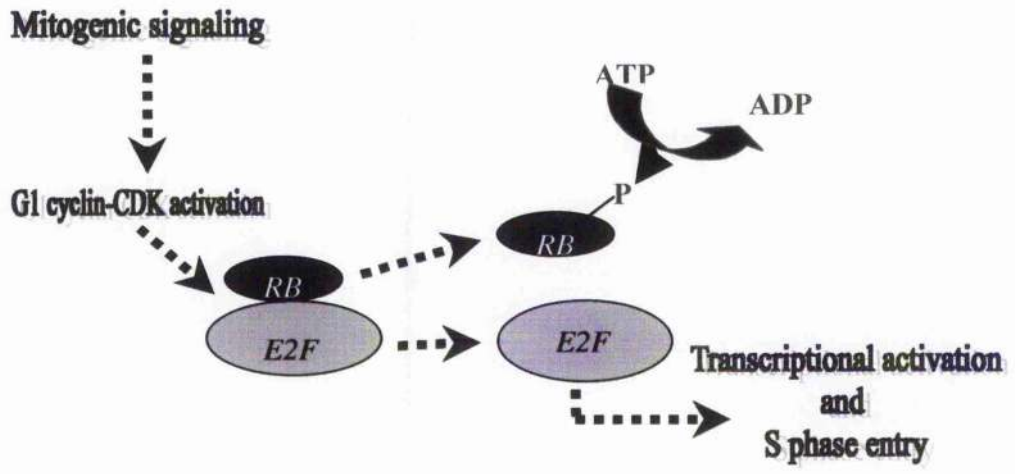


Figure 6. Schematic diagram showing the regulation of E2F by RB and the G1 cyclin/CDK complex.
For details see text.

Cyclin/CDK mediated phosphorylation of RB is the mechanism by which the growth suppressive function of RB is turned off during the G1/S phase transition, and in the following phases of the cell cycle. Thus, a loss of function of physiological inhibitory constraints of cyclin/CDK complexes can produce a state of uncontrolled cell proliferation (Bartek and Lukas 2001).

The D type cyclins recruit CDKs to their substrates and are unique in their ability to physically interact with RB (Matsushime et al. 1994). As synthesis of D-type cyclins in many cells is growth factor dependent, they are the first cyclins to be expressed after mitogenic stimulation of quiescent cells, and are perceived as a link between the cell cycle machinery and growth factor-induced signalling (Lukas et al. 1996). Once these cyclins have been accumulated, they associate to form complexes with CDK4 and CDK6 to regulate their function. Cyclin D1 may also have additional functions as a possible transcriptional regulator, which modulates the activity of transcription factors (Coqueret 2002).

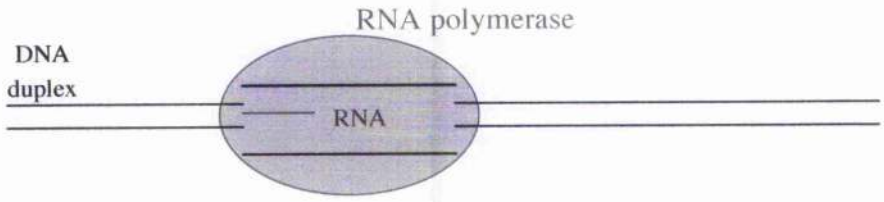
As the cell proceeds through the R point into late G1, other cyclins, such as cyclin E and cyclin A, form complexes with CDK2, which specifically target RB phosphorylation sites to maintain RB phosphorylation (Bartek et al. 1996). p107 and p130 also associate with the G1- specific cyclin E/CDK2 and cyclin A/CDK2 complexes to form E2F-containing complexes still able to bind E2F. Thus, RB, p107 and p130 co-operate to confine the activation of E2F, and thus expression of E2F-responsive genes to precise stages of the cell cycle (Bartek and Lukas 2001).

1F Eukaryotic transcription

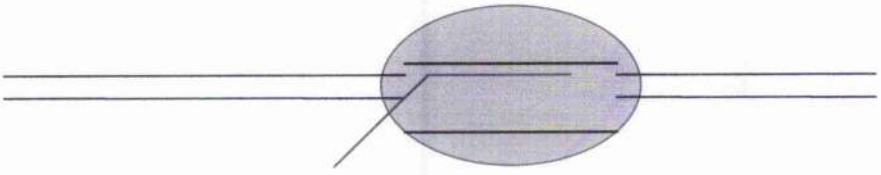
I *Transcription mechanisms*

Transcription is the process by which one strand of a double-stranded DNA molecule is used as a template for the synthesis of a complementary single-stranded RNA strand. This is the crucial stage in the overall process of gene expression, which ultimately leads to synthesis of the protein encoded by a gene. RNA polymerase and transcription factors form the complex that initiates transcription. Therefore, RNA polymerase is the crucial enzyme responsible for catalysing transcription (Turner et al. 2000).

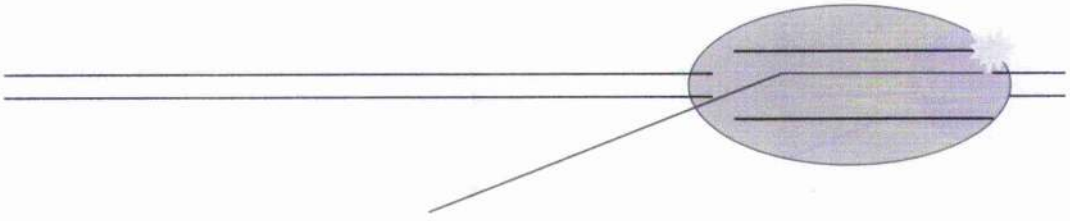
Transcription occurs in multiple steps in the following way:



INITIATION: RNA polymerase initiates a new nucleic acid strand when it finds a specific initiation site, called a promoter, on duplex DNA. The polymerase binds the promoter DNA, temporarily separates the two strands in that region (usually unwinding about 17 base pairs of template DNA), and begins generating a new RNA strand. The position of the first synthesised base of the RNA is called the start site and is designated as position +1.



ELONGATION : DNA is unwound ahead of the moving polymerase, and the helix is reformed behind the polymerase as it moves along the DNA and sequentially synthesises the RNA chain.



TERMINATION : This is the dissociation of the transcription complex and the ending of RNA synthesis. It involves the recognition by the polymerase of a specific sequence known as the 'terminator' sequence.

There are three different RNA polymerases identified so far in eukaryotic cells, designated I, II and III. Each eukaryotic RNA polymerase catalyses transcription of genes encoding different classes of RNA. RNA polymerase I is responsible for the synthesis of precursor ribosomal RNA (rRNA). RNA polymerase

II catalyses transcription of all protein-coding genes by producing mRNAs and also produces four small RNAs that take part in RNA splicing. Lastly, RNA polymerase III transcribes the genes encoding a whole range of small, stable RNAs including tRNAs and some smaller rRNAs. The RNA polymerases contain two large sub-units and 12-15 smaller sub-units, some of which are present in two or all three of the polymerases.

Combinations of short sequence elements in the immediate vicinity of a gene act as recognition signals for transcription factors to bind to the DNA in order to guide and activate the polymerase, as RNA polymerases are not able to initiate transcription by themselves. A major group of such short sequence elements is often clustered upstream of the coding sequence of a gene, where they collectively constitute the promoter. After general transcription factors bind to the promoter region, an RNA polymerase binds to the transcription factor complex and is activated to initiate the synthesis of RNA from a unique location.

Transcription factors are *trans*-acting elements, which recognise and bind specific *cis*-acting promoter DNA sequence elements. Promoters are a recognised class of *cis*-acting sequence element for individual genes, and are present a short distance upstream of the open reading frame. Other examples of *cis*-acting sequences include enhancer elements that can initiate transcription and enhance the transcriptional activity of specific genes. Enhancer elements may be located at a great distance, either, upstream, downstream or even within the midst of a transcribed gene that they controls. They can even exert their stimulatory actions over distances of several thousand base pairs, and their function is independent of their orientation. Binding of specific proteins to an enhancer element either stimulates or decreases the rate of transcription of the associated gene. Enhancer elements appear to work by binding to gene regulatory proteins and, subsequently, the DNA between the promoter and enhancer loops out, allowing the proteins bound to the enhancer to interact with the transcription factors bound to the promoter, or with the RNA polymerase. Enhancers are effective only in certain cells, for example the immunoglobulin enhancer functions in B-lymphocytes, but not elsewhere (Turner et al. 2000).

Another class of *cis*-acting elements are called "response" elements. These are found only in selected genes whose expression is controlled by certain external

factors, such as a hormone, a growth factor, or by an internal signalling molecule such as cAMP. They are often located a short distance upstream of the promoter elements (often within 1 kb of the transcription start site). Finally, so-called "silencers", the most recently discovered *cis*-acting elements, negatively control expression. They have been reported to be found both upstream and downstream of transcriptional start, and occasionally lie within introns (Lodish et al. 1995).

Many genes are also controlled by multiple promoter proximal elements. Regulatory DNA sequences, located within 200 base pairs of the promoter, bind specific proteins, thereby modulating transcription of the associated protein-coding gene (Turner et al. 2000).

II *RNA polymerase II*

Genes actively transcribed by RNA polymerase II have a promoter element, which always includes a TATA box. This consensus sequence is a heptanucleotide of A and T residues, flanked by GC rich sequences, and is present in nearly all eukaryotic genes giving rise to mRNA. A single base change in this nucleotide sequence drastically decreases *in vitro* transcription of TATA containing promoters. The TATA box is not always sufficient when strong promoter activity is necessary, and additional elements can be located between -40 and -110 base pairs, relative to the ATG. Many promoters also contain a CAAT box at about –80 base pairs, which is usually the strongest determinant of promoter efficiency. Constitutive genes also contain a GC box in their promoter, containing variants of the consensus sequence GGGCGG. The positions of these upstream sequences vary amongst promoters. Both the CAAT box and GC boxes appear to be able to function in either orientation, although their sequences are asymmetrical (Naar et al. 2001).

In order to initiate transcription RNA polymerase II is guided to the start site by a set of transcription factors known collectively as TFII (transcription factor RNA polymerase II). Initiation begins with the binding of a component of TFIID, TATA box binding protein (TBP), to the TATA box, which is the heart of the initiation complex. The surface of the TBP provides docking sites for the binding of other

components of TFII whereby TFIIA is recruited, followed by TFIIB (Reinberg et al. 1998). RNA polymerase II and then TFIIE join the other factors to form a complex called the basal transcription apparatus. The assembly of additional transcription factors occurs on this nucleus and DNA can be looped so that sites further apart in the linear sequence are brought closer together. This enables proteins bound to distant enhancers to interact with the TBP and participate in the formation of a functional transcription complex (Dynlacht et al. 1991).

1G Yeast

I *Introduction to yeast*

The discovery that all eukaryotes have similar mechanisms underlying the regulation of their cell cycles has validated the proposal to study it in the simplest possible experimental system that can be manipulated with the most powerful genetic tools. Thus, the application of yeast genetics to the cell division cycle has proved to be of significant benefit, as it has allowed the identification of a number of genes critical for controlling cell division (Qin and Li 2003). Yeasts have been especially useful for isolation of mutants that are blocked at specific steps in the cell cycle, or that exhibit altered regulation of the cycle. Temperature and cold-sensitive mutants (*ts* and *cs* mutants, respectively) with defects in specific proteins essential to progress through the cell cycle are readily recognised microscopically (Hartwell 1974; Nurse et al. 1976). This makes it possible to learn about the function of a conserved regulator rapidly in yeast and use the knowledge to understand complex metazoan systems, in which experimental procedures are more difficult to perform.

Although a considerable amount of knowledge regarding the regulation of eukaryotic cell cycle stems from the budding yeast *Saccharomyces cerevisiae*, a substantial amount of data has also been generated from the fission yeast *Schizosaccharomyces pombe*. Both these yeast species have been used with great success over the past years as model organisms to elucidate eukaryotic cell cycle control mechanisms (Hartwell 1974; Nurse 1985; Lee and Nurse 1987; Qin and Li 2003).

I I *Budding yeast*

S. cerevisiae is a single celled organism that reproduces by budding, with the bud growing in size throughout the cell cycle, providing a morphological indicator of cycle progress. A fundamental step in understanding the cell division cycle in budding yeast was the isolation of so called cell division cycle (*cdc*) mutants. By definition, mutations in genes required for cell division are lethal for the cell, as they are essential, which precludes the use of conventional mutants to identify such genes. The identification of *ts* conditional lethal cell cycle mutants was a major step in cycle studies, as it allowed the identification of cell cycle genes. These mutants only display their mutant phenotype at the non-permissive temperature, when they eventually die. At the permissive temperature, however, they behave essentially like wild-type cells and can be manipulated to allow experiments to be performed (Hartwell 1974). Consequently, studies with these genes allowed the identification of genes critical for cell cycle progression in budding yeast, and ultimately contributed significantly to our current knowledge of cell cycle mechanisms in higher eukaryotes and mammalian models.

In addition, the mutants demonstrated that the initiation of certain steps in the cell division cycle is dependent on the completion of one or more preceding steps. For instance, onset of mitosis and nuclear division is dependent on the completion of DNA replication, and the completion of mitosis is dependent on the assembly of the mitotic spindle. These observations were the first evidence for the existence of checkpoint control mechanisms (Hereford and Hartwell 1974).

III *Fission yeast*

S. pombe is also a single celled organism, but is rod shaped and increases in length before dividing in the middle by medial fission, to form two daughter cells. DNA and RNA sequence analyses have been used to demonstrate that fission yeast is phylogenetically as distant from budding yeast as it is from humans. The *Schizosaccharomyces* lineage separated about 1 billion years ago to form an ancestral branch of the ascomycetes (Figure 7) (Sipiczki 2000).

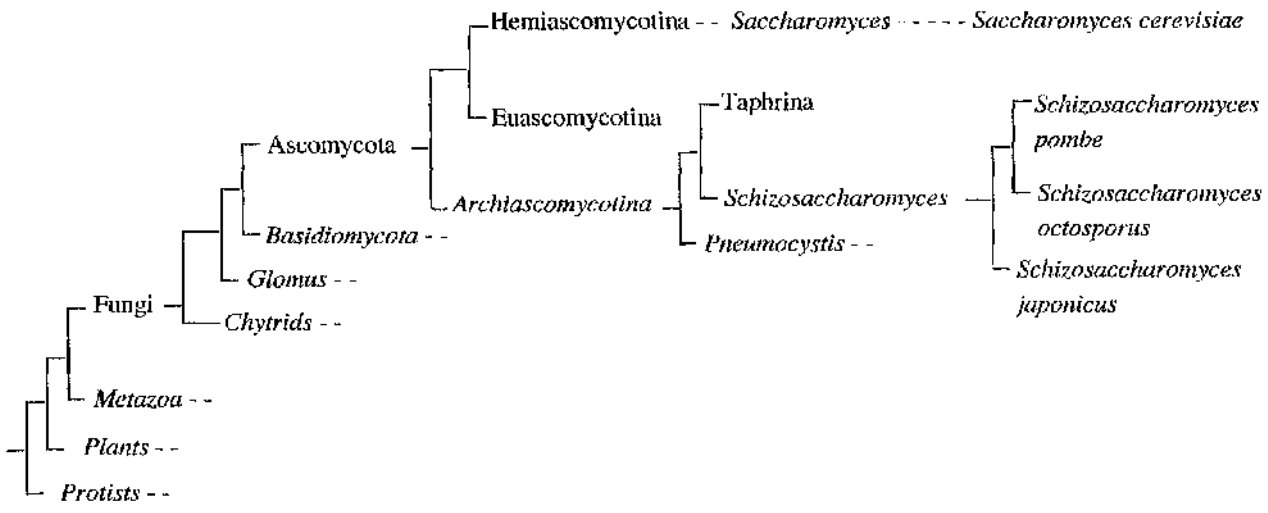


Figure 7. A consensus phylogeny of fission yeast.

Times are estimated as (1) 1,200 million years ago (Ma); (2) 1,100 to 1,000 Ma; (3) 600 to 500 Ma; (4) 400 Ma; (5) 420 to 330 Ma; (6) 250 Ma. Modified from Sipiczki (2000).

Fission yeast cells are usually haploid, with a genome size similar to budding yeast of approximately 14 million base pairs (Mb) in size. In budding yeast this is organised as 17 chromosomes, whereas in fission yeast it is organised as three chromosomes, namely chromosome I (5.7 Mb), II (4.6 Mb), and III (3.5 Mb). The gene density in fission yeast is approximately 1 gene per 2,300 base pairs and the whole genome is predicted to contain approximately 5,400 genes (Wood et al. 2002).

Fission yeast temperature-sensitive conditional lethal *cdc* mutants have also been isolated, which enabled the identification of genes required for cell cycle progress in this organism (Nurse et al. 1976; Nasmyth and Nurse 1981). These *cdc* mutants grow without dividing, and so form elongated cells at the non-permissive temperature, which creates a distinct phenotype when examined microscopically (Figure 8).

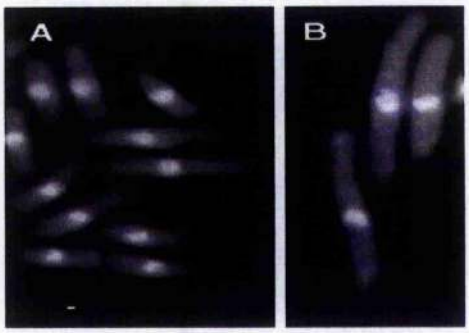


Figure 8. Growth morphology of fission yeast.
A. Wild-type *S. pombe* cells. **B.** *S. pombe* temperature sensitive *cdc*$^-$ mutant cells, after incubation at 36°C for 4 hours.

Another class of *S. pombe* cell cycle mutants, called '*wee*' (from the Scottish word for small) divide before the parental cell has grown to the normal length, forming daughter cells that are thus, shorter than normal (Nurse and Thuriaux 1980). These genes encode regulators of the cell cycle.

IV *Cloning of cell cycle genes*

Temperature sensitive mutations in particular *cdc*$^-$ mutants block progression through the cell cycle at the non-permissive temperature, and eventually cause cells to die (Nurse et al. 1976). The wild-type version of a *cdc*$^+$ gene can be isolated by transforming mutant *cdc*$^-$ cells with a wild-type fission yeast plasmid DNA library. These transformed cells, cultured at the permissive temperature, are then grown at the non-permissive temperature. The mutant cells that take up a plasmid containing the wild-type version of the mutant *cdc*$^-$ gene are complemented, allowing the cell to

replicate and form a colony at the non-permissive temperature. The plasmid bearing the wild-type allele can then be recovered from these cells.

As many of the proteins regulating the cell cycle are highly conserved, it has also been possible to rapidly isolate human genes encoding cell cycle control proteins using yeast as model systems (Nurse 1990). Human cDNAs libraries in yeast expression vectors when transformed into fission yeast *cdc*⁻ mutants, have been found to complement yeast function (Lee and Nurse 1987). A number of human homologues to yeast *cdc* genes have been found to complement yeast *cdc*⁻ mutants, thus permitting their isolation (Sanchez-Diaz et al. 2001) (Introduction, page5).

1H Control points

There are two major control points during the cell cycle of the two yeast species, one in G1 called START (equivalent to the Restriction point "R" in mammals (Pardee 1989) (Introduction, page 3), and another in late G2, just prior to mitosis. At START the cell makes a decision to enter a sexual or vegetative cell cycle. Before START cells are competent to enter either life cycle, but in the absence of nutrients haploid cells stop progression through the mitotic cycle and enter stationary phase. In the presence of cells of the opposite mating type, cells can enter the sexual cycle of conjugation, meiosis and sporulation.

Another major control point for the mitotic cell cycle occurs at the G2/M transition, when processes are activated to assess whether DNA replication is completed, and to ensure that DNA is not damaged. The mechanisms regulating this step are conserved among higher eukaryotes from yeasts to mammalian cells (Doree and Hunt 2002).

In yeasts, a number of checkpoint responses to DNA damage or S phase arrest have been identified. These include the DNA damage checkpoint, which prevents mitosis when DNA damage occurs, and the intra-S phase checkpoint, which causes a delay of progression of DNA replication in response to DNA damage (Humphrey 2000).

1 I DNA Replication in yeast

I Budding yeast

DNA replication is accurately and temporally regulated during the cell cycle in all eukaryotes. In yeasts, as in mammalian cells, each chromosome contains autonomously replicating sequences (ARS) which, depending on their location in the chromosome, initiate replication either in early or late S phase. Pre-replicative complexes (pre-RC) are assembled during M and G1 phase (Nasheuer et al. 2002). Budding yeast ARS elements are typically short, only about 100 to 200 base pairs, compared to fission yeast ARS, which are much longer at about 500 to 1000 base pairs and appear to be more diffuse and functionally less efficient (Dubey et al. 1996; Clyne and Kelly 1997).

In budding yeast the pre-RC, essential for initiation of replication, contains the origin-recognition complex (ORC), the minichromosome-maintenance complex (MCM) and Cdc6p (Kelly and Brown 2000). The pre-replication complex is formed at the end of mitosis. pre-RC formation is initiated by the association of Cdc6p with the ORC, followed by the binding of a set of six related proteins, Mcm2p to Mcm7p (Tanaka et al. 1997). After complex formation the components of the complex are phosphorylated by the S phase CDKs Clb5p or Clb6p, in association with the Cdc28p, and the Dbf4p/Cdc7p kinases, that leads to initiation of DNA replication (Donaldson and Blow 1999).

ORC, consisting of six polypeptides, remains bound to ARS throughout the cell cycle and is essential for DNA replication and cell division (Klein and Kreuzer 2002). Cdc6p, when over-expressed, can bind to ORC throughout the cell cycle, although the binding of MCM proteins to the ORC-Cdc6p complex during G2 and M phase is inhibited, most likely due to inhibitory effects from S-CDK protein kinases. This strongly suggests that Cdc6p, which is also known to have sequence similarity to the large sub-unit of ORC (Drury et al. 1997), plays a central and limiting role in the onset of DNA replication in *S. cerevisiae*. Cdc6p is synthesised during the cell cycle in two peaks, initially in late mitosis after anaphase, and secondly in late G1 and is targeted for proteolysis at onset of S phase (Donovan et al. 1997).

II *Fission yeast*

Similar mechanisms regulating DNA replication are proposed to operate in other organisms, including fission yeast, since homologues of pre-RC proteins and its regulators have been found in many organisms (Dutta and Bell 1997). However, one notable difference is that the ARS in fission yeast and mammalian cells are not discrete sequence specific regions of DNA as in budding yeast, but are spread out over large AT rich regions. In fission yeast the proteins involved in the initiation of DNA synthesis include Cdc18p (a homologue to budding yeast Cdc6p), Cdt1p (Yanow et al. 2001), ORP proteins (homologous to ORC proteins) (Leatherwood et al. 1996), MCM proteins (Maiorano et al. 1996), and the Hsk1-Dfp1p/Him1p complex (a counterpart of Cdc7-Dfb4p) (Takeda et al. 1999). Many of these components are also needed for restraining mitosis during S phase in the cell cycle (Moser and Russell 2000).

Initiation of DNA synthesis occurs once pre-RC has been formed and is triggered by cell cycle regulated protein kinases. Protein kinase activity is stimulated by the binding of Dfp1p (the homologue of Dfb4p in budding yeast) and Hsk1p (the homologue to Cdc7p in budding yeast), which phosphorylate MCM2p (Cdc19p) (Masai et al. 1995; Brown and Kelly 1998; Takeda et al. 1999). The cell cycle regulated accumulation of Dfp1p at the G1/S boundary initiates activation of Hsk1p, and the actual trigger for replication initiation is the phosphorylation of pre-RC components by this kinase and another cell cycle specific protein kinase, Cdc2p (Brown and Kelly 1999b).

At the onset of S phase the binding complex of Cdc18p, Cdt1p and ORP, essential to load the MCM protein complex onto DNA, is recruited to the replication start sites to licence DNA for replication (Kearsey et al. 2000). Cdt1p interacts with Cdc18p and enhances the ability of Cdc18p to induce continuing DNA synthesis, suggesting that both proteins work together to promote DNA replication (Nishitani et al. 2000). Cdc18p is phosphorylated late in G1, possibly by the kinase complex Cdc2/Cig2p, resulting in its dissociation from DNA and subsequent degradation. The down regulation of Cdc18p, due to *cdc18*$^{+}$ transcription being switched off during S phase, prevents re-initiation of DNA synthesis until completion of M phase (Yanow et al. 2001).

In addition, regulation of both these proteins in G2 plays a crucial role in preventing the re-initiation of DNA synthesis until the next cell cycle (Yanow et al. 2001). Cdt1p has been detected in *S. cerevisiae*, and its importance for DNA licensing in eukaryotes has been confirmed by its discovery in *Xenopus* oocyte lysates (Maiorano et al. 2000; Tanaka and Diffley 2002).

The ORP complex in fission yeast also consists of six sub-units similar to ORC in budding yeast, SpOrc1p-SpOrc6p, which are encoded by the *orp1-orp6* genes, respectively (Lygerou and Nurse 1999; Moon et al. 1999). These sub-units form a complex throughout the cell cycle and their nuclear localisation and chromatin association remain constitutive. The SpOrc2 sub-unit is phosphorylated in cells arrested in M phase, when Cdc2p activity is high, and its de-phosphorylation accompanies completion of mitosis and cell cycle progression to G1 (Leatherwood et al. 1996).

1J Cyclins and CDKs

I *Activity*

Passage through control points and checkpoints in yeast, as in mammalian cells during the cell cycle, is controlled by the activity of CDKs. However, unlike higher eukaryotic cells which have multiple CDKs that regulate cell cycle progression, a single CDK in association with B-type cyclins regulates both S phase and mitosis in budding and fission yeast. Thus, the specific cyclins and CDKs required for the transition of mammalian cells through G1 and S phase differ to those needed for similar processes in yeast (Clarke and Gimenez-Abian 2000).

Initiation of DNA synthesis and nuclear divisions in budding and fission yeast is attributed to the activity of the single CDK catalytic sub-unit encoded by the *CDC28* and *cdc2*$^{+}$ genes, respectively (Doree and Hunt 2002). Other CDKs may play a role in certain circumstances (Nishizawa et al. 1998). Dominant forms of Cdc2p advance entry into mitosis (Nurse and Thuriaux 1980), whereas a loss of Cdc2p

kinase activity results in cells arresting before START in G1 and in late G2 before M phase (Nurse and Bissett 1981a).

The yeast Cdc2p/Cdc28p kinase, required for entry into both S phase and mitosis in yeasts, is the homologue to human CDK1, which is only essential for mitotic entry in mammalian cells (Riabowol et al. 1989; Hamaguchi et al. 1992). The amino acid sequences of human and yeast Cdc2p proteins are 65% identical - so similar that a mutant yeast lacking *cdc2*$^{+}$ can be rescued by insertion of the homologous human gene (Lee and Nurse 1987). Thus, the regulatory control over Cdc2p is highly conserved in eukaryotic evolution, with its activity being regulated by the binding of B-type cyclin and reversible phosphorylation that control the activity of the complex (Dunphy 1994; Doree and Hunt 2002).

II *Fission yeast*

In fission yeast once START has been traversed Cdc2p is activated when complexed with the B-type cyclins Cdc13p and Cig2p. Cdc2p is also known to associate with two other cyclins, Puc1p and Cig1p, which may monitor cell growth or cell size during G1 phase of the cell cycle, and induce the G1/S transition indirectly (Martin-Castellanos et al. 2000).

The amount of Cdc2p protein remains constant throughout cell cycle, while cyclin protein levels oscillate (Moser and Russell 2000). A proposed model for cell cycle specific regulation of Cdc2p is shown in Figure 9. Cig2p, the major S phase cyclin, plays the principal role in promoting entry into DNA replication (Fisher and Nurse 1996), whereas Cdc13p is needed for Cdc2p activity at the onset of M phase (Moreno et al. 1989). Consequently, the level of Cig2-Cdc2p complex activity peaks around G1/S (Mondesert et al. 1996). Cdc13p levels are low in G1 phase, but increase in G2 phase and are maintained until the end of M phase (Booher et al. 1989).

The inactive state of the Cdc2p complexes in G1 is maintained by both direct binding of a CKI, Rum1p, and proteolytic degradation of the cyclin sub-unit (Kominami et al. 1998). Rum1p accumulates in mitotic anaphase and persists through

G1, inhibiting the kinase activities of Cdc2/Cdc13p and Cdc2/Cig2p and targets Cdc13p for degradation. At the G1/S transition phosphorylation of Rum1p itself by a ubiquitination dependent mechanism results in its degradation, allowing Cdc2/Cig2p to induce S phase entry (Benito et al. 1998).

In mammalian cells, as in yeast, the CKIs are marked for degradation by a ubiquitin-conjugating enzyme system consisting of E1, E2 and E3. E1 is the ubiquitin-activating enzyme, E2 the ubiquitin conjugation enzyme and E3 the ubiquitin ligating enzyme (Ciechanover et al. 2000). Whilst E1 enzyme initiates the first step in the reaction, a variety of E2 enzymes in conjunction with E3 enzymes seem to determine the specificity for the proteins targeted for ubiquitination. Once the target proteins are ubiquinated they are readily degraded (Hilt and Wolf 1996). This ubiquitin-dependent proteolysis is also responsible for the destruction of cyclins, contributing to periodic changes in their levels during cell cycle (Deshaies et al. 1995). It also plays a key regulatory function during the G2/M transition (Smits and Medema 2001). Completion of mitosis is regulated by APC, which in fission yeast functions to specifically target the mitotic B type cyclins Cdc13p and Cig1p for degradation in G1 (Blanco et al. 2000). On passage through START APC-mediated degradation is inhibited and the Cdc2/Cdc13p complex accumulates, increasing to peak in late G2.

The transcription factor complex DSC1 (DNA synthesis control 1) plays a key role in the inactivation of Cdc13p by controlling the periodic production of APC regulators (Tournier and Millar 2000). Thus, Cdc13p protein levels accumulate from S phase to the end of mitosis when it is degraded by APC, and Cig2p protein and mRNA levels both peak around G1/S, when the level of Cdc13p protein is low (Mondesert et al. 1996). Translational inhibition of Cdc13p and Cig2p represents a mechanism that contributes to Cdc2p inactivation as cells exit from the mitotic cell cycle and prepare for meiosis (Grallert et al. 2000). This acts as another cell cycle control mechanism.

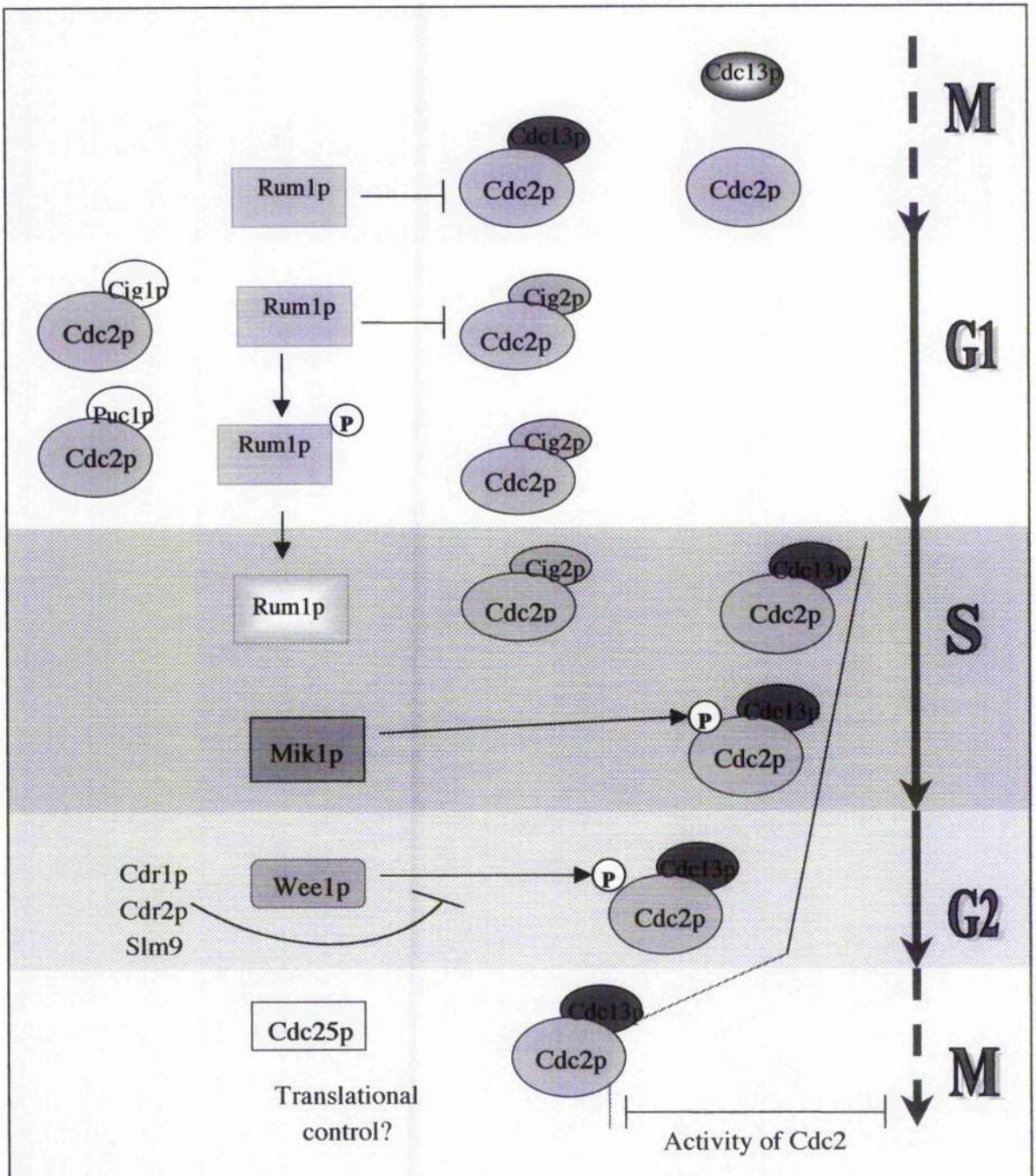


Figure 9. Model of cell-cycle specific regulation of Cdc2p in fission yeast.

Cdc2p activity is inhibited when the cells exit mitosis by degradation of associated Cdc13p. Accumulation of the Cdc2p inhibitor Rum1p ensures that Cdc2p activity is kept low throughout late M phase and G1-phase. Phosphorylation then targets Rum1p for degradation, resulting in a rise in Cdc2p-Cig2p activity, which induces entry into S phase. Cdc13p accumulates during S phase and remains associated with Cdc2p until it is degraded upon exit from M phase. In S and G2, its activity is down regulated through the inhibitory phosphorylation mediated by Mik1p during S phase, and Wee1p during G2 phase. Wee1p is inhibited by the various protein kinases. In contrast Cdc25p phosphatase accumulates during interphase, probably through translational up-regulation. The counter-balance of Wee1p and Cdc25p activity changes as cell size increases: at the proper cell size, Cdc25p is able to bring about Cdc2p activation, driving cells into M phase.

Although Cdc2p associates with four cyclins, only Cdc13p is indispensable for cell cycle progression and is sufficient to trigger both S phase and the initiation of mitosis in the absence of the other cyclins (Stern and Nurse 1996). The *S. pombe* *cdc13*$^{+}$ gene has homology to sea urchin and *Xenopus* cyclin B. Further studies showed that a heterodimer of Cdc13p and Cdc2p form *S. pombe* MPF and, like mammalian MPF, this heterodimer has protein kinase activity (Nurse 1990).

Cdc2p is phosphorylated by Wee1p and Mik1p on the inhibitory residue tyrosine-15 (Tyr-15), in order to restrain activity during S phase and G2. Cdc2p Tyr-15 phosphorylation regulates the timing of mitosis during the normal cell cycle and is also thought to be the method of controlling mitosis following activation of the DNA replication and the DNA damage checkpoints (Rhind and Russell 2001). Mik1p regulation helps to ensure that the onset of mitosis is coupled to the completion of DNA replication during the normal cell cycle or completion of DNA repair in cells that have suffered DNA damage (Baber-Furnari et al. 2000; Ng et al. 2001). It has also been found that Mik1p can be moderately induced in G2 cells in a Chk1p dependent manner, independently of the Mik1p responses in S phase. Chk1p is an important kinase in the DNA replication checkpoint (Christensen et al. 2000).

De-phosphorylation of the Tyr-15 residue is carried out by the Cdc25p tyrosine phosphatase and to a lesser extent by the Pyp3p phosphatase (Millar and Russell 1992). Full activation of Cdc2p by tyrosine de-phosphorylation brings about entry into mitosis. The kinase activity of Tyr-15 phosphorylated Cdc2p is about 30% compared with that of the de-phosphorylated form in fission yeast interphase cells. This activity is sufficient to bring about DNA replication but not mitosis (Fisher and Nurse 1996).

A newly discovered gene, *slm9*$^{+}$, identified in a genetic screen looking for genes that control the timing of the entry into mitosis, regulates Cdc2p activity through Wee1p and is involved in multiple signal transduction pathways that affect cell growth (Kanoh and Russell 2000).

In fission yeast the G2 DNA damage checkpoint promotes cell cycle delay through a double control mechanism, through both up-regulation of Wee1p and down-regulation of Cdc25p, to ensure cell cycle arrest and genomic stability. Due to the

high degree of homology of G2/M cell cycle and checkpoint controls, this model may be applicable to all eukaryotes (Raleigh and O'Connell 2000). Indeed, an analogous inhibitory pathway to tyrosine-19 phosphorylation of Cdc28p (equivalent to Tyr-15 of Cdc2 in fission yeast) has been described in budding yeast. This residue is phosphorylated by Swe1p - a homologue of Wee1p, and de-phosphorylated by Mih1p - a homologue of Cdc25 (Booher et al. 1993). When a bud is not formed properly or the actin cytoskeleton is defective a morphogenesis checkpoint, which requires Swe1p-mediated phosphorylation of Cdc28p, operates to delay nuclear division (McMillan et al. 1999). However, there is no evidence that tyrosine phosphorylation determines the cell cycle timing of mitosis in budding yeast, as in fission yeast.

III *Budding yeast*

The Cdc28p CDK and the G1 cyclins, Cln1p, Cln2p and Cln3p are essential for passage through START in *S. cerevisiae* (Clarke and Gimenez-Abian 2000). START requires the activation of Cln1/Cdc28p and Cln2/Cdc28p. The transcription of the two genes *CLN1* and *CLN2*, like many other genes acting in late G1, is dependent on the accumulation of Cln3p, whose association with Cdc28p rises in late G1 (Koch and Nasmyth 1994). When a threshold concentration of the Cln3p/Cdc28p kinase complex is achieved, a burst of late G1 specific gene transcription occurs, including *CLN1* and *CLN2* (Dirick et al. 1995). Active Cln1p/Cdc28p and Cln2p/Cdc28p complexes act as a positive feedback loop to further stimulate the transcription of *CLNs*. In G1 both B-type cyclins Clb5p and Clb6p also associate with Cdc28p (Nasmyth 1993). Their transcription is concurrent with that of *CLN1* and *CLN2* in late G1 (Schwob and Nasmyth 1993). Two additional budding yeast G1 cyclins, *PCL1* and *PCL2* (formerly called Hcs26p and Orfdp, respectively) are also maximally expressed in G1 (Tyers et al. 1993). Pcl1p and Pcl2p complex with another cyclin-dependent kinase, Pho85p, to promote cell cycle progression (Measday et al. 1994). The Cln3p/Cdc28p-dependent transcription of late G1 specific genes is mediated by two related heterodimeric transcription factors, MBF (*Mlu*I cell-cycle box binding factor) and SBF (Swi4/Swi6 cell cycle box binding factor) (Iyer et al. 2001).

IV *Regulation*

Although proteolysis is of major importance for the regulation of cyclin periodicity, cyclin expression can also be regulated at the level of translation. In budding yeast, expression of the G1 cyclins, Cln2p and Cln3p, is inhibited by means of translational repression under certain conditions (Hall et al. 1998; Philpott et al. 1998), thereby linking translation to regulation of progression through the G1 phase of the cell cycle.

In fission yeast it has been suggested that translational inhibition of *cdc13*$^{+}$ expression contributes to Cdc2p inactivation as cells exit from the mitotic cell cycle and prepare for meiosis (Grallert et al. 2000). This translational regulation of B-type cyclins is important in higher eukaryotes for both cell cycle control and development.

CDK activity is also essential for the regulation of cell cycle progression and is tightly regulated through different mechanisms: binding by activating cyclins, binding by inhibitory CDK inhibitors (CKIs), inhibitory phosphorylation of the CDK and activating phosphorylation of the CDK by a CDK-activating kinase (CAK) (Kaldis 1999). CAK phosphorylation stabilises cyclin-CDK interaction, enhances substrate-binding (Russo et al. 1996), and is essential for CDK activation as a loss of CAK activity causes cell cycle arrest (Larochelle et al. 1998).

In *S. pombe* the CAK, Mcs6p/Mop1p/Crk1p, works with a divergent CDK family member, Csk1p, to activate Cdc2p (Lee et al. 1999). Homologues to CAK in budding yeast, Kin28p, and metazoans, CDK7p (Buck et al. 1995; Damagnez et al. 1995), have been identified suggesting that similar mechanisms exist in all higher eukaryote.

1K Checkpoint controls

Genetic screens in fission yeast and budding yeast have identified many genes needed for G2 checkpoint control, which appear to be highly conserved in evolution. Both DNA damage and S phase arrest are accompanied by the formation of DNA structures that are able to recruit checkpoint kinases into catalytically active complexes (O'Connell et al. 2000).

In all eukaryotic organisms each characterised DNA-damage and replication checkpoint pathway depends completely on one or more members of the phosphoinositide-3-kinase-related (PIK) family (Zhou and Elledge 2000). Mec1p is the PIK in budding yeast and in fission yeast and mammalian cells they are Rad3p and ATM, respectively. Mec1p and Rad3p are needed for both S phase arrest and the DNA damage checkpoints. A protein with similarity to a subgroup of PIK, ATM, is the catalytic sub-unit of the DNA dependent protein kinase (DNA-PK) (Smith and Jackson 1999). DNA-PK is needed for immunoglobulin gene rearrangement, recombination and repair of radiation induced double-stranded breaks (DSBs). Two regulatory sub-units, Ku70 and Ku86, bind to DSBs are needed for DNA-PK to function as a serine/threonine protein kinase, which then plays a key role in non-homologous end joining of the damaged DNA (DeFazio et al. 2002).

Other genes involved in DNA damage processing in yeasts, include fission yeast *rad1*$^{+}$ and the budding yeast homologue *RAD17*, which encode putative 3-5' exonucleases needed for DNA-repair and arrest. Furthermore, *RAD24* in *S. cerevisiae* and *rad17*$^{+}$ in *S. pombe*, display limited homology to human replication factor C, which is required for DNA polymerase sub-unit binding to primed DNA (Boddy and Russell 2001).

Genomic integrity requires that chromosomes are not only fully replicated each cell cycle, but also that sister chromatids are properly segregated at mitosis. Recent data suggests that Trf4p and Trf5p, two closely related proteins in *S. cerevisiae*, act as a DNA polymerase to couple cohesin to replicatioin (Wang et al. 2000b). Two related *TRF4/5* genes in *S. pombe* are *cid1*$^{+}$ and *cid13*$^{+}$. Cid1p and Cid13p are representative of a class of cytoplasmic proteins with poly(A) polymerase

activity (Wang et al. 2000a; Saitoh et al. 2002). Poly(A) polymerase is responsible for bulk mRNA polyadenylation in the nucleus following the site-specific cleavage of primary polII transcripts (Proudfoot et al. 2002). Poly(A) tail length is associated with stability of the mRNA and efficient translation after export to the cytoplasm, as the poly(A) binding protein is an important component of the translation pre-initiation complex (Pestova et al. 2001).

Cytoplasmic Cid1p and Cid13p act to extend the poly(A) polymerase of cytoplasmic mRNAs and increase the levels of corresponding protein products by promoting mRNA stability and/or translation efficiency (Read et al. 2002). In addition Cid1p is found to be specifically required to inhibit mitosis, promoting cell survival when certain DNA polymerases are inhibited (Wang et al. 2000a) and like Cid13p is suggested to be essential during DNA damage checkpoint in order to maintain dNTP pools (Read et al. 2002). Multiple Cid1p/Cid13p-related proteins are also identified in distantly related eukaryotes including plants and humans. At least one of the human Cid1p-like proteins is constitutively cytoplasmic (Read et al. 2002), suggesting similar mechanisms for DNA replication checkpoint control may be present in higher eukaryotes (Wang et al. 2000a).

The proteins encoded by checkpoint genes are mainly protein kinases and in many cases they act by phosphorylating, and thus activating, one or both of the downstream protein kinases. In the case of fission yeast the essential checkpoint proteins are the Chk1p and Cds1p protein kinases. The activation and function of these two proteins is cell cycle dependent (Rhind and Russell 2000b). Chk1p and Cds1p function downstream of a group of checkpoint 'Rad' proteins. These are essential for the S/M replication checkpoint when DNA replication is slowed in order to prevent mitosis by maintaining Cdc2p in an inhibited, tyrosine phosphorylated state (Figure 6) (Saka et al. 1997; Rhind and Russell 1998). The protein kinase Cds1p functions downstream of the checkpoint Rad proteins in order to enforce the S/M checkpoint (McGowan 2002).

Chk1p is not found to be essential for normal yeast cell growth but is important for yeast to survive radiation exposure (Liu et al. 2000a). Chk1p forms a complex with and phosphorylates Cdc25p during S and G2 phases (Furnari et al. 1997; Peng et al. 1997). This keeps Cdc25p sequestered in an inactive complex,

thereby preventing it from de-phosphorylating and activating Cdc2p, and so preventing the G2/M phase transition. This part of the checkpoint pathway is species specific, as it occurs in *S. pombe* and human systems, whereas no *chk1*$^{+}$ related genes have been found in *S. cerevisiae* (Figure 10) (Liu et al. 2000a).

The human homologues of the *chk1*$^{+}$ and *cds1*$^{+}$ genes inhibit Cdc25p, and become activated or hyper-phosphorylated in response to DNA damage via ATM (Blasina et al. 1999; Brown et al. 1999a). In *S. pombe* Rad3p is responsible for phosphorylating the different targets in response to DNA damage and S phase arrest. The functions of most Radp proteins are usually interdependent and so central to DNA-damage and replication checkpoint signalling that deletion of the gene encoding any one of them usually completely eliminates checkpoint signalling (Edwards et al. 1999).

In recent findings it has been suggested that DNA damage-inducible recombination and/or repair processes may compete with and thus slow down replication (Foiani et al. 2000; Liberi et al. 2000; Rhind and Russell 2000b). In addition, it has been suggested that downstream protein kinases in fission yeast, such as Chk1p and Cds1p, only activate the intra-S phase checkpoint when replication forks encounter DNA damage (Rhind and Russell 2000b).

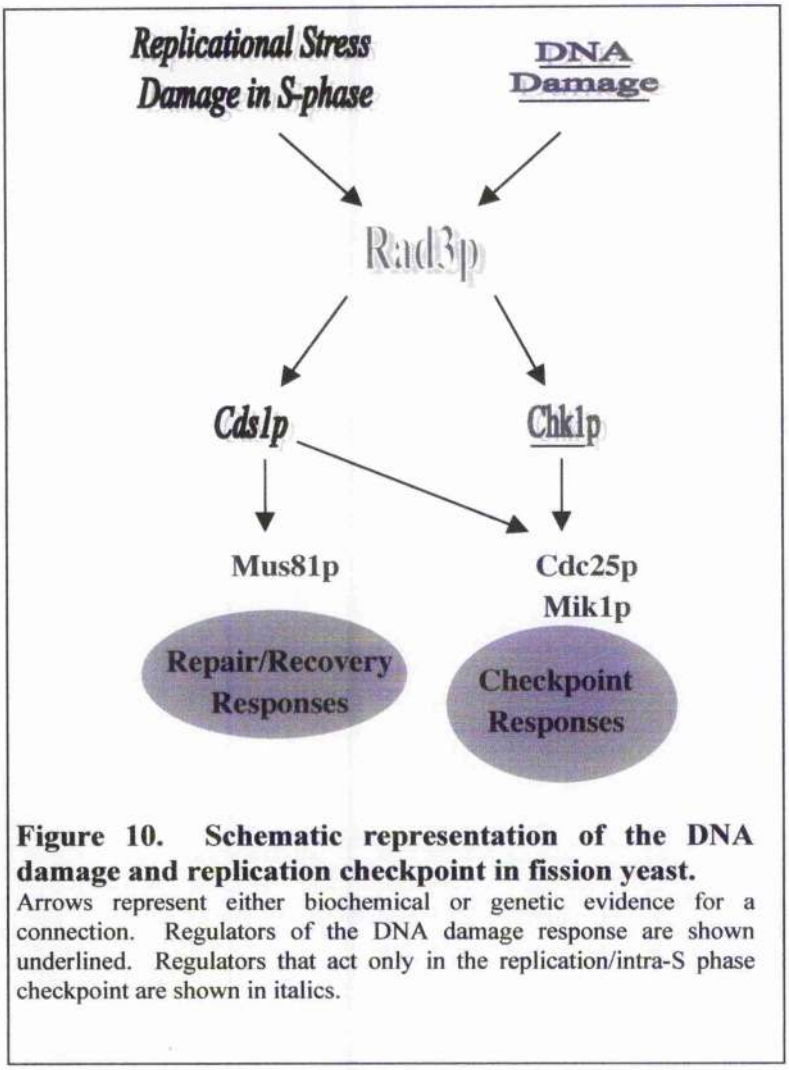


Figure 10. Schematic representation of the DNA damage and replication checkpoint in fission yeast.
Arrows represent either biochemical or genetic evidence for a connection. Regulators of the DNA damage response are shown underlined. Regulators that act only in the replication/intra-S phase checkpoint are shown in italics.

In *S. cerevisiae* the Rad53p protein kinase, homologous to *S. pombe* Cds1p (Tercero and Diffley 2001), is activated by trans-phosphorylation in a Mec1p-dependent manner in response to both DNA damage and S phase arrest (Figure 11) (Gilbert et al. 2001; Pellicioli et al. 2001).

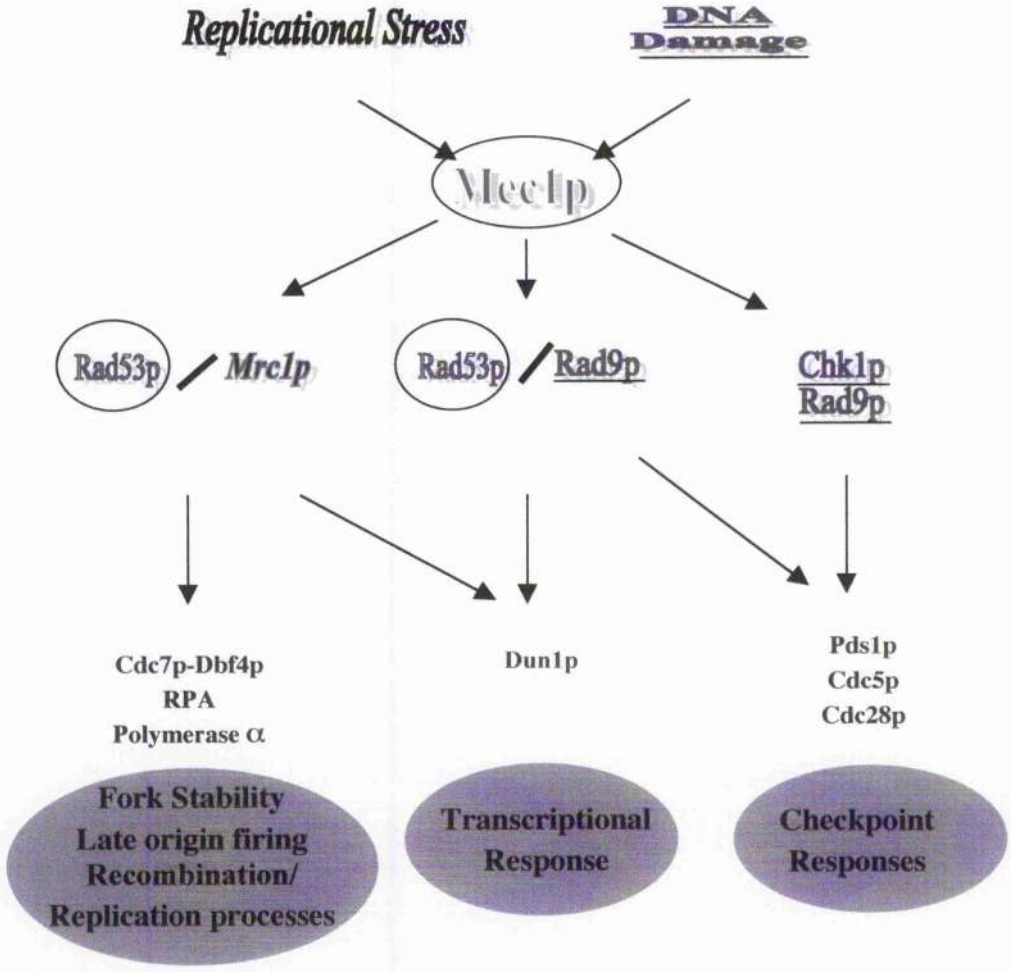


Figure 11. Schematic representation of the DNA damage and replication checkpoints in budding yeast.

Arrows represent either biochemical or genetic evidence for a connection. Regulators of the DNA damage response are indicated underlined. Regulators that act only in the replication/intra-S phase checkpoint are indicated in italics. Protein kinases involved in both branches of the pathway are circled.

1L Transcriptional control at START

I *Budding yeast*

The *Mlu*I cell cycle box binding factor (MBF) and Swi4/Swi6 cell cycle box binding factor (SBF) are two large protein complexes involved in cell cycle specific transcriptional regulation in *S. cerevisiae*. Specific binding of these complexes to DNA sequences in promoters regulates transcription of many genes during the G1/S transition period of the cell cycle. Cyclin-dependent activation and inactivation causes MBF and SBF mediated transcription to be cell cycle regulated (Horak et al. 2002).

Both complexes contain the Swi6p regulatory protein and one of two distinct DNA binding proteins, Swi4p in SBF (Andrews and Herskowitz 1989b; Andrews 1992) or Mbp1p in MBF (Koch et al. 1993). SBF is necessary for the expression of a number of genes involved in cell wall biosynthesis, budding and morphogenesis, and G1 cyclins including *CLN1*, *CLN2*, *PCL1* and *PCL2* (Cho et al. 1998; Spellman et al. 1998). The SBF complex binds to a DNA sequence during G1 known as the SCB motif (Swi4/Swi6 dependent cell-cycle box), that consists of a consensus sequence that is often present in multiple copies of CACGAAA (Iyer et al. 2001).

Genes encoding proteins for DNA replication are regulated by the binding of MBF to sequences called MCBs (*Mlu*I cell-cycle box), that have a similar consensus sequence - ACGCGTNA - to the *Mlu*I restriction site (McIntosh et al. 1991; McIntosh 1993; Iyer et al. 2001). It has been suggested that SBF is not as specific for SCBs as was originally thought, but rather can bind, at least in some cases, to motifs more closely matching the MCB consensus sequence (Partridge et al. 1997). Activation of these transcription factor complexes at the G1/S boundary requires Cdc28p kinase associated with one of the four Clnp cyclins (Wijnen et al. 2002).

Cyclin associated Cdc28p kinase regulates the ability of SBF to bind SCB, or the ability of previously bound SBF to activate transcription in a positive feedback manner (Taba et al. 1991; Koch et al. 1996). Any one of the Clnp cyclins is capable of activating late G1 specific transcription of SBF or MBF when ectopically

expressed, although *CLN3* is believed to activate SBF and MBF *in vivo* under normal conditions (Wijnen et al. 2002).

Transcription factors homologous to the MBF complex have been identified in other eukaryotes, including fission yeast and mammalian systems. In mammalian cells it is believed that E2F is the functional homologue of MBF, although E2F transcription factors are not structural homologues of the transcription factors associated with the MBF complex (Nurse 1990). The E2F factors are key components in a cell cycle checkpoint that determines whether a cell will arrest in G1 to differentiate or enter into S phase (Macleod 1999; La Thangue 2001).

To date a structural homologue of RB has not been identified in yeast, but proteins that are similar to mammalian targets of RB function have been noted. For example it was found that when RB was ectopically expressed in *S. cerevisiae* it was phosphorylated prior to the initiation of DNA synthesis, coincident with the cell cycle checkpoint START (Weinberg 1995). It has also been found that RB family of proteins can function as direct transcriptional repressors in yeast with properties similar but distinct from those observed in mammalian cells (Arneric et al. 2002). The transcriptional response of a subset of RB-regulated genes is dependent on GC-rich promoter elements termed retinoblastoma control elements (RCE's) (Chen et al. 1994; Udvadia et al. 1995). The RCEs share limited sequence homology GCGC-CACC with yeast SCB and MCB motifs, and it has been hypothesised that RCEs might represent a related family of cell cycle-regulated yeast promoter elements. Furthermore, a novel cell cycle regulated RCE-binding protein, p180p, when synthesised in *S. cerevisiae* has been found to have a binding domain functionally homologous to members of a family of mammalian transcription factors (Cuevo et al. 1997). It has therefore been suggested that p180p may regulate the transcription of a subset of yeast genes whose expression is coincident with the onset, and or progression, of DNA replication.

II *Fission yeast*

DSC1

In fission yeast a transcription factor complex, similar to budding yeast MBF has been discovered and named DSC1 (DNA synthesis control 1). DSC1 binds to MCB sequences and regulates transcription at START (Lowndes et al. 1992). DSC1 may be directly or indirectly under the control of CDK/cyclin activity, as it is found to disappear at the onset of mitosis and reappears during S phase of the next cell cycle (Reymond and Simanis 1993). More recently it has been suggested that a new cyclin named Pas1p, structurally similar to the budding yeast Pclp family members, may be able to contribute to the control of the cell cycle START in fission yeast (Moffat et al. 2000). Pas1p cyclin resembles Cln1/2/3p of budding yeast in function and promotes cell cycle START by specifically activating DSC1 through its association with a kinase, Pef1p, and Cdc2p (Tanaka and Okayama 2000). However, Cdc2p kinase does not appear to be essential for the induction, maintenance and repression of target genes of DSC1 (Baum et al. 1997).

In *S. pombe* the first genes to be identified as required for the passage of START were *cdc2*$^+$ and *cdc10*$^+$ (Nurse and Bissett 1981). Cdc10p is found to be an essential component of DSC1 (Lowndes et al. 1992), which also contains products of the *res1*$^+$, *res2*$^+$, *rep2*$^+$ and *rep1*$^+$ genes (Caligiuri and Beach 1993; Miyamoto et al. 1994; Sugiyama et al. 1994; Nakashima et al. 1995; Zhu et al. 1997; Tahara et al. 1998; White et al. 2001). Interestingly, Swi6p, Swi4p and Mbp1p of budding yeast together with Cdc10p, Res1p and Res2p form a closely related family of transcription factors that share several regions of sequence homology (Taylor et al. 2000). It is found that the N-terminal regions of Swi4p and Mbp1p are highly homologous to those of Res1p and Res2p. In addition, Swi4p and Swi6p have homology to one another and also to Cdc10p (Breeden and Nasmyth 1987; Andrews and Herskowitz 1989a). This homology is most significant within two 33 amino acid regions, known as the ankyrin repeats, that also occur in a number of metazoan proteins (Andrews and Herskowitz 1990). However, it is worth noting that although Swi6p and Cdc10p are found to have similar roles (Breeden and Nasmyth 1987), they are not functionally interchangeable (Lowndes et al. 1992). Furthermore, homologous segments have also

been identified in several other transcriptional regulators indicating that this motif may also be commonly used in other species (Foord et al. 1999).

Like the N-terminal regions of Swi4p and Mbp1p, which are necessary for the DNA binding activity of SBF and MBF (Primig et al. 1992; Koch et al. 1993), the DNA binding function of DSC1 is also found to be accomplished by Res1p and Res2p (Whitehall et al. 1999). Res1p and Res2p also contain highly similar N-terminal DNA binding domains and have centrally located ankyrin repeats, which interact with Cdc10p via their C-terminus. The C-terminal region of Res2p is essential for the specific function of Res2p in meiosis and is also found to confer requirement for the co-activator Rep2p (Sturm and Okayama 1996) which itself has a potent activation domain (Tahara et al. 1998). Rep2 shares a limited but significant structural and functional similarity with Rep1p (Nakashima et al. 1995), which has recently been suggested to be an important factor involved in the regulation of DSC1 during mitosis (White et al. 2001).

Despite being highly related in structure, Res1p and Res2p are functionally non-identical. Cells deleted for *res1*$^+$ (*res1Δ*) have deficiencies in the mitotic cycle, and have a cold and heat sensitive phenotype resulting in a G1 arrest (Tanaka et al. 1992). In contrast, *res2Δ* cells have no obvious defects in the mitotic cell cycle, but are severely impaired in their ability to enter into pre-meiotic DNA synthesis and meiosis, indicating that Res2p has roles in the sexual differentiation process (Miyamoto et al. 1994; Zhu et al. 1994; Ayte and DeCaprio 1997). In addition it was found that over-expression of *res2*$^+$ can rescue the mitotic defects of *res1Δ* cells, but that the meiotic phenotypes of *res2Δ* cells are unable to be rescued by over-expression of *res1*$^+$ (Miyamoto et al. 1994; Zhu et al. 1997).

Consequently, the phenotypes of mutations in the two genes led to a model whereby two different but overlapping DSC1 complexes were thought to operate, with Cdc10/Res1p acting during the mitotic cycle and Cdc10/Res2p acting during meiotic cell cycle. However, it has emerged that Res1p and Res2p can heterodimerise in a Cdc10p-dependent manner *in vitro*, and that the mitotic DSC1 complex, detectable in native extracts by gel retardation experiments contains, Res1p, Res2p and Cdc10p (Miyamoto et al. 1994; Ayte et al. 1995). Additional genetic analysis has

shown that both Resp sub-units are needed for mitotic regulated transcription and that Res2p, like Res1p, is an active component of DSC1 in mitosis (Sturm and Okayama 1996; Baum et al. 1997; White et al. 2001). Therefore, a revised model suggesting that both Res proteins remain associated to Cdc10p throughout the mitotic cell cycle, and are needed for periodic cell cycle-regulated transcription, has been proposed (Figure 12) (White et al. 2001).

Mutations of *cdc10*$^{+}$, *res2*$^{+}$ and *rep1*$^{+}$ have also been found to affect meiotic progression, suggesting additional roles for all these components in meiosis (Beach et al. 1985; Smith 1994; Zhu et al. 1994).

It has been suggested that N-terminal fragments of Res2p bind to MCB elements as dimers (Zhu et al. 1997). However, titration experiments demonstrated that the N-terminal domains of Swi4p and Mbp1p bind to MCB and SCB sequences, with a stoichiometry of one protein molecule per recognition site (Taylor et al. 2000). Given the degree of sequence conservation within this family of proteins, it is likely that this will be true for all members.

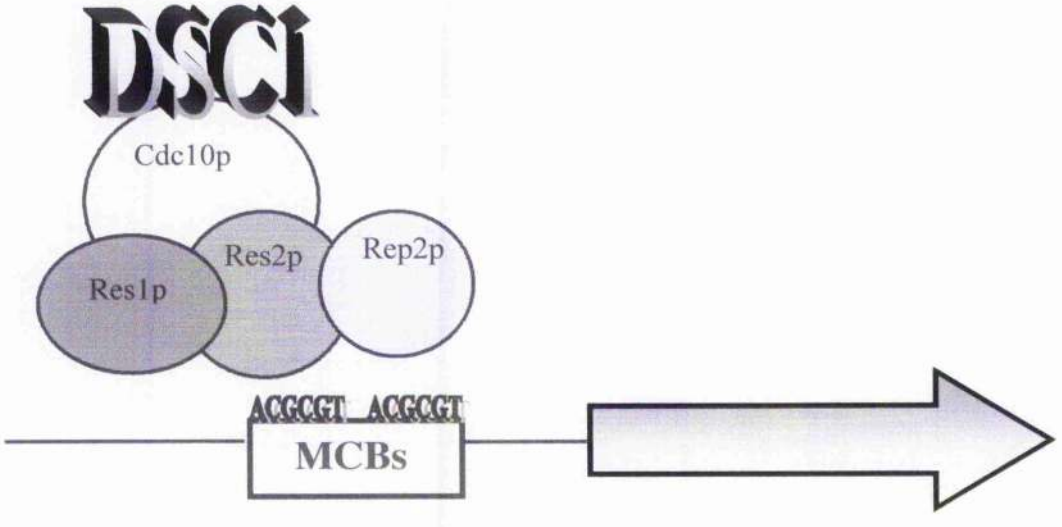


Figure 12. Schematic representation of the fission yeast transcription factor complex. DSC1 (DNA synthesis control), binding MCB (*Mlu*I cell cycle box) promoter motifs that operate during the G1/S transition.

III *Fission yeast*

MCBs

DSC1 is essential for the periodic expression of genes during the mitotic G1/S transition (Lowndes et al. 1992; Kelly et al. 1993), whose products are needed for the onset of DNA synthesis in fission yeast. These genes include, *cdc22*$^{+}$, *cig2*$^{+}$, *cdt1*$^{+}$, *cdc18*$^{+}$ and *mik1*$^{+}$ (McIntosh et al. 1991; Connolly and Beach 1994; Hoffmann and Beach 1994; Baum et al. 1997; Nishitani et al. 2000; Ng et al. 2001). Each of these genes is found to contain an upstream activation sequence (UAS) motif that is common to the 5' non-transcribed regions of these genes (Gordon and Fantes 1986; Lowndes et al. 1991; McIntosh et al. 1991). The conserved core of this motif consists of one or more hexameric sequence that coincides with the *Mlu*I restriction site (ACGCGT), and is referred to as the *Mlu*I cell cycle box, or MCB motif. Each of the MCB-regulated genes contains one or more MCB motif in its promoter, which binds specifically to DSC1 and forms an integral component of the transcription system (Gordon and Fantes 1986; Ng et al. 2001). DSC1 also forms an inactive complex, with MCBs responsible for the transcriptional repression of genes in late S/G2 (McInerny et al. 1995).

MCBs have highly related promoter sequences present in the phylogenetically distinct budding yeast (McIntosh 1993). It is also interesting to note that the recognition sequence for E2F, the functional homologue to DSC1 in mammalian systems, TTTTGCGCG or CGCGCAAAA is present in the promoters of many genes induced at the beginning of S phase (Mudryi et al. 1990; Johnston and Lowndes 1992). This sequence is found in close proximity to the transcription start site in cell cycle regulated genes that are important for DNA replication (Kel et al 2001), which suggests that such types of control may also be present amongst all eukaryotes.

In budding yeast some MCB-containing genes expressed at G1/S during mitosis are also induced during meiosis (Johnston et al. 1986). However, although no role for MCB sequences in meiotic transcription in budding yeast has been established (Cole and Mortimer 1989), more recently it has been suggested that MCB sequences are physiologically relevant during meiosis in fission yeast and are likely to control transcription during pre-meiotic S phase (Cunliffe et al. unpublished data).

At present there is little understanding of how DSC1 and MCBs work together to confer cell cycle regulation of genes transcribed at the G1/S boundary in fission yeast. Cdc10p is believed to play a central role in this regulation as it is absolutely needed for cell cycle progression (Nurse and Fantes 1981b). Various *cdc10* mutants are found to have profound effects on MCB-dependent genes, suggesting both positive and negative roles for Cdc10p in controls (Kelly et al. 1993; Hoffmann and Beach 1994; McInerny et al. 1995; Baum et al. 1997). More recently, it has been shown that over-expression of $cdc10^{+}$ had no effect on MCB-regulated gene transcription, arguing that it is not the gross level of Cdc10p that controls MCB gene expression but other components of DSC1, which ultimately contribute to cell cycle activation (White et al. 2001).

Partners to Cdc10p in DSC1, Res1p and Res2p, have roles in cell cycle-regulated transcription, with Res1p activating the Res2p repressing transcription (Baum et al. 1997; Whitehall et al. 1999). In addition, over-expression of $rep1^{+}$ and $rep2^{+}$ results in loss of cell cycle regulated transcription of MCB genes, suggesting that both these genes have important regulatory roles in controlling MCB gene expression during mitosis (White et al. 2001). Other experiments have also suggested that Rep2p confers an important positive function to DSC1, possibly through its direct contact with Res2p (Nakashima et al. 1995; Sturm and Okayama 1996; Tahara et al. 1998).

IV *Ribonucleotide reductase in the two yeast species*

The two yeast species are good model systems for studies of ribonucleotide reductase (RNR) regulation at the transcriptional level and its role in the cell division cycle. In addition they have provided a good system for the study of the DNA damage response pathway, which is largely conserved from yeast to humans (Lozano and Elledge 2000; Tanaka and Okayama 2000).

Budding yeast

There are four genes in budding yeast encoding the large and small sub-units of RNR, which are all located on different chromosomes (V, IX, X and VII) (Huang et al. 1998). Unlike the mammalian enzyme, the major control of budding yeast RNR appears to focus on the large sub-unit, encoded by two alternative genes, named *RNR1* and *RNR3*. Expression of *RNR1* is essential for mitotic viability and is primarily cell cycle regulated with maximal mRNA levels present during S phase. *RNR3* is not essential for mitotic viability, but is instead highly induced by DNA damage (Elledge and Davis 1990). Its transcript, almost absent during normal growth, appears after DNA damage. Thus during normal growth the large sub-unit is an $\alpha 2$ homodimer containing only Rnr1p (Domkin et al. 2002).

The small sub-unit of RNR is encoded by the essential *RNR2* gene (Hurd et al. 1987; Elledge and Davis 1987; Huang and Elledge 1997). Rnr2p cannot fold correctly by itself and is unable to form an iron-radical centre. Instead, another protein, Rnr4p, an R2-like protein (Wang et al. 1997), has the crucial role of correctly folding and stabilising an active Rnr2p-Rnr4p complex (Chabes et al. 2000). Thus, in contrast to other class I RNRs, the active form of the budding yeast small sub-unit is a $\beta\beta'$ heterodimer containing Rnr2p and Rnr4p, the only active form of the small sub-unit.

Although transcription of all *RNR* genes is inducible by DNA damage/replication blocks, the effect is most pronounced for *RNR3* (de la Torre Ruiz and Lowndes 2000). It is also found that *RNR3* is not an essential gene, as gene disruption studies with *rnr3* mutants have no phenotype under all studied conditions (Elledge and Davis 1990). Such features have meant this gene is frequently used in the study of the DNA damage checkpoint pathway in *S. cerevisiae*. It has consequently permitted the identification of a number of important genes involved in the DNA damage checkpoint function such as *CRT1*, *TUP1*, *SSN6* and *DUN1* (Zhou and Elledge 1992; Zhou and Elledge 1993; Huang et al. 1998; Li and Reese 2001).

Over-expression of *RNR1/RNR3* suppresses the lethality of *mec1Δ* and *rad53Δ* null mutants, genes encoding proteins essential to arrest cell cycle progression in the presence of DNA damage/DNA replication blocks (Huang et al. 1998). This is

explained by the observation that the DNA damage response in budding yeast activates the Mec1p and Rad53p protein kinase pathways, which in turn activates the transcription of the *RNR* genes. In parallel, this leads to the degradation of the Sml1p protein, a specific inhibitor of the yeast RNR large sub-unit (Chabes et al. 1999). This is achieved through the action of Dun1p, a downstream checkpoint kinase of the Mec1p/Rad53p checkpoint, which removes Sml1p during S phase and after DNA damage (Introduction, page 40 and Figure 11) (Zhoa et al. 2001).

Fission yeast

In fission yeast the larger sub-unit of RNR is encoded by *cdc22*$^{+}$ (homologous to *RNR3*) and the smaller sub-unit is encoded by *suc22*$^{+}$ (homologous to *RNR2*) (Fernandez-Sarabia et al. 1993). As in budding yeast, both genes are located on different chromosomes (I and II, respectively). *cdc22*$^{+}$ encodes a single transcript, that is periodically expressed during the cell cycle with a peak at G1/S, whereas two transcripts have been identified for *suc22*$^{+}$ (Gordon and Fantes 1986). The smaller *suc22*$^{+}$ transcript, of about 1.5 kb in size, is sufficient to contain the predicted ORF 1.2 Kb in size. It is, however, present at an essentially constant level throughout the cell cycle (Gordon and Fantes 1986; Fernandez-Sarabia et al. 1993). The larger transcript, 1.9 Kb in size, which also derives from the *suc22*$^{+}$ gene has start sites ~550 nucleotides upstream of those of the smaller transcript. Its expression is periodic during the cell cycle, with a maximum at the G1/S boundary, coincident with *cdc22*$^{+}$ (Harris et al. 1996).

Both *cdc22*$^{+}$ and *suc22*$^{+}$ large transcript are induced after treatment with hydroxyurea, which suggested this is a response to DNA damage (Fernandez-Sarabia et al. 1993). Hydroxyurea (HU) is a known inhibitor of RNR, which scavenges the essential RNR tyrosyl free radical and converts it to an abnormal tyrosine residue (Thelander et al. 1985). After exposure to HU, cells arrest in S phase and do not enter mitosis and at the level of transcription, RNR gene expression is elevated during S phase (Elledge et al. 1992). This is due to the transcriptional activation of RNR being tightly linked to the DNA replication (S-M) checkpoint response (Harris et al. 1996; Huang et al. 1998). Central to this response is Rad3p in fission yeast (Bentley et al. 1996), a protein kinase analogous to ATR in humans (Introduction, page 40) (Keegan et al. 1996) and Mec1p in budding yeast (Zhou and Elledge 2000) (Introduction, page 38). Rad3p activates programmes that arrest cell cycle progression, modulate repair

and promote recovery from replication arrest (Boddy and Russell 2001). Part of this response mechanism involves regulation of RNR (Huang et al. 1998; Zhoa et al. 2001).

Since HU arrests cells in S phase it is possible that the observed induction of RNR genes is solely due to accumulation of cells in S phase, rather than DNA damage. In order to confirm that the induction of *cdc22*$^{+}$ and *suc22*$^{+}$ large transcript can be due to a DNA damage response, the G2 arrest checkpoint resulting from UV-induced DNA damage was exploited. Wild-type cells were treated with the UV-mimetic agent 4-NQO (4-nitroquinoline oxide) and it was demonstrated that induction can occur outside S phase. Thus, induction of *suc22*$^{+}$ large transcript and *cdc22*$^{+}$ can occur both in response to DNA damage and through inhibition of DNA synthesis (Harris et al. 1996), as in budding yeast (Elledge et al. 1992).

In *S. pombe* another DNA checkpoint gene, *rad1*$^{+}$, effects the induction of the small sub-unit of RNR in response to HU and also triggers cell cycle arrest (Introduction, page 40). The *rad1*$^{+}$ gene product, needed for the induction of *suc22*$^{+}$ large transcript (Harris et al. 1996), is an essential component of the mitotic checkpoint system, which responds by blocking DNA replication and DNA damage (Introduction, page 37). Thus it is needed for checkpoint mediated G2 arrest (Lydall and Weinert 1995).

The large transcript of *suc22*$^{+}$, in contrast to *cdc22*$^{+}$, is also inducible by heat shock and unlike induction by DNA damage, this response does not need *rad1*$^{+}$ (Harris et al. 1996). This response may be mediated by the heat shock factor (HSF), a universal eukaryotic transcription factor involved in heat shock responses, which binds to a variable number of inverted nGAAn motifs (Amin et al. 1994; Fernandes et al. 1994). This sequence is found upstream of the start site of *suc22*$^{+}$ large transcript, adjacent to the MCB motifs (Fernandez-Sarabia et al. 1993).

In *S. pombe cdc22*$^{+}$ was the first cell cycle regulated gene to be discovered that is expressed at the G1/S interval (Gordon and Fantes 1986). It was identified by the isolation of two mutant alleles in collections of temperature-sensitive lethal mutants,

showing the classical cell cycle arrest phenotype of cell elongation (Nasmyth and Nurse 1981).

cdc22^{+} has an interesting array of MCB motifs in its promoter region (McIntosh et al. 1991; Lowndes et al. 1992; Fernandez-Sarabia et al. 1993). It was found that it contains two clusters of MCB motifs, which appear to bind to DSC1 (Figure 13) (Lowndes et al. 1992). Within these clusters there are three MCB motifs that are identical to the *Mlu*I recognition sequence, while the remaining five all contain the central CGCG core thought to be essential for function as defined in budding yeast (McIntosh 1993). There is also a single core MCB motif between the two MCB clusters.

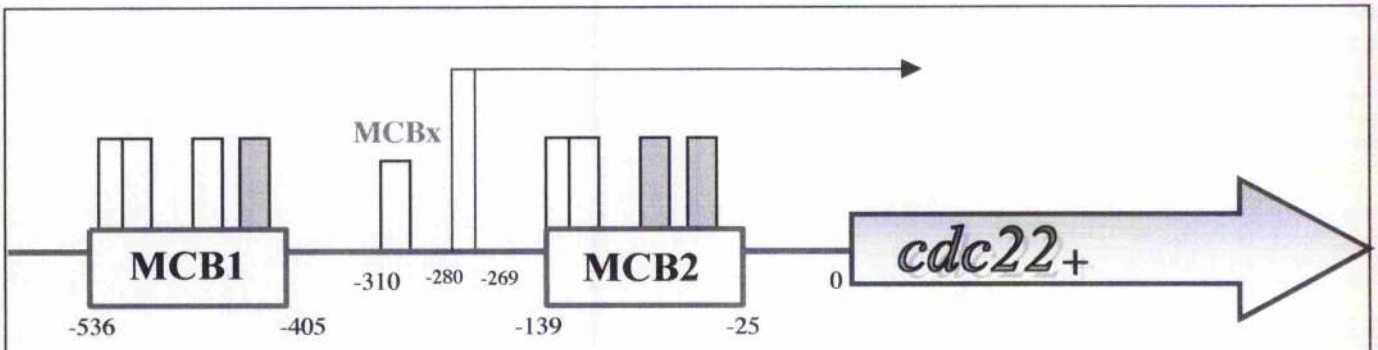


Figure 13. Schematic representation of the fission yeast *cdc22*$^{+}$ promoter region.

*Mlu*I sites (filled boxes), *Mlu*I $^{5}/_{6}$ matches (empty boxes) and transcriptional start sites (arrow) are indicated. The position of the MCB (*Mlu*I cell cycle box) motifs and the transcriptional start site relative to the open reading frame are indicated.

1M Research aims

I am interested in understanding the mechanisms that control the cell division cycle in fission yeast, as I hope studies in this model eukaryotic organism may contribute to current knowledge of cell division in human cells.

Specifically, we planned to study the molecular processes that regulate the expression of genes needed for DNA synthesis, or S phase, to occur. An important part of this mechanism in fission yeast involves the DSC1 transcription factor complex binding to MCB motifs present in the promoters of genes specifically transcribed at G1/S (Lowndes et al. 1992). We aimed to understand further how DSC1 and MCB interact to regulate G1/S transcription in fission yeast.

To this end, we studied the *cdc22*$^{+}$ gene, which was the first G1/S transcribed gene to be discovered in fission yeast (Gordon and Fantes 1986). Previous work had shown that *cdc22*$^{+}$ contains a complicated and interesting array of MCB motifs in its promoter region, with two clusters of MCB motifs present. This array is not untypical of other MCB-regulated genes in fission yeast, so we hope new information about this gene's regulation may be true for fission yeast MCB-regulated genes in general.

In previous experiments, mapping of the *cdc22*$^{+}$ gene using primer extension and Northern blot analysis led to the finding that the transcription start point of the gene lies between the two clusters of MCBs (Figure 12) (Lowndes et al. 1992; Maqbool et al. 2003). In addition, *in vitro* gel retardation analysis and *in vitro* DNAseI footprint suggested that DSC1 can bind to either MCB cluster, implying that both MCB clusters binds to DSC1 and may be involved in the transcriptional initiation of the gene (Maqbool et al. 2003).

In this thesis, I describe a series of experiments to investigate the role of the two MCB clusters in binding of DSC1 that elucidate the contribution of these factors to the cell cycle regulation of *cdc22*$^{+}$.

Chapter 2: Materials and Methods

Escherichia coli

Bacterial cell culture and strains

Basic *E. coli* culture and growth was as described in Sambrook *et al.* 1989. Vectors, oligonucleotides and bacterial strains used in this study are described in Appendix A, Appendix B and Appendix C, respectively.

Preparation of plasmid DNA from *E. coli*

For plasmid DNA preparation from *E. coli*, a single isolated bacterial colony was selected from a fresh solid medium plate and used to inoculate 10 ml of LB medium containing appropriate selective antibiotic (50 μg ml^{-1} ampicillin). Following overnight incubation at 37°C in an orbital shaker, 1.5 ml of cells were transferred to a micro-centrifuge tube and harvested by centrifugation at 13,000 rpm, for 1 minute. The bacterial pellet was re-suspended in 150 μl of buffer P1 (50 mM tris.Cl pH 8.0, 10 mM EDTA, 100 μg ml^{-1} RNAse A) and then mixed with 150 μl buffer P2 (0.2 M NaOH, 1% SDS), to initiate alkaline lysis of the cells. This reaction was allowed to proceed for 5-10 minutes at room temperature before neutralising the lysate by the addition of 150 μl of chilled buffer P3 (2.55 M KOAc pH 4.8), which subsequently resulted in the formation of a precipitate of potassium dodecyl sulphate. The SDS-denatured proteins and chromosomal DNA were co-precipitated with the detergent whilst the plasmid DNA remained in solution. The precipitate was pelleted by centrifugation for 5 minutes at 13,000 rpm. The supernatant containing plasmid DNA was transferred to another micro-centrifuge tube and the DNA precipitated by adding 1 ml of 100 % ethanol with centrifugation for 5 minutes at 13,000 rpm. The plasmid DNA pellet was then washed with 70% ethanol, dried at room temperature for 5-10 minutes, and re-suspended in a 40 μl volume of TE buffer pH 8 (10 mM tris.Cl pH 8, 1 mM EDTA).

Bacterial transformation (Sambrook *et al.* 1989)

E. coli XL-1 Blue "Super-competent" cells (Stratagene; used for colour selection) or "One shot" competent DH5α *E. coli* cells (Stratagene) were transformed using either a chemical or

electroporation method, for plasmid storage and propagation. In both cases bacterial cells, stored at -70°C, were thawed on ice to prevent loss of transformation efficiency.

The chemical method used 2 µl of 0.5 M β-mercaptoethanol, which enhanced transformation efficiency, and was added to 50 µl of *E. coli* cells. Typically 10-20 µl of plasmid DNA was then gently mixed into the chilled cells. The contents were occasionally tapped during a 30 minute incubation on ice, before being heat shocked for 30 seconds in a 42°C water bath, and then transferred to ice for a further 2 minutes (care was taken not to shake the samples at this point). Cells were incubated in an orbital shaker (225-250 rpm) for one hour at 37°C following the addition of 250 µl of SOC medium (LB broth, 0.04% glucose, 10 mM $MgCl_2$).

When the electroporation method was used, 40 µl of cell suspension was mixed with 1-2 µl of DNA. The cells were left on ice for 0.5-1 minutes, before transfer to an ice-chilled 0.2 ml cuvette and pulsed using the electroporator apparatus (Biorad *E. coli* pulser, settings: 2.25 kV, 200 Ω). 1 ml of SOC was immediately added and the cells were quickly but gently re-suspended with a pasteur pipette (this step was important in maximizing the recovery of the transformants) before being incubated in an orbital shaker (225-250 rpm) for one hour at 37°C.

After both transformation methods, 50 µl and 200 µl aliquots of the transformation mixture were then plated on LB agar (2% LB, 2% agar) plates containing 50 µg ml^{-1} of ampicillin. With *E. coli* XL-1 Blue "Super-competent" cells, 40 µl of 20 mg ml^{-1} X-Gal, was also added at this stage to permit colour selection. The plates were incubated at 37°C overnight to allow growth and colony-formation of transformed cells.

S. pombe

Fission yeast cell culture and strains

Media used for the propagation of *S. pombe* were as described by Moreno et al (1991). Standard genetic procedures of Gutz et al (1974) and Kohli et al (1977) were followed. Fission yeast strains used in this study are described in Appendix D.

To resuscitate yeast strains from -70°C glycerol stocks (made by adding 850 µl of cells, from a 10 ml pre-culture of cells to 15% glycerol) cells were streaked onto complete rich medium (YE) plates (Moreno et al 1991). The cells were then grown at the permissive temperature (25°C) for 2/3 nights and checked microscopically to ensure no contamination had

occurred, and that the cells were growing normally. A few individual colonies were then picked and streaked onto a YE master-plate, which was incubated overnight at 25°C. The master plate was used to replica-plate colonies, using a velvet cloth, onto selective medium to confirm the yeast strain genotype.

For physiological experiments cells were routinely grown in minimal medium (EMM) with shaking at 25°C or 32°C (Moreno et al 1991). Temperature sensitive mutants were incubated at the restrictive temperature of 36°C to display their mutant phenotype.

Cell number per ml of liquid culture was determined from a sample added to Isoton (Becton Dickinson); following sonication cells were counted electronically with a Z2 Coulter Counter. Typically 100 µl of cells was added to 10 ml of Isoton and counted twice and added together (each count was for 500 µl) and multiplied by 100 (the dilution factor) to give the final cell count per ml^{-1} of cells. A cell count of 1 to 2 x 10^6 cells ml^{-1} indicated cells were at exponential phase of growth.

Synchronisation of cells by transient temperature shifts in the *cdc25-22* mutants was achieved by growing the cells in EMM to mid-exponential growth at 25°C, before shifting to 36°C for 4 hours to cause cells to arrest in G1. Cells were then shifted back to 25°C to enter the mitotic cell cycle in synchrony. Samples were subsequently removed at 15 minute time intervals both for RNA extraction (35 ml) and to measure septation indices (90 µl (added to 10 µl of formaldehyde)) by microscopic examination.

Fission yeast transformation

Fission yeast cells from glycerol stocks were awoken and transformed using a chemical (Bahler et al. 1998) or electroporation method (Biorad instructions), for plasmid storage and propagation. For both methods cells for transformation were prepared by growth in EMM to mid-log phase with a density of about 10^7 cells ml^{-1}, at 25°C.

In the chemical method (Bahler et al. 1998) cells were harvested by centrifugation at 3,000 rpm for 5 minutes at 20°C and washed once with an equal volume of ice cold sterile water. Cells were then re-suspended in 1 ml of water and transferred to a 1.5 ml micro-centrifuge tube before being washed again with LiAc/TE made from 10 x filter-sterilised stocks (10 x LiAc: 1 M Lithium acetate adjusted to pH 7.5 with diluted acetic acid, 10 x TE: 0.1 M tris.Cl pH 7.5, 0.01 M EDTA, pH 7.5). The cell pellet was then re-suspended in LiAc/TE at 2 x 10^9 cells ml^{-1} and a 100 µl of the concentrated cells were mixed with 2 µl of

sheared hearing testes DNA (10 mg ml^{-1} yeast marker carrier DNA; Clontech Laboratories) and 10 µl of the transforming DNA. After incubation at room temperature for 10 minutes, 260 µl of 40% PEG/LiAc/TE (dissolved 8g of PEG 4000 in 2 ml of LiAc, 2 ml of 10 x TE and 9.75 ml sterile dH_2O, solution was filter sterilised and could be stored up to one month) was added and the cell suspension gently mixed and incubated at 30°C for 30-60 minutes. Cells were heat shocked for 5 minutes at 42° after adding 43 µl of DMSO (dimethyl sulfoxide) and washed once with 1 ml of sterile water before being re-suspended in 0.5 ml of sterile water. The cells were then plated onto selective EMM, left to air dry, and incubated at permissive temperature for 3/4 days.

When using the electroporation method the cell pellet was re-suspended in 40 ml of ice-cold 1 M sorbitol before being finally re-suspended in 2 ml of ice-cold 1 M sorbitol, to a density of about 1.5 x 10^9 cells ml^{-1}. 200 µl of the cell suspension was then added to a chilled 1.5 ml micro-centrifuge tube containing 1-2 µl of DNA and incubated on ice for 5 minutes. The cells and DNA were transferred to an ice chilled 0.2 ml cuvette and pulsed using an electroporator (Biorad *E. coli* pulser, settings: 1.5 kV, 200 Ω). 1 M sorbitol was then immediately added to the cuvette, the cell suspension returned to the micro-centrifuge tube and placed on ice. The transformation mixture was then plated out onto selective EMM, left to air dry, and incubated at permissive temperature for 3/4 days.

Plasmid stability test (Moreno et al. 1991)

To confirm the instability of a plasmid in transformed yeast strains (as opposed to stable integrated vectors), transformed cells were streaked to single colony on minimal medium and incubated for 2/3 days at the permissive temperature of 25°C. Single colonies were then selected and re-streaked on YE plates and incubated at the permissive temperature for 2/3 days, before being replica-plated onto selective minimal medium plates. Instability of transformed plasmids was shown by loss of ability of transformed strains to grow after relaxation of nutritional selection, due to plasmid loss.

Mating fission yeast cells (Moreno et al. 1991)

To mate two different yeast strains of opposite mating types, a loop-full of freshly growing cells from a master-plate of each strain was mixed together on an ME agar plate with 100 µl of dH_2O. The cells were air dried before incubation at below 30°C for 3/4 days. A portion of the mating mix was then added, in a micro-centrifuge tube, to 1 ml of dH_2O and 20 µl of β - glucoronidase (Sigma G0876, 100 units μl^{-1}) before incubation and left over-night at 37°C. The cells were then pelleted and re-suspended in 1 ml of dH_2O, and pelleted again, and then re-suspended in 500 µl of dH_2O. 100 µl of this cell culture were spread onto a YE plate and left at 25°C for 2/3 days and dilutions with 10 µl and 1 µl of cells with 100 µl of dH_2O were also spread onto separate YE plates.

Isolation of chromosomal DNA from fission yeast (Moreno et al. 1991)

For plasmid DNA preparation, an isolated fission yeast colony was selected from a master-plate and used to inoculate 10 ml of YE. Once the cells were grown to saturation for 2/3 overnights at the permissive temperature, they were harvested by centrifugation at 2000 rpm for 5 mins. The cells were then re-suspended in 0.5 ml dH_20 and transferred to a 1.5 ml micro–centrifuge tube and pelleted by a 5 second spin. The pellet was re-suspended in its residual liquid before adding 0.2 ml of solution A (10% Triton, 10% SDS, 1 M NaOH, 1 M tris.Cl pH 7.5, 0.5 M EDTA), 0.2 ml of phenol/chloroform (1:1 ratio) and 0.3 g of acid washed glass beads (425 – 600 micron, Sigma G 9268). The sample was vortexed for 3-5 minutes and 0.4 ml of TE buffer was added before being centrifuged at high speed for 5 minutes. The aqueous layer, containing the RNA and DNA was transferred to a new tube and the nucleic acids were precipitated by adding 1 ml of 100% ethanol and centrifuging the sample for 2 minutes. The pellet was re-suspended in 0.4 ml of TE buffer and 30 µg of RNAse A (dissolved at 10 mg ml^{-1} in 0.01 M sodium acetate pH 5.2) and incubated at 37°C for 5 minutes to remove the RNA. DNA was precipitated by adding 8 µl of 5 M NH_4Ac, 1 ml of 100% ethanol, and centrifuged at high speed for 2 minutes. The supernatant was carefully discarded and the pellet left to air dry before being re-suspended in 100 µl of TE buffer.

RNA preparation from fission yeast (McInerny et al. 1995)

For RNA extraction from fission yeast, 200 ml cultures of mid-exponential stage of growth fission yeast cells were prepared and harvested by centrifugation at 3000 rpm for 5 minutes. Each cell pellet was re-suspended in 1 ml of STE (0.32 M sucrose, 20 mM tris.Cl pH 7.5, 10 mM EDTA pH 8.0) and transferred to a screw cap micro-centrifuge tube and centrifuged at high speed for 5 seconds (cells could be stored at –70°C at this stage). The cell pellets were re-suspended in 200 µl of STE before adding acid washed glass beads (425 – 600 micron, Sigma G 9268) to just beneath the meniscus, followed by 600 µl of NTES (100 mM NaCl, 5 mM EDTA, 50 mM tris.Cl pH 7.5, 1% (w/v) SDS). 500 µl of H_2O saturated hot phenol at 65°C was added next and the cells were disrupted using a Ribolyser (Hybaid Ltd, UK) with 3 bursts of 40 seconds, at setting 4, and centrifuged for 5 minutes at high speed. This resulted in the separation of the samples into three phases: a lower and red organic phase containing protein, a middle white interphase containing precipitated DNA, and an upper colourless aqueous phase containing the RNA. The upper and middle interface were carefully removed and transferred to a second 500 µl aliquot of hot (65°C) phenol and again homogenised in the Ribolyser with 1 burst at 40 seconds, setting 4. Samples were centrifuged for 5 minutes at high speed and the aqueous phase only was transferred to an aliquot of 400 µl of phenol/chloroform at room temperature and given another burst of 40 seconds in the ribolyser. The aqueous phase was again removed and transferred into a second aliquot of 400 µl phenol/chloroform at room temperature, and homogenised in the Ribolyser, 1 burst for 40 seconds at setting 4, before being centrifuged at high speed for 5 minutes. (This step was repeated if any protein was found present at the phenol/aqueous interface).

The aqueous phase was precipitated with 1/10 volume of 3 M sodium acetate and 3 volumes of 100% ethanol. Following an overnight incubation at 20°C, the RNA was pelleted by centrifugation at high speed for 10 minutes at room temperature and washed with 70% ethanol in RNAse-free H_2O. The RNA pellet was re-suspended in 100 µl of RNAse-free H_2O and dissolved by incubating at 65°C for 3 minutes, with repeated pipetting to facilitate the re-suspension of the RNA.

5 µl of each sample was diluted in 500 µl of dH_2O and was used to estimate the amount and quality of RNA by spectrophotometry at A_{260}. The concentration of RNA in µg μl^{-1} was calculated by using the formula: (A_{260} x dilution factor x 40) ÷ 1000. Usually such

dilutions gave readings of about 0.5, within the accurate range of the spectrophotometer. If the RNA was too concentrated, it was diluted further before measurement. The RNA samples were stored at –70°C.

Protein extraction from fission yeast (Ng et al. 2001)

200 ml cultures of fission yeast cells, in mid-exponential stage of growth, were prepared and harvested by centrifugation at 5000 rpm for 10 minutes in screw cap centrifuge tubes. The cell pellet was re-suspended in 200 µl of ice cold lysis buffer [50 mM KCl, 50 mM tris.Cl pH 8, 25% glycerol, 2 mM DTT (dithiothreitol, Sigma), 0.1% Triton X-100, 5 µg of protease inhibitors: chymostatin, pepstatin, antipain, leupeptin, aprotonin (Sigma), 0.2 mM PMSF (phenylmethanesulfonylfluoride)] in 2 ml screw capped micro-centrifuge tubes. The cells were pelleted at 13,000 rpm for 1 minute in a high speed micro-centrifuge and again re-suspended in 200 µl of lysis buffer. Acid washed glass beads (425 – 600 micron, Sigma) were added to just beneath the meniscus and the tubes were chilled on ice for 2-3 minutes before being disrupted using a Ribolyser (Hybaid Ltd, UK) with 1 burst at 40 seconds, setting 4. The cell debris was pelleted by centrifugation at 13,000 rpm for 5 minutes at 4°C and the protein supernatants were transferred to a fresh chilled micro-centrifuge tube, and clarified by centrifugation at 13,000 rpm for 30 minutes at 4°C.

Supernatants were transferred to a fresh chilled micro-centrifuge tube and 5 µl was removed to determine protein concentration, which was estimated using Bradford's reagent (Biorad) according to the manufacturer's instructions, and the remainder of the protein sample was snap frozen on solid CO_2, and stored at –70°C.

General molecular techniques

Acrylamide gel purification of DNA

Small polymerase chain reaction (PCR) products (100-300 base pairs) were commonly separated on a 6% acrylamide gel and purified using the following method. The acrylamide gel was stained with ethidium bromide and DNA visualised under short-wave UV, and the desired DNA fragment was excised with a scalpel. The acrylamide pieces were then

transferred to a 500 µl pipette tip (sealed with a bunsen flame and containing a siliconized glass wool plug) and mashed with a tooth pick. The mashed acrylamide was suspended in 400 µl of elution buffer and the top of the 500 µl tip was sealed using Nesco-film. Following an overnight incubation at 37°C, the tip of pipette was removed and the solution eluted into a 1.5 ml micro-centrifuge tube, by adding 400 µl more elution buffer. To remove excess acrylamide the sample was micro-centrifuged for 5 minutes. Two volumes of ethanol and 1 µl of carrier glycogen (20 mg ml^{-1}, Roche) were added to the supernatant and left at –70°C for 5-10 minutes to precipitate the DNA. The DNA was pelleted at 13,000 rpm for 30 minutes, washed with 90% ethanol and was re-suspended in 30-50 µl dH_2O. 5-10 µl of the sample was loaded onto a 1% agarose gel next to a 1 kb ladder of known concentration and visualised under short-wave UV to determine the concentration of the sample DNA.

Northern blot analysis (Sambrook et al. 1989)

Typically RNA samples of 10 µg were added to 20 µl of RNA sample buffer [600 µl Formaldehyde, 200 µl formamide, 240 µl 5 x MNE (0.12M MOPS pH 7, 25 mM NaOAc, 5 mM EDTA), 160 µl dH_2O]. Samples were heated at 65°C for 5 minutes to denature the RNA secondary structure, and were immediately transferred to ice to prevent re-naturation. To each sample 1 µ l of a 1/30 dilution of ethidium bromide (10 mg ml^{-1}) was added, and the samples were loaded onto a formaldehyde denaturing gel (1% agarose, 20 ml 5 x MNE, 38 % formaldehyde, 63 ml dH_20). The gel was electrophoresed for 3-4 hours at 60 v in 1 x MNE to separate the different species of RNA according to size. The gel was visualised under a UV trans-illuminator to confirm successful separation and photographed.

The gel was then washed for 15 minutes in 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.5) [100 ml stock: 3.9 ml 1 M NaH_2PO_4, 6.1 ml 1 M Na_2HPO_4, 90 ml dH_2O] prior to capillary transfer as described by Sambrook et al (1989). The transfer procedure required the prepared gel to be placed, inverted, on a bridge of Whatman 3 MM chromatography paper supported on a glass plate and suspended over a reservoir of 10 mM Na_2HPO_4/NaH_2PO_4 (pH6.5). An appropriate size of Genescreen membrane (NEN Life Science) was pre-soaked in 10 mM Na_2HPO_4/NaH_2PO_4 (pH6.5) and positioned over the gel, followed by a further two layers of pre-soaked Whatman paper. Care was taken to ensure removal of air bubbles. A stack of paper towels and a weight were added on top of the arrangement to allow efficient capillary

action. During transfer the migration of the RNA from the gel to the membrane was facilitated by the passive movement of the solution through the gel. Plastic strips were placed along each side of the gel to prevent direct contact between the paper towels and the Whatman bridge; this ensured movement of the buffer was only through the gel. To achieve high transfer efficiency, the capillary action was allowed to proceed overnight. Following transfer, the RNA was fixed to the membrane by UV-cross-linking at 1200 MJ and was then washed for 5 minutes in dH_20.

Radio-labelled DNA probes complementary in sequence to a particular RNA of interest were used to locate it on the membrane. The probes were routinely amplified by PCR using fission yeast genomic DNA as a template. The end product was a region of about 1 kb of each ORF, and the oligonucleotides used to make the various probes are listed in Appendix B.

The DNA probes were labelled using random hexanucleotide (N_6) primed method of Feinberg and Volgelstein (1983). This involved the addition of 10-15 µg DNA to DEPC water to give a final volume of 11 µl, which was then denatured for 5 minutes at 95°C followed by the addition of 2 µl of N_6 random hexanucleotide at 100 pm μl^{-1} (MWG oligonucleotide service). Labelling was carried out at 37°C for 1 hour or more in a 1 x reaction buffer (containing 500 mM tris.Cl pH7.5 with 100 mM $MgCl_2$) following the addition of 2 µl of 250 µM:dCTP, dGTP, dATP (Promega), 40 µCi of [α^{32}P] dCTP (300 Ci $mmol^{-1}$) and 2U DNA polymerase I Klenow fragment (Boehringer Mannheim).

The probe was purified by addition of 100 µl of dH_2O, and passing the mixture through a Sephadex G-50 size exclusion column. The Sephadex G-50 (Pharmacia) was prepared by autoclaving in two volumes of TE. A plunger from a 1 ml disposable syringe (Plastipak) was removed and a small amount of siliconised glass wool (Sigma 20411) was pushed to the bottom of the tube and the column was placed in a 50 ml plastic screw cap tube with a micro-centrifuge tube placed at the end. The G-50 was added to fill the column, which was then spun for 2-5 minutes at 2,000 rpm. The remaining TE was discarded and, if necessary, the column was spun again. The labelled DNA was added and the column was spun again for 2-5 minutes at 2,000 rpm with a fresh micro-centrifuge tube and the separation of labelled DNA fragment from unincorporated radio-nucleotide was confirmed by monitoring the radioactive profile with a Geiger counter.

The probe was added to the Genescreen membrane with bound RNA after the membrane had been pre-hybridised. This involved rotation in a hybridisation oven at 42°C for 2-4 hours in 18 mls of pre-hybridization buffer [10 ml formamide, 4 ml P buffer (1% BSA, 1% pyrolidone, 1% ficoll, 250 mM tris.Cl pH8, 0.5% sodium pyrophosphate, 5% SDS), 4 ml 50% $DXSO_4$ (10 g dextran sulphate, 20 ml dH_2O)] these ingredients were heated together at 42°C for 10 minutes before the addition of 1.16 g NaCl and 200 µl salmon sperm (this was added after incubation at 95°C for 5 minutes and ice for 5 minutes). The radio-labelled probe was then added to the pre-hybridisation buffer, and membrane incubated with rotation at 42°C for 16 hours. The nylon membrane was then rinsed in 2 x SSPE [20 x SSPE in 1 litre: 175.3 g NaCl, 27.6 g NaH_2PO_4, and 40 ml EDTA pH 8] at room temperature for 30 minutes. This was followed by rinsing with 2 x SSPE + 0.5% SDS at 65°C for 15 minutes. A final wash in 0.1% SSPE at room temperature was completed to remove residual SDS, before being exposing the membrane to auto-radiography film overnight at –70°C.

The membranes were stripped of probe DNA by incubating at 70°C for 30-120 minutes in strip buffer [1 M tris.Cl pH 8, 0.5M EDTA pH 8, 0.5 M sodium pyrophosphate, 0.02% polyvinylyrolidone, 0.02% ficoll, 0.02% BSA (Helena Biosciences)], until no radioactivity was detected.

Quanitification of Northern blot transcripts

To allow comparison between transcript levels in different samples detected by Northern blot analysis, the transcripts were quantified using NIH image software. A box was drawn around each transcript and the calculated measurement of the intensity of each band was noted using the software. To minimise error the same sized box was drawn around each transcript before the measurement was taken. Occasionally, background signal had to be measured and subtracted from the counts. The invariant *adh1*$^{+}$ transcript level was also quantified using the same method, and the ratio of transcript levels to *adh1*$^{-}$ calculated and plotted for each experiment.

Electophoretic mobility shift assay (EMSA) (Ng et al. 2001)

Probe preparation

DNA fragments were 5' end-labelled using T4 polynucleotide kinase (PNK). To 5-10 μg of DNA in 5 μl of dH_2O on ice was added 10 U of T4 PNK in 1 x T4 PNK buffer (Promega), followed by the addition of 10 μCi of [γ-^{32}P] dATP (Amersham, 10 μCi μl^{-1}) to give a total volume of 8 μl. The reaction was incubated at 37°C for 1 hour, and the DNA was purified using the Sephadex G-50 column (p.x).

EMSA Assay

Each shift assay was performed by adding 10 μl of sample buffer [1 M tris.Cl pH 7.5, 1 M KCl, 50 % Glycerol, 100 mM DTT, 100 mM protease inhibitors: chymostatin, pepstatin, antipain, leupeptin, aprotonin (Sigma), 100 mM PMSF, 1 M $MgCl_2$] to 20 μg of protein, 1 μg of dIdC (1 mg ml^{-1}; Pharmacia) and 1-2 μl of labelled probe. If required non-specific or specific competitor DNA was also added. The samples were left for 5 minutes on ice before the addition of each reagent. Analysis of the formation of protein-DNA complexes was achieved by electrophoresis of samples on a 10% acrylamide gel in 1 x TAE buffer (40 mM tris-acetate, 1 mM EDTA pH 8) for 1.5-2 hours at 180v. The gel was dried for 1.5 hours at 80°C and exposed to auto-radiography film at -70°C.

Site directed mutagenesis

A series of PCR amplification reactions were used to introduce mutated base pairs into the *cdc22*$^+$ promoter region, using the appropriate integration vector, pPJK7 or pPJK10 as a DNA template.

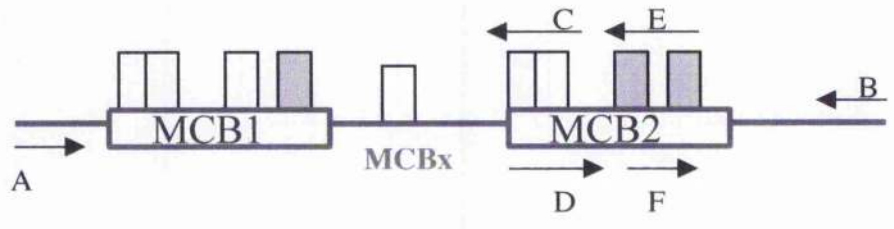


Figure A. A schematic representation of the oligonucleotides designed relative to the *cdc22*$^+$ promoter region to mutate the MCB2 motifs in pPJK7 and pPJK10. Filled boxes, MCB motifs exact matches to *Mlu*I site; empty boxes, core CGCG sequence. Arrows indicate oligonucleotides, which are labelled as A to F (Appendix B). Note that C and E are reverse complements to D and F.

Oligonucleotide primers are labelled A-F for clarity (Figure A; Appendix B). Oligonucleotides C–F contained the mutations to be included in the final product. Oligonucleotides A and B incorporated the restriction sites *Hind*III and *Kpn*I, respectively, which allowed the ligation of the new DNA fragment into the integration vector. Separate PCRs were used to amplify two DNA products with the oligonucleotides, A+C and D+B. The amplified DNA products were run on a 1% agorose gel and purified using agorse gel purification kit (Qiagen). Using these purified DNA products as DNA templates, a fusion PCR was performed using the oligonucleotides A+B. This gave an amplified DNA product containing the mutations introduced by the oligonucleotides C+D. The newly amplified product was then purified with the agarose gel extraction , and used as a DNA template for another two separate PCRs using oligonucleotides A+E and F+B. These amplified DNA products were then band purified. A fusion PCR was carried out again using the oligonucleotides A and B, this time using the new amplified PCR products as templates, and this final mutated product cloned into pPJK7 or pPJK10. All mutations were confirmed by sequencing.

Chapter 3: Results

A *cdc22*$^{+}$ MCB clusters confer G1/S transcription

I *Introduction*

Progression through the cell division cycle in fission yeast is dependent on passage through START, which is controlled in part by the transcription factor complex DSC1 that regulates the expression of genes essential for DNA synthesis (Lowndes et al. 1992; Connolly and Beach 1994; Hofmann and Beach 1994; Baum et al. 1997; Nishitani et al. 2000; White et al. 2001). The DSC1 transcription factor complex is essential for the periodic expression of genes during the G1/S transition (Lowndes et al. 1992; Kelly et al. 1993). DSC1 activates G1/S transcription by binding MCB UAS motifs present in the promoters of a group of genes expressed at this cell cycle time. The conserved core of this element consists of one or more hexameric sequence that coincides with the *Mlu*I restriction site (ACGCGT), and is referred to as the *Mlu*I cell cycle box, or MCB motif. Each of the MCB-regulated genes contains one or more MCB motifs in its promoter, which bind specifically to DSC1 to form the transcription control system (Lowndes et al 1992; Ng et al 2002).

The MCB motif was originally identified in budding yeast, where a consensus sequence ACGCGTNA was found in the promoter region of cell cycle regulated genes essential for DNA synthesis (McIntosh et al. 1991). It has also been identified in fungi (McIntosh et al. 1993) and the most similar sequence currently found in mammalian genes is the E2F transcription factor site TTTGCGC or CGCGCAAAA. Similar to yeast, the E2F binding site can be found in the promoter of cell cycle regulated genes, whose expressioin peaks at the G1/S boundary and are required for DNA replication (Kel et al. 2001). As transcriptional mechanisms involving passage through START and commitment to a new cell cycle appear to be conserved from yeast to mammals (Qin and

Li 2003), it is possible that MCB motifs may also be an integral component of a transcription system that is conserved in other eukaryotic organisms.

cdc22^{+}, encoding the large sub-unit of ribonucleotide reductase, was the first gene to be discovered in fission yeast whose expression is cell cycle regulated at the G1/S interval (Gordon et al. 1986). *cdc22*$^{+}$ has been found to contain an interesting array of MCB motifs in its promoter, implicated in regulating its G1/S specific expression (McIntosh et al. 1991; Lowndes et al. 1992; Fernandez-Sarabia et al 1993, McInerny et al. 1995). The promoter region contains two clusters of MCB motifs, for clarity named in this thesis "MCB1" and "MCB2" (Figures 13 and 14). Within MCB1 and MCB2, there are three *Mlu*I motifs that are identical to the *Mlu*I recognition sequence, while the remaining five motifs all contain the central CGCG core thought to be essential for function (McIntosh et al. 1991). There is also a single core *Mlu*I site between the two MCB clusters, named in this thesis "MCBX" (Figures 13 and 14).

In previous experiments, the mapping of transcriptional start of *cdc22*$^{+}$, using primer extension and Northern blot analysis revealed that the start point of the gene lies between the two MCB clusters (Figures 13 and 14) (Maqbool et al, 2003). Furthermore, *in vitro* DNAseI footprint analysis showed there are protected regions over MCB2, the downstream cluster of MCBs (Maqbool et al, 2003). These results imply that either one or both of the MCB clusters is essential for the binding of DSC1 and activating transcription of *cdc22*$^{+}$. The possibility that MCB2 may participate in the initiation of the transcription of *cdc22*$^{+}$ is interesting, because this would be the first evidence for a UAS downstream to the transcriptional start site of a RNA polymerase II regulated gene in yeasts. This potential observation is especially interesting in light of recent observations that reveal mammalian E2F binding sites occur in close proximity, upstream or downstream, to the transcriptional start site of cell cycle regulated genes (Kel et al. 2001).

The role of MCB motifs in fission yeast G1/S specific transcription was first suggested by the observation that three adjacent synthetic *Mlu*I sequences placed in the UAS reporter plasmid pSPΔ178 conferred G1/S specific transcription to the *lacZ* gene (Lowndes et al. 1992). This result demonstrated that MCB motifs can confer expression

to a heterologous gene in a cell cycle dependant manner in fission yeast, implicating the MCB motifs in the *cdc22*$^{+}$ promoter in regulating its cell cycle transcription. The presence of MCB motifs in the promoters of other fission yeast G1/S regulated genes is also consistent with this hypothesis.

However, these initial experiments were limited, as the native promoter of the endogenous *cdc22*$^{+}$ gene contains 9 *Mlu*I motifs, which are dispersed along the promoter and are not immediately adjacent to each other. In addition, they occur as two main clusters, MCB1 and MCB2, within which there are three *Mlu*I motifs that are identical to the *Mlu*I recognition sequence, and six that only contain the central CGCG core (Figure 14). These initial experiments did not show, therefore, which of the MCB clusters in the endogenous *cdc22*$^{+}$ promoter binds to DSC1, nor did they confirm if other elements may contribute to promoter activity.

Thus, the main aim of the research presented in this thesis is to understand the role and contribution of the two MCB motifs in the *cdc22*$^{+}$ promoter in regulating its expression at the G1/S boundary. It is hoped that such information will contribute towards a better understanding of cell cycle regulated transcription in fission yeast and ultimately other eukaryotic systems including mammalian cells.

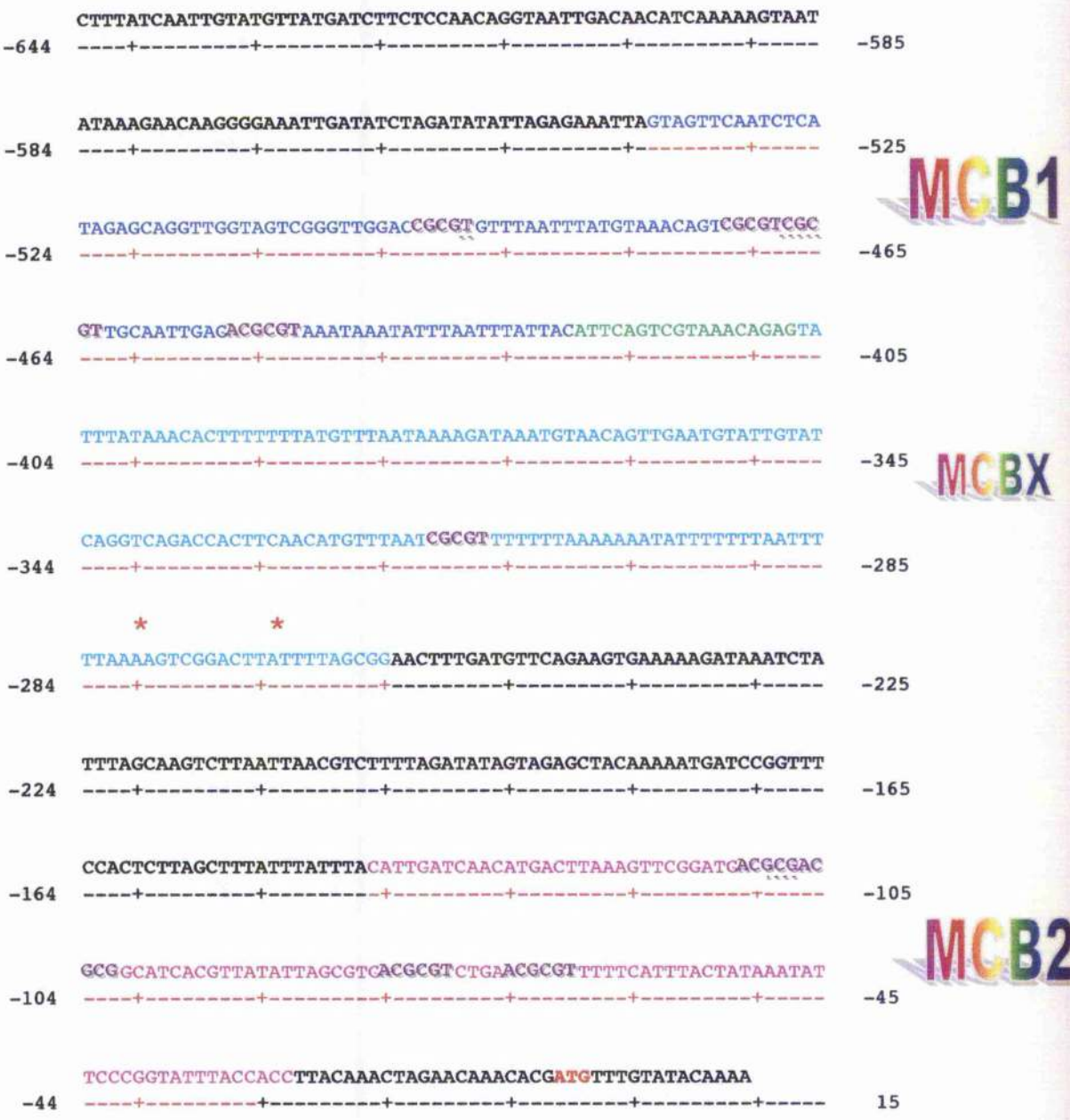


Figure 14. The 5' nucleotide sequence of the *S. pombe cdc22*$^{+}$ promoter region

MCB cluster fragments used in subsequent experiments are indicated: MCB1 (dark blue), MCB2 (pink), MCBX (light blue) and overlapping region between MCB1 and MCBX (green). MCB motifs (purple) and transcriptional start sites (asterisks), are also indicated. Numbers are relative to the predicted start codon (red).

II *Cloning of cdc22⁺ MCB clusters into pSPΔ178*

We initially extended previous observations analysing the role of MCB motifs in fission yeast by testing if native MCB motifs from the *cdc22*⁺ promoter could confer G1/S transcription to *lacZ*. We separately cloned the two MCB clusters, MCB1 and MCB2 (Figure 14), from the promoter of the *cdc22*⁺ into the UAS reporter plasmid pSPΔ178 (Appendix A.1) in both the forward and reverse orientations (Figure 15). We then tested the ability of these four constructs to confer G1/S expression to *lacZ* in fission yeast.

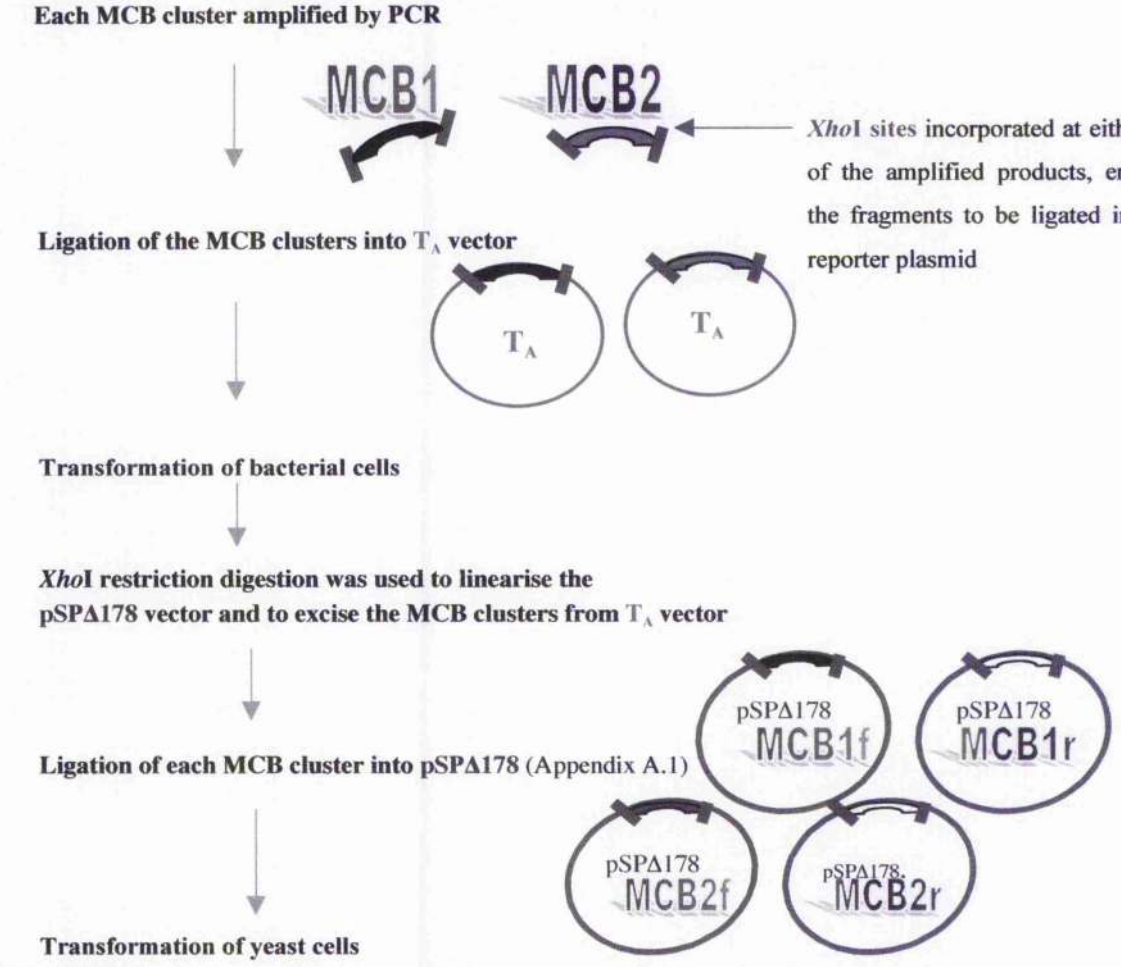


Figure 15. Schematic representation of the construction of four vectors containing the two *cdc22*⁺ promoter MCB clusters in different orientations in the UAS reporter plasmid pSPΔ178.

pSPΔ178.MCB1f (GB 73) and pSPΔ178.MCB1r (GB 74) each contain MCB1 in the forward and reverse orientation, respectively. pSPΔ178.MCB2f (GB 57) and pSPΔ178.MCB2r (GB 58) each contain MCB2 in the forward and reverse orientations, respectively.

III *MCB1 and MCB2, in the correct orientation, can each confer cell cycle expression*

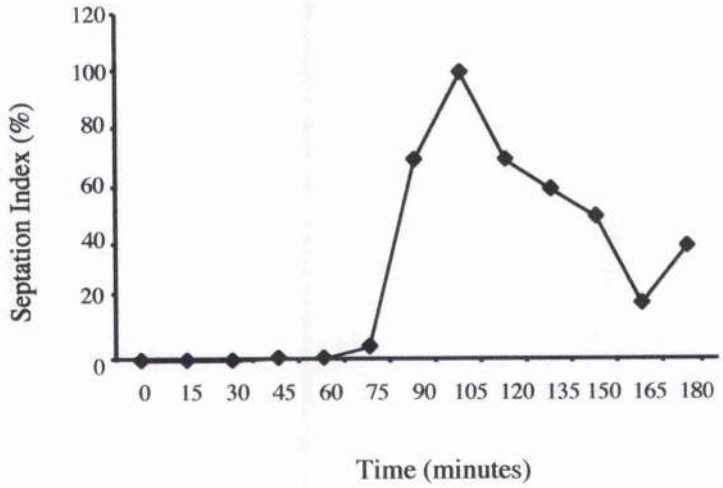
To test the ability of each MCB cluster from the *cdc22*$^{+}$ promoter to confer mitotic cell cycle transcription to *lacZ* each of the four constructs, containing either MCB1 or MCB2 in either orientation, was transformed into the fission yeast mutant strain *cdc25-22* (GG 193; Materials and methods, pages 57-58). This temperature sensitive cell cycle mutant strain permits the synchronisation of fission yeast cells by transient temperature arrest. Routinely, we would transfer exponentially dividing cells grown at 25°C, to the non-permissive temperature of 36°C, for 4 hours. At this temperature the cells continued to grow but stopped dividing, and accumulated at G2/M. The cells were then shifted back to the permissive temperature of 25°C, when they were able to proceed through highly synchronous cell cycles, as determined by measuring septation indices. Quantification of each transcript was completed using NIH image software (Material and methods, page 64): measurements were taken for each transcript and these ratioed to *adh1*$^{+}$ mRNA levels as a loading control in each sample. Two control lanes were included, containing RNA from asynchronous fission yeast mutant *cdc10-C4* cells, containing (i) the pSPΔ178 plasmid with a triple MCB, known to confer *lacZ* transcription and (ii) pSPΔ178 empty vector, known not to confer *lacZ* transcription. *cdc10-C4*, mutated in a component of DSC1, results in deregulated expression of all known MCB regulated genes throughout the cell cycle, which in asynchronous cells manifests as over-expression relative to wild-type (McInerny et al. 1995; Ng et al. 2001).

Northern blot analysis of RNA obtained from the cells containing pSPΔ178 with MCB1 in the forward orientation, in three identical experiments, revealed specific induction of *lacZ* mRNA, similar to the *cdc22*$^{+}$ profile (Figure 16). MCB1 conferred one prominent peak of G1/S transcription to *lacZ* during the first cell cycle, and a weaker second peak during the second cell cycle. Similarly, Northern blot analysis of cells containing MCB2 in the forward orientation also revealed a *lacZ* profile similar to *cdc22*$^{+}$ (Figure 18). However, MCB2 was able to drive two peaks of *lacZ* transcription over the two synchronous cell cycles. Interestingly, no *lacZ* transcript was observed in cells

undergoing synchronous cell cycles containing either MCB cluster in the reverse orientation (Figures 17 and 19).

These experiments allowed us to conclude that both MCB clusters are capable of contributing to *cdc22*$^{+}$ cell cycle expression independently of each other, as each can confer G1/S transcription to a heterologous *lacZ* gene. At least for MCB2 this result was surprising, as the cluster lies downstream to transcriptional start of the native *cdc22*$^{+}$ promoter. These data implicate MCB2 as a downstream UAS (here termed a DAS, for a downstream activating sequence), the first example of such an element in fission yeast for a RNA polymerase II regulated gene. We were also surprised by the fact that both MCB1 and MCB2 appeared to have no ability to initiate transcription in the reverse orientations, as the "perfect" MCB motifs in each cluster are palindromic, having the sequence ACGCGT. However, unlike MCB motifs in budding yeast (McIntosh 1993) this result suggested that the activity of MCB clusters in the promoter of *cdc22*$^{+}$ are orientation dependent.

A


Septation Index (%)
120
100
80
60
40
20
0
0 15 30 45 60 75 90 105 120 135 150 165 180
Time (minutes)

B

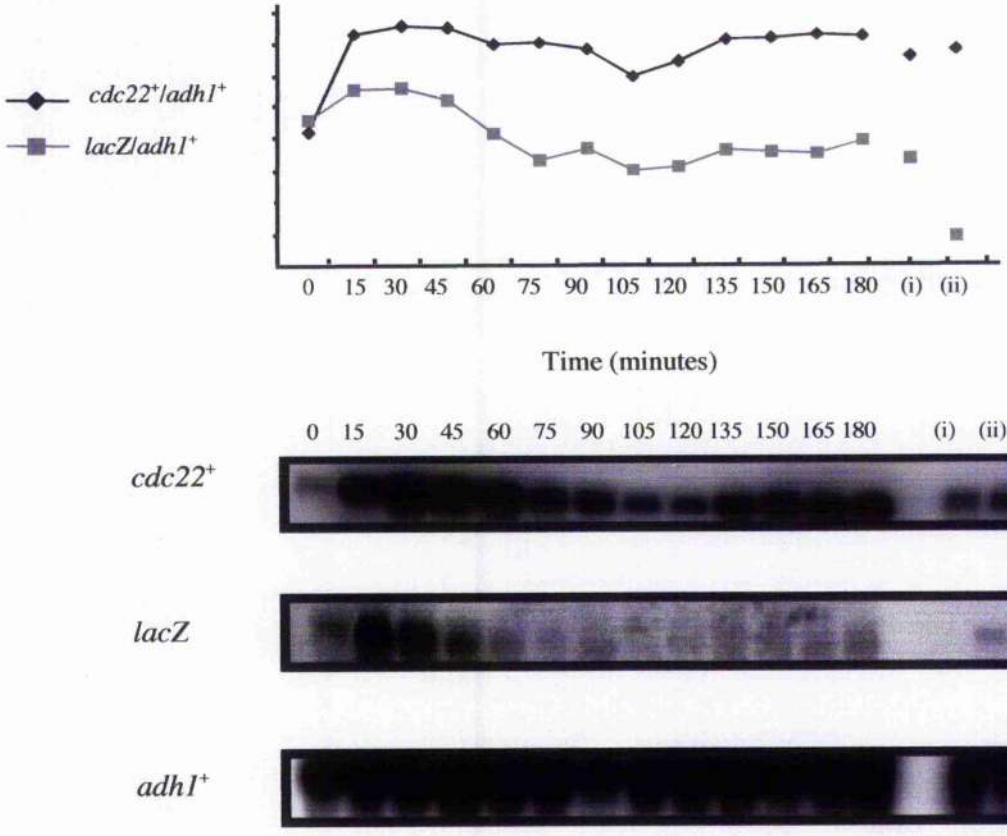
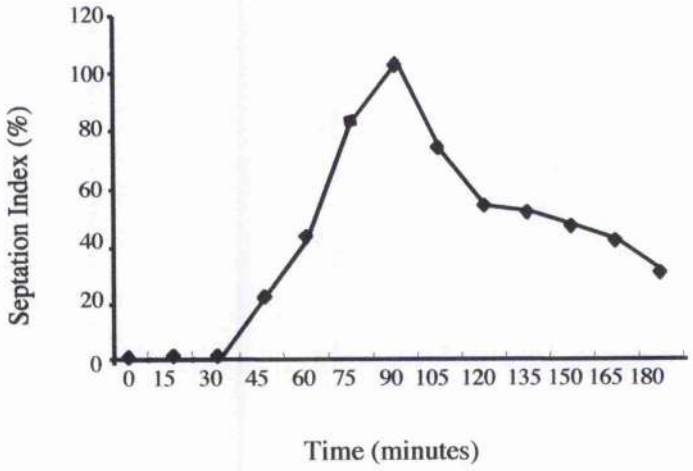

cdc22+/adh1+
lacZ/adh1+
0 15 30 45 60 75 90 105 120 135 150 165 180 (i) (ii)
Time (minutes)
0 15 30 45 60 75 90 105 120 135 150 165 180 (i) (ii)
cdc22+
lacZ
adh1+

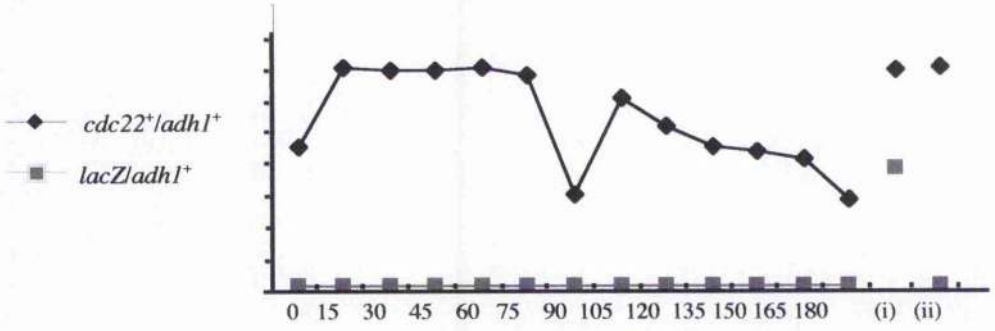
Figure 16. MCB1 confers G1/S expression to *lacZ* in the forward orientation.

(A) An exponentially growing culture of *cdc25-22* cells containing pSPΔ178.MCB1f (GG 251) was grown in minimal media and arrested at G2 by incubation at 36°C for 4 hours. Cells were shifted to 25°C and samples collected every 15 minutes for RNA extraction, and examined microscopically for the appearance of septa. (B) The RNA was subjected to Northern blot analysis and the blot was hybridised with *cdc22*$^{+}$, *lacZ* and *adh1*$^{+}$ probes, which were amplified by PCR using genomic DNA (oligo's described in Appendix B). Two control lanes were included containing RNA from asynchronous *cdc10-C4* cells containing the plasmid (i) pSPΔ178 empty vector (GG 258) and (ii) pSPΔ178.3M (GG 257). Quantification of each transcript against *adh1*$^{+}$ using NIH image software is shown. Data presented are representative of three identical experiments.

A


Septation Index (%)
120
100
80
60
40
20
0
0 15 30 45 60 75 90 105 120 135 150 165 180
Time (minutes)

B


cdc22+/adh1+
lacZ/adh1+
0 15 30 45 60 75 90 105 120 135 150 165 180 (i) (ii)
Time (minutes)

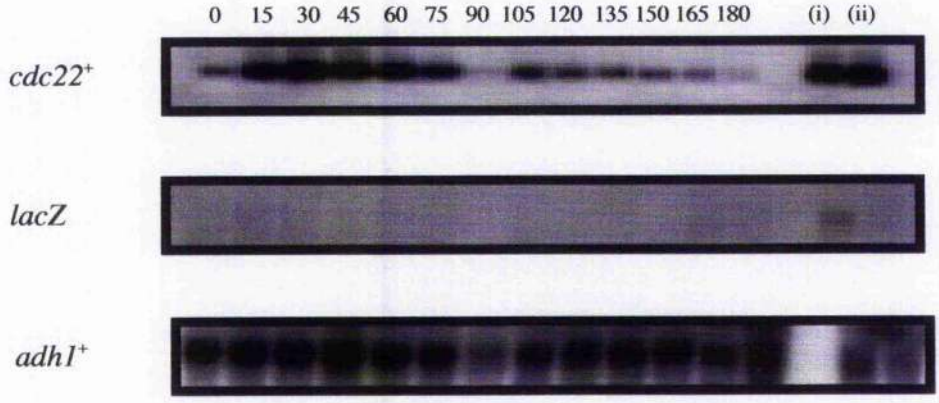
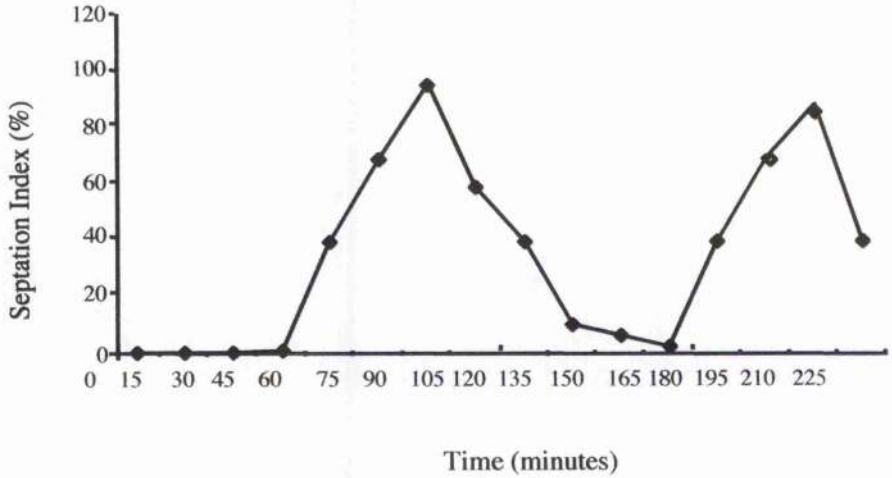

0 15 30 45 60 75 90 105 120 135 150 165 180 (i) (ii)
cdc22+
lacZ
adh1+

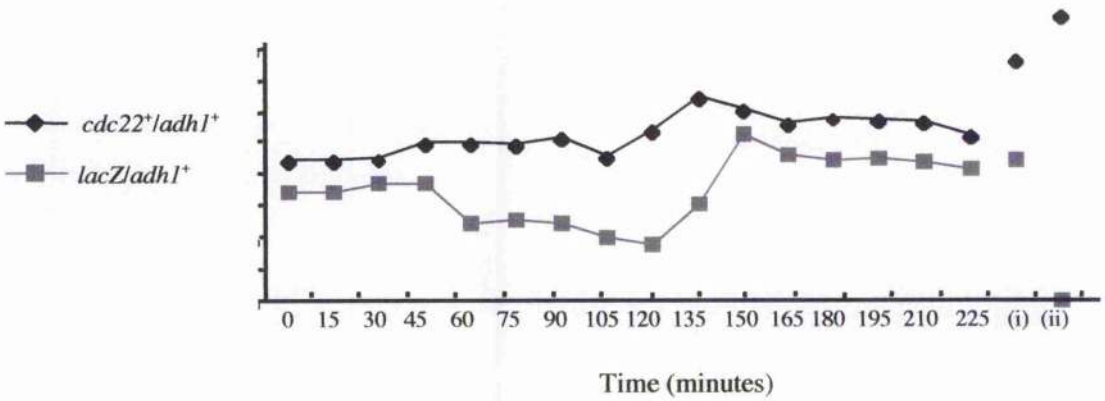
Figure 17. MCB1 does not confer expression to *lacZ* in the reverse orientation.

(A) An exponentially growing culture of *cdc25-22* cells containing pSPΔ178.MCB1r (GG 265) was grown in minimal media and arrested at G2 by incubation at 36°C for 4 hours. Cells were shifted to 25°C and samples collected every 15 minutes for RNA extraction, and examined microscopically for the appearance of septa. (B) The RNA was subjected to Northern blot analysis and the blot was hybridised with *cdc22*$^{+}$, *lacZ* and *adh1*$^{+}$ probes, which were amplified by PCR using genomic DNA (oligo's described in Appendix B). Two control lanes were included containing RNA from asynchronous *cdc10-C4* cells containing the plasmid (i) pSPΔ178.3M (GG 257) and (ii) pSPΔ178 empty vector (GG 258). Quantification of each transcript against *adh1*$^{+}$, using NIH image software is shown. Data presented are representative of three identical experiments.

A


Septation Index (%)
120
100
80
60
40
20
0
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225
Time (minutes)

B


cdc22+/adh1+
lacZ/adh1+
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 (i) (ii)
Time (minutes)

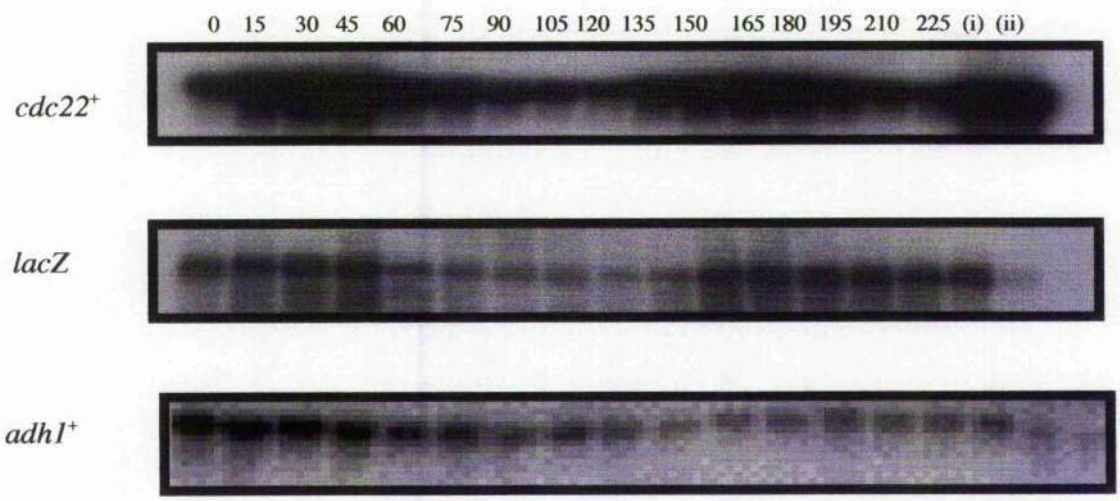

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 (i) (ii)
cdc22+
lacZ
adh1+

Figure 18. MCB2 confers G1/S expression to *lacZ* in the forward orientation

(A) An exponentially growing culture of *cdc25-22* cells containing pSPA178.MCB2f (GG 252) was grown in minimal media and arrested at G2 by incubation at 36°C for 4 hours. Cells were shifted to 25°C and samples collected every 15 minutes for RNA extraction, and examined microscopically for the appearance of septa. (B) The RNA was subjected to Northern blot analysis and the blot was hybridised with *cdc22*$^{+}$, *lacZ* and *adh1*$^{+}$ probes, which were amplified by PCR using genomic DNA (oligo's described in Appendix B). Two control lanes were included containing RNA from asynchronous *cdc10-C4* cells containing the plasmid (i) pSPA178.3M (GG 257) and (ii) pSPΔ178 empty vector (GG 258). Quantification of each transcript against *adh1*$^{+}$, using NIH image software is shown. Data presented are representative of three identical experiments.

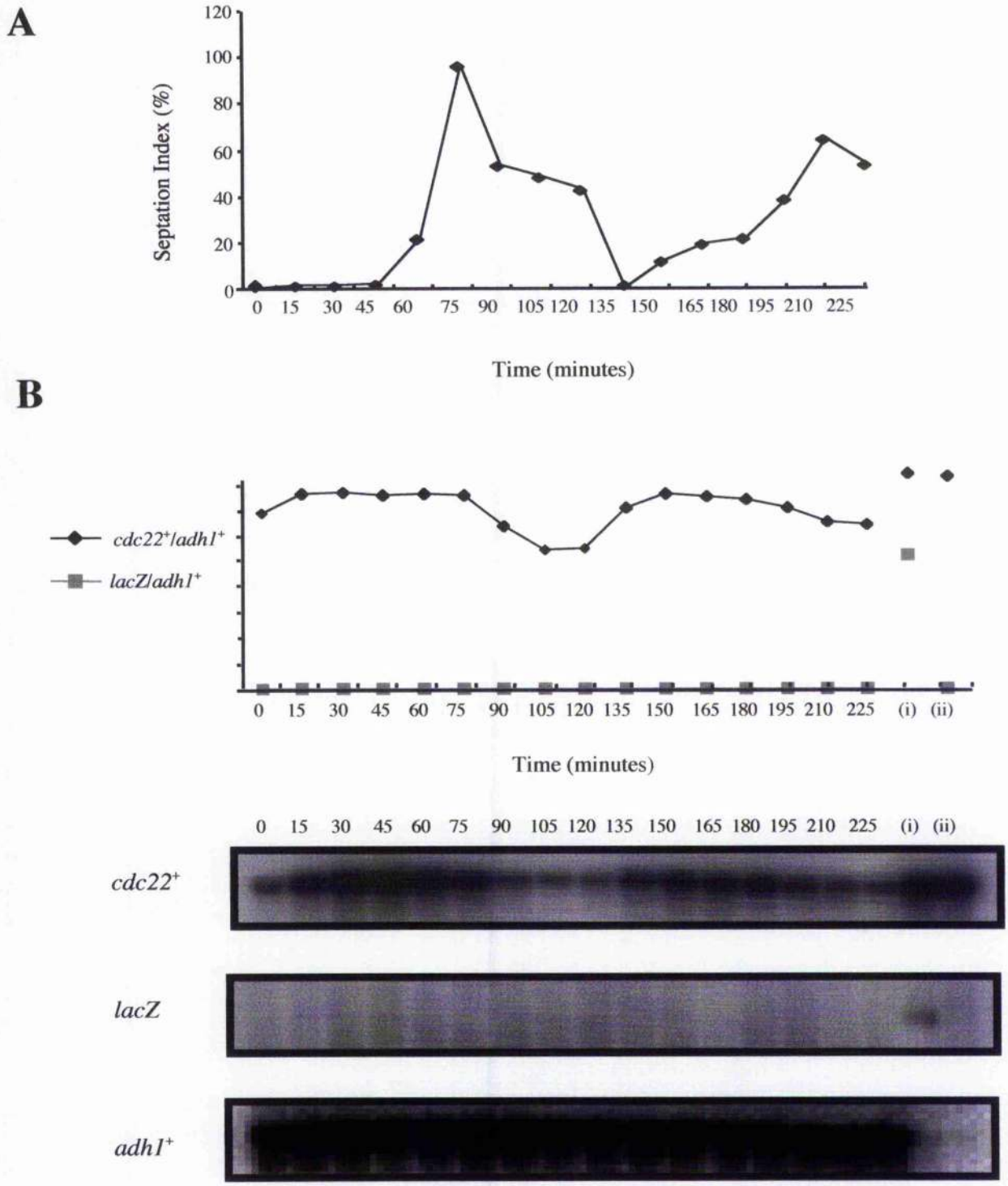

A
Septation Index (%)
120
100
80
60
40
20
0
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225
Time (minutes)
B
cdc22+/adh1+
lacZ/adh1+
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 (i) (ii)
Time (minutes)
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 (i) (ii)
cdc22+
lacZ
adh1+

Figure 19. MCB2 does not confer expression to *lacZ* in the reverse orientation.

(A) An exponentially growing culture of *cdc25-22* cells containing pSPΔ178.MCB2r (GG 253) was grown in minimal media and arrested at G2 by incubation at 36°C for 4 hours. Cells were shifted to 25°C and samples collected every 15 minutes for RNA extraction, and examined microscopically for the appearance of septa. (B) The RNA was subjected to Northern blot analysis and the blot was hybridised with *cdc22*$^{+}$, *lacZ* and *adh1*$^{+}$ probes, which were amplified by PCR using genomic DNA (oligo's described in Appendix B). Two control lanes were included containing RNA from asynchronous *cdc10-C4* cells containing the plasmid (i) pSPΔ178.3M (GG 257) and (ii) pSPΔ178 empty vector (GG 258). Quantification of each transcript against *adh1*$^{+}$, using NIH image software is shown. Data presented are representative of three identical experiments.

IV *MCB1 and MCB2 bind to a DSC1-like complex*

Previous experiments have implicated a transcription factor complex called DSC1 in regulating *cdc22*$^{+}$ cell cycle expression. DSC1 was first identified by gel retardation studies using a synthetic DNA fragment containing three adjacent MCB sequences as labelled substrate (Lowndes et al. 1992). These studies also revealed that MCB1 and MCB2 independently bind to DSC1, whereas MCBX cannot, when used as unlabelled competitor DNAs *in vitro* (Lowndes et al. 1992).

We next confirmed and extend these experiments by using MCB1, MCB2 and MCBX DNA instead as labelled substrates in gel retardation experiments (Materials and methods, pages 64-65). With MCB1 and MCB2 as labelled probes, we detected a binding activity in fission yeast wild-type cells, which produced a single prominent band of similar low mobility to DSC1 (Figure 20, lane 2, and lane 7, large arrow) (Lowndes et al. 1992). The low mobility of this complex suggested that it was likely to be DSC1. In both cases the complex was specifically competed when the same DNA was added as unlabelled competitor to the reaction mixtures (Figure 20, lanes 3-5 and 8-10). However, when MCBX was used as labelled substrate no retarded complex was detected (Figure 20, lanes 12-15), suggesting this lone MCB motif did not bind to DSC1.

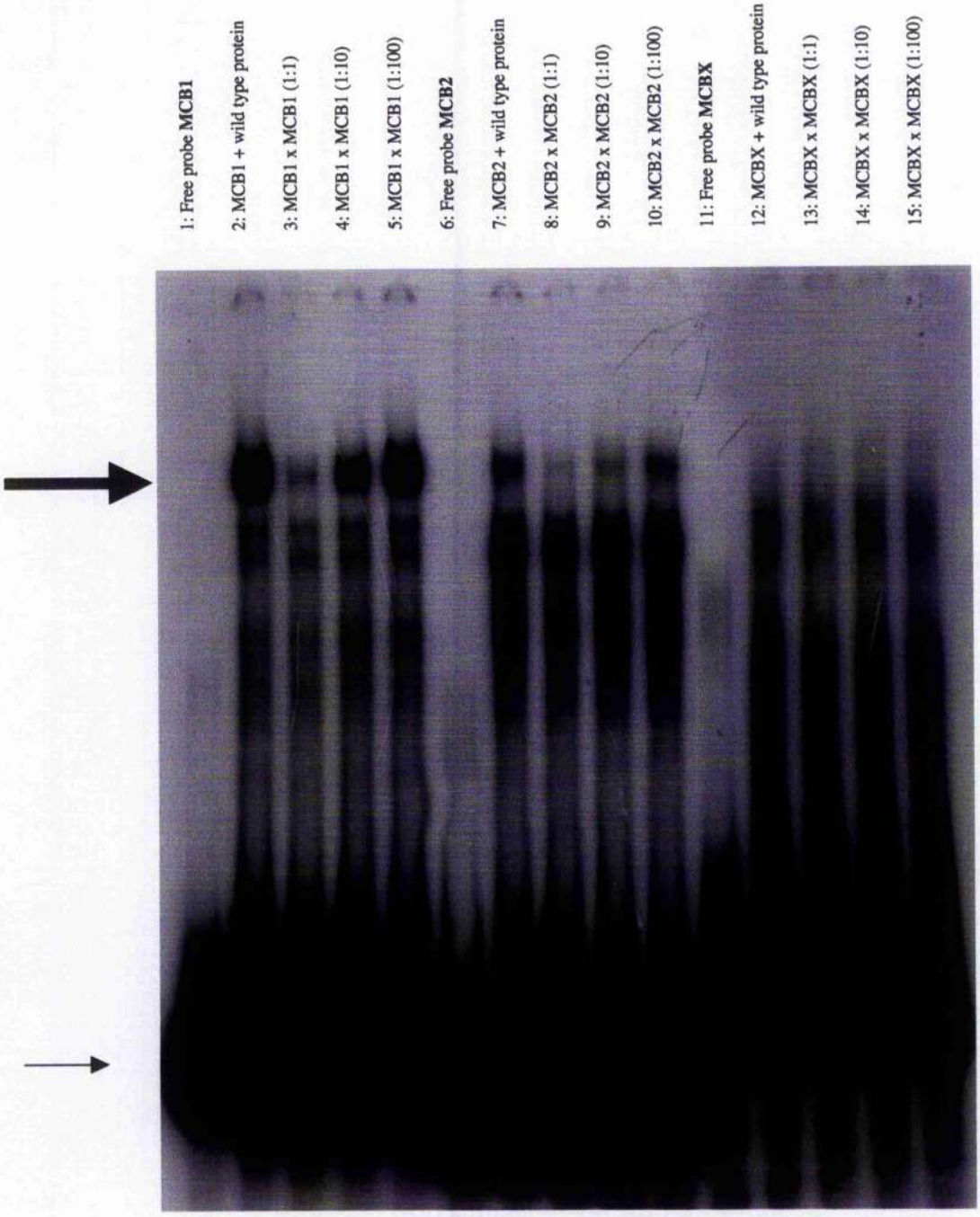


Figure 20. Both MCB1 and MCB2, but not MBCX, bind to a DSC1-like complex *in vitro*.

Gel retardation assay using MCB1, MCB2 and MCBX DNA promoter fragments as labelled probes, with protein extracts from wild-type cells (GG 217). In alternate lanes we added equal amounts (1:1) and 1 in 10 (1:10) and 1 in 100 (1:100) dilutions of excess unlabelled self-competitor MCB DNAs to the reaction mixture prior to electrophoresis. Large arrow indicates DSC1-like complex, small arrow free probe. Data presented are representative of three identical experiments.

V *The complex that binds to MCB1 and MCB2 is DSC1*

To confirm that the low mobility complex detected in the previous experiment was DSC1, gel retardation analysis with various strains containing mutations in components of DSC1 was completed. Protein extracts were prepared from *res1Δ*, *res2Δ*, *rep2Δ* and *cdc10-129* cells. *cdc10-129* is a temperature sensitive *cdc10* mutant which, when grown at the permissive temperature of 25°C, behaves like wild type, but at the restrictive temperature of 36°C, when the cells are cell cycle arrested, results in the loss of DSC1 binding affinity *in vitro* (Lowndes et al. 1992).

When MCB1 was used as a labelled substrate, the single retarded band of low mobility was detected in protein extracts from wild-type (Figure 21, lane 2) and *cdc10-129* cells at permissive temperature (Figure 21, lane 6). This band disappeared in protein extracts from cells containing deletions of components of DSC1, and in *cdc10-129* cells grown at the restrictive temperature of 36°C for four hours (Figure 21, lanes 3-5 and 7). Similarly, when MCB2 was used as labelled substrate, the same low mobility complex was detected in protein extracts from wild-type (Figure 21, lane 2) and *cdc10-129* cells at permissive temperature (Figure 21, lane 6). This low mobility complex was also lost in protein extracts from cells containing deletions of DSC1, and in *cdc10-129* cells grown at the restrictive temperature of 36°C (Figure 21, lanes 3-5 and 7). Combined, these results confirm that the retarded complex of low mobility that binds to both MCB1 and MCB2 *in vitro* is DSC1.

Interestingly, in protein extracts from *res1Δ* and *cdc10-129* cells grown at the restrictive temperature an additional retarded band of higher mobility than DSC1 was identified, when MCB2 was used as labelled substrate (Figure 22, red arrow; lanes 7 and 3). To establish if this band was a non-specific/specific complex binding to MCB2 (possibly DSC1 containing a reduced number of components), gel retardation analysis was carried out using MCB2 DNA as labelled substrate and protein extracts were prepared from wild-type, *res1Δ* and *cdc10-129* cells at the restrictive temperature. It was consequently found that the higher mobility band did not disappear when MCB1 or MCB2 DNAs were added as unlabelled competitor (Figure 23, lanes 5-10). This observation suggests that the higher mobility band has no binding specificity for MCB motifs, and is therefore a non-specific protein complex binding to MCB2 DNA that is not DSC1.

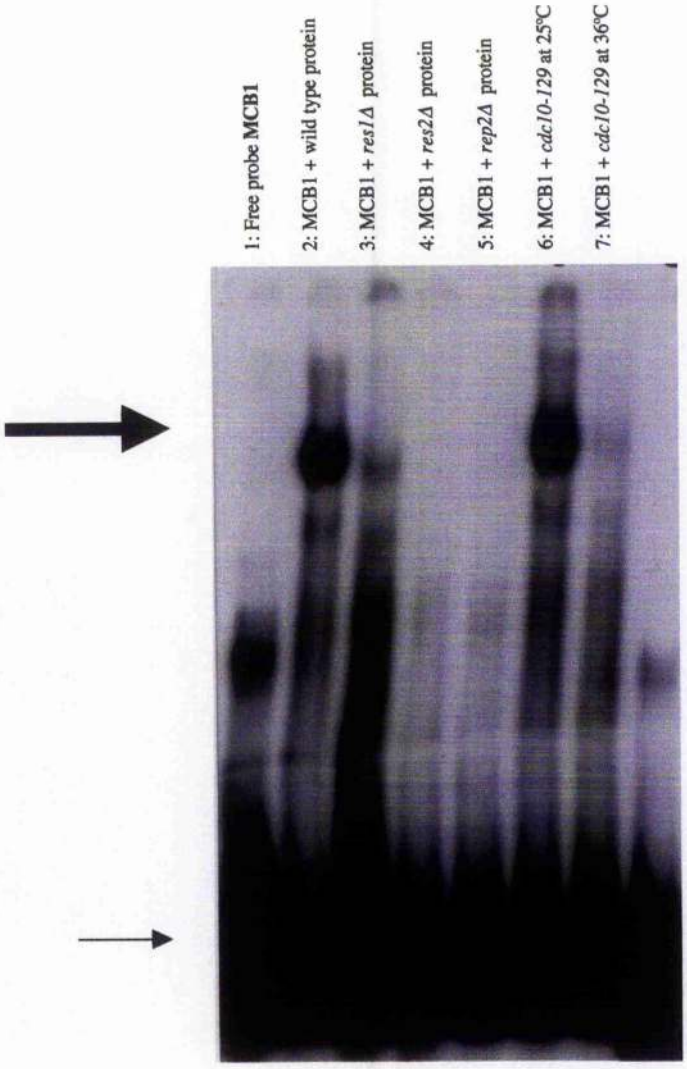


Figure 21. Loss of complex binding to MCB1 in DSC1 mutant strains..

Gel retardation assay using MCB1 DNA promoter fragment as labelled probe with protein extracts from various DSC1 mutants. In lanes 2-7 protein extracts were prepared from wild-type (GG 217), *res1Δ* (GG 155), *res2Δ* (GG 156), *rep2Δ* (GG 158), and *cdc10-129* (GG 28) cells grown at 25°C and 36°C, respectively. Large arrow indicates DSC1, small arrow free probe. Data presented are representative of three identical experiments.

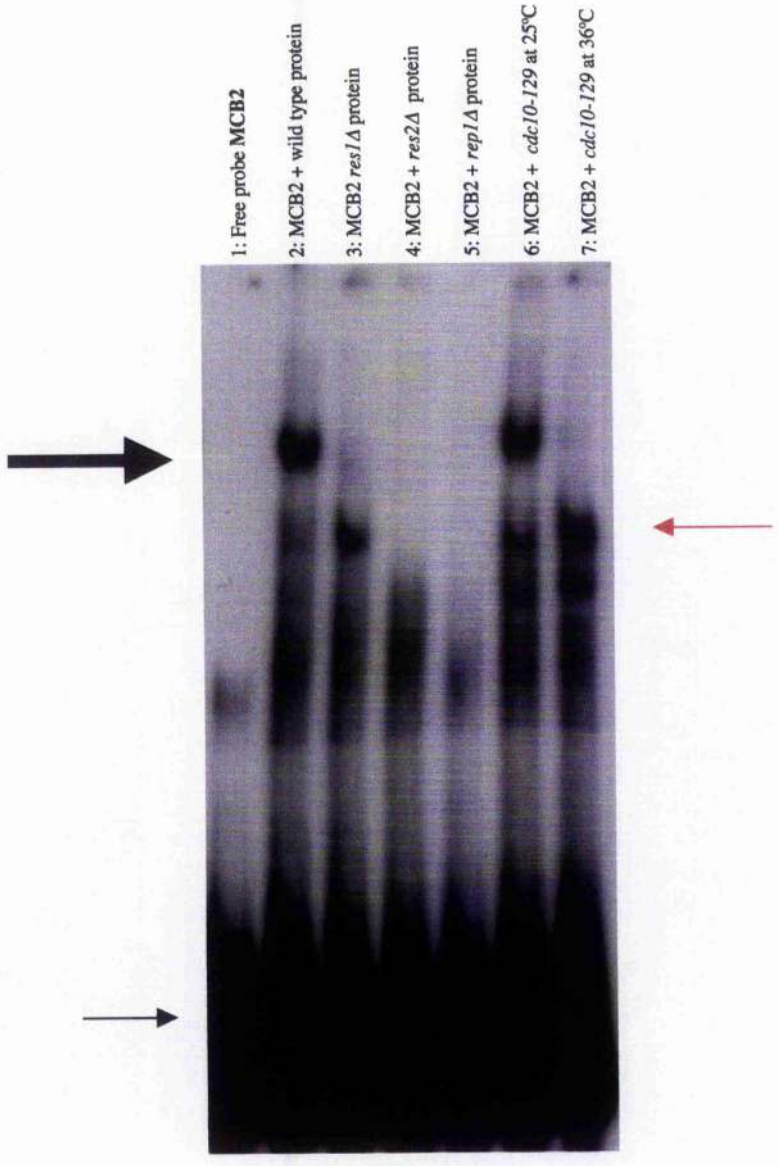


Figure 22. Loss of complex binding to MCB2 in DSC1 mutant strains. Gel retardation assay using MCB2 DNA promoter fragment as labelled probe with protein extracts from various mutants. In lanes 2-7 protein extracts were prepared from wild-type (GG 217), *res1Δ* (GG 155), *res2Δ* (GG 156), *rep2Δ* (GG 158), and *cdc10-129* (GG 28) cells grown at 25°C and at 36°C, respectively. Large arrow indicates DSC1; red arrow indicates non-specific complex; small arrow free probe. Data presented are representative of three identical experiments.

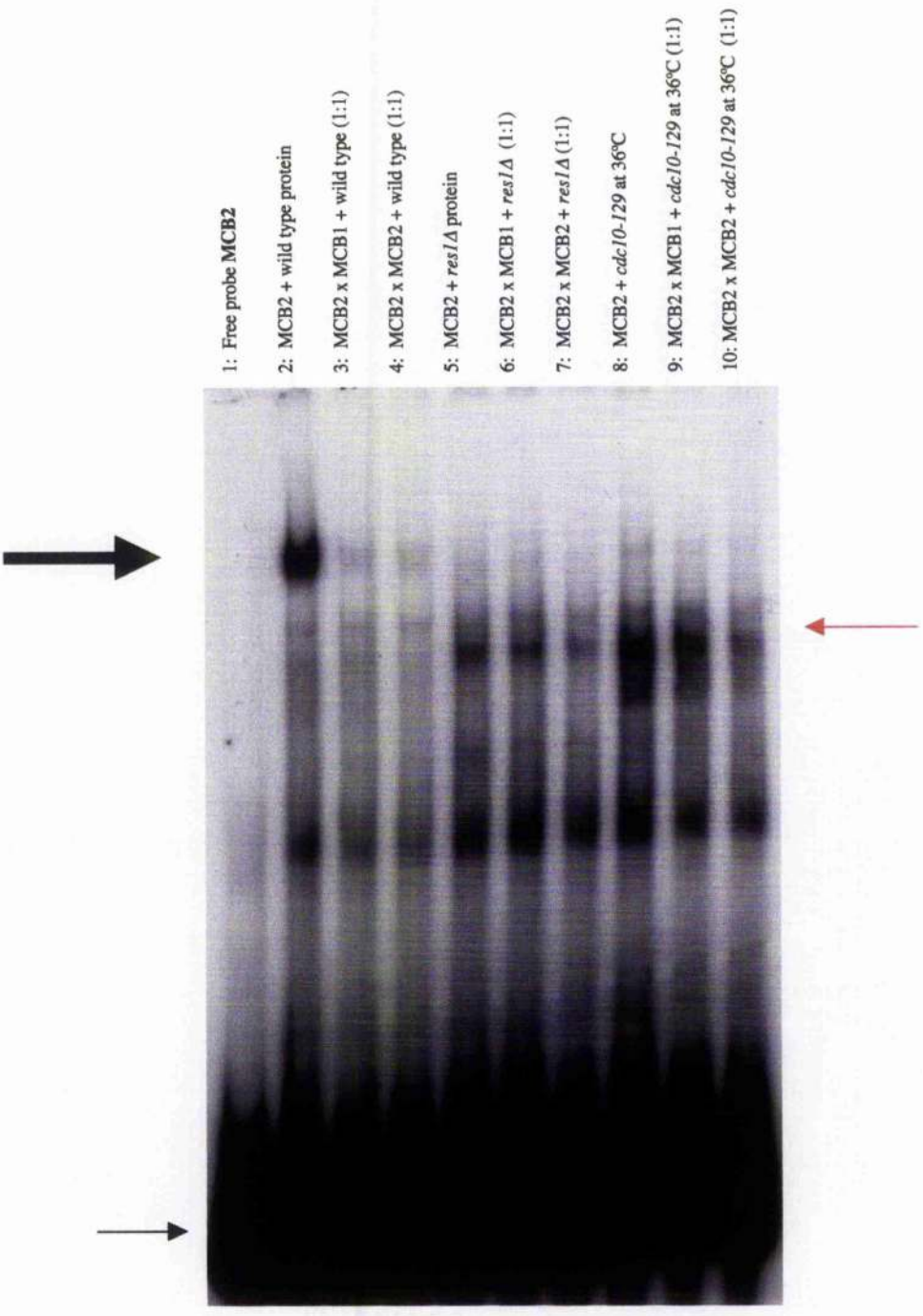


Figure 23. Higher mobility band detected binding to MCB2 in *res1Δ* and *cdc10-129* cells is not DSC1.

Gel retardation assay using MCB2 DNA promoter fragment as labelled probe with protein extracts from wild-type and various mutant cells. In lanes 2-10 protein extracts were prepared from wild-type (GG 217), *res1Δ* (GG 155), and *cdc10-129* (GG 28) cells grown at grown at 36°C. Large arrow indicates DSC1; small arrow free probe. In alternate lanes we added equal amounts (1:1) of excess unlabelled self-competitor/MCB1 DNAs to the reaction mixture prior to electrophoresis. Data presented are representative of three identical experiments.

VI *MCB1 has a stronger binding affinity for DSC1 than MCB2*

We next sought to determine if the different MCB clusters were capable of competing with each other *in vitro*, to give an indication of their relative binding affinities for DSC1.

MCB1 DNA, when used as labelled substrate in gel retardation studies, revealed DSC1 binding, which disappeared when the same DNA or MCB2 DNA were added as unlabelled competitors (Figure 24, lanes 2-4, lanes 5-7). However, as expected, adding MCBX as unlabelled competitor had no effect on DSC1 binding (Figure 24, lanes 8-10).

Similarly, gel retardation studies using MCB2 DNA as labelled substrate revealed the retarded complex that is DSC1, which disappeared when the same DNA or MCB1 DNA were added as unlabelled competitors (Figure 25, lanes 2-4 and 5-7). Again, when MCBX was used as unlabelled competitor, DSC1 did not disappear (Figure 25, lanes 8-10), confirming that MCBX does not bind DSC1 *in vitro*.

Combined these results confirm that both MCB1 and MCB2 bind to DSC1 *in vitro* and compete with each other for its binding activity. Furthermore, it appears that MCB1 has a stronger binding affinity for DSC1 than MCB2 as, in both cases, MCB1 was the stronger competitor for DSC1. These gel retardation results by themselves suggest that MCB1 has a more important role in regulating *cdc22*$^{+}$ expression, if *in vitro* binding affinity reflects *in vivo* activity. This is surprising, as MCB2 conferred G1/S expression to *lacZ* more efficiently than MCB1 *in vivo* (Figures 16 and 18).

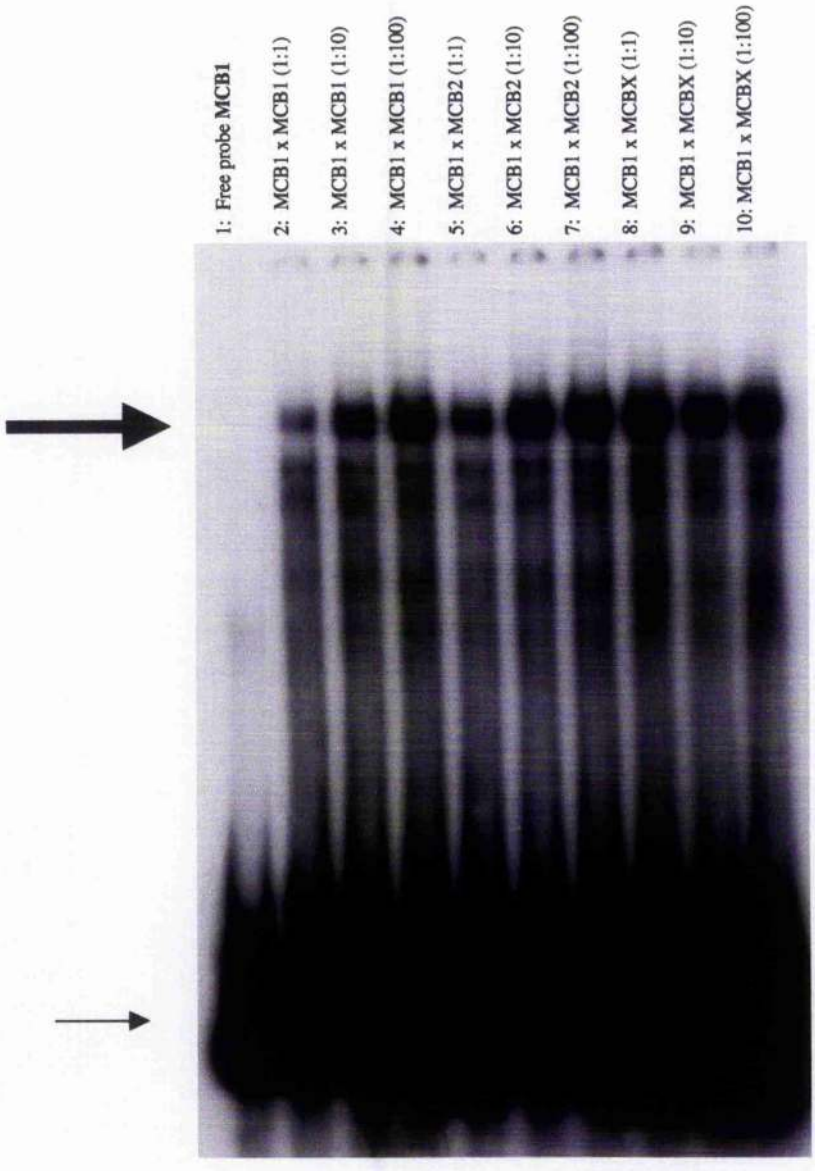


Figure 24. MCB1 can compete with MCB2 to bind DSC1 *in vitro*.

Gel retardation assay using MCB1 DNA promoter fragment as labelled probe with protein extracts from wild type (GG 217) cells. In alternate lanes we added equal amounts (1:1), 1 in 10 (1:10) and 1 in 100 (1:100) dilutions of excess unlabelled self-competitor MCB DNAs to the reaction mixture prior to electrophoresis. Large arrow indicates DSC1, small arrow free probe. Data presented are representative of three identical experiments.

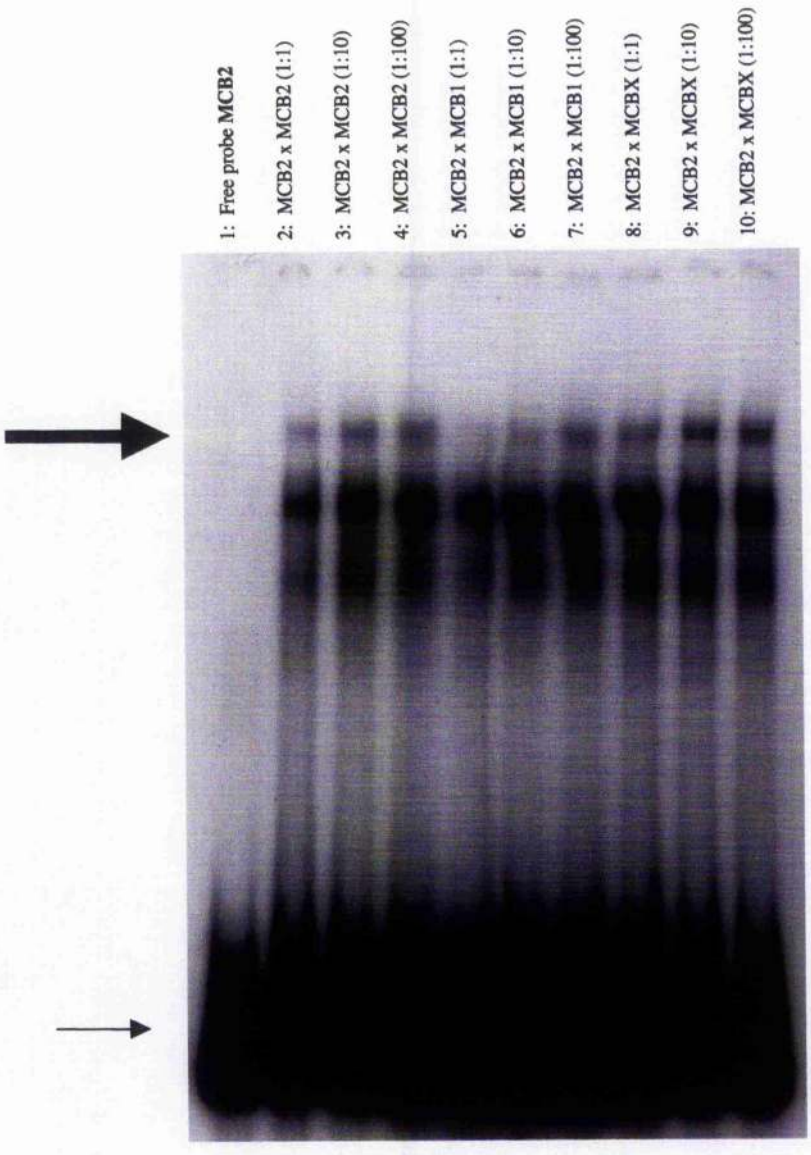


Figure 25. MCB2 can compete with MCB1 to bind DSC1 *in vitro*.

Gel retardation assay using MCB2 DNA promoter fragment as labelled probe with protein extracts from wild-type (GG 217) cells. In alternate lanes we added equal amounts (1:1), 1 in 10 (1:10) and 1 in 100 (1:100) dilutions of excess unlabelled competitor MCB DNAs from MCB2, MCB1 and MCBX to the reaction mixtures prior to electrophoresis. Large arrow indicates DSC1; small arrow free probe. Data presented are representative of three identical experiments.

3B *cdc22*$^{+}$ MCB clusters are required for G1/S transcription

1 *cdc22*$^{+}$ *promoter confers G1/S cell cycle regulation to lacZ in an integration vector*

The previous experiments have established that native MCB clusters, present in the *cdc22*$^{+}$ promoter, can both confer G1/S transcription to *lacZ in vivo* and bind to DSC1 *in vitro*, strongly implicating them in regulating cell cycle specific expression of this gene in fission yeast. As another way to address the role of the MCB clusters in the *cdc22*$^{+}$ promoter, we next sought to examine the effect of mutating the motifs in the context of the complete promoter on G1/S specific transcription.

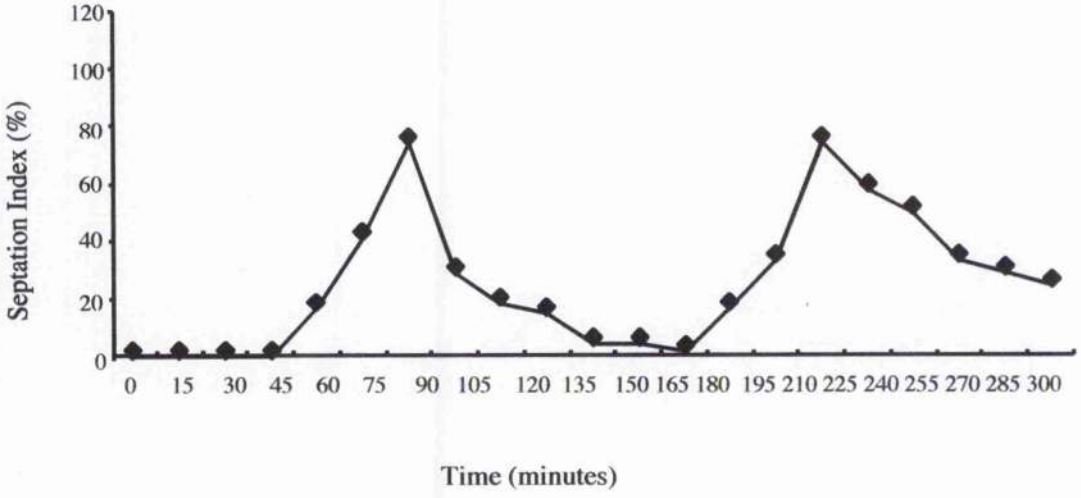
To do this we initially used a vector named pPJK7 made by Dr. Kersey in Dr. Fantes's laboratory, at the University of Edinburgh (Kersey 1995). pPJK7 contains the entire *cdc22*$^{+}$ promoter linked in frame to the *lacZ* gene (Appendix A.2). This vector also contains the *ura4*$^{+}$ nutritional gene, which allows the plasmid to be integrated into the fission yeast *ura4* genomic locus in single copy, by homologous recombination. pPJK7 also contains the *amp*^{R} gene and an *E. coli* origin of replication, to permit the growth and maintenance of the vector in bacteria.

In the experiments carried out with pPJK7 by Dr. Kersey, the construct was integrated into wild-type cells, which were synchronised for mitotic division by elutriation (Kersey 1995). This method permits the size selection of small fission yeast cells in early G2, which subsequently divide synchronously. When *cdc22*$^{+}$ and *lacZ* transcript levels were monitored in such cells it was observed that *lacZ* mRNA was present in a higher proportion of the cell cycle than the *cdc22*$^{+}$ mRNA, although the two transcript levels appeared to peak at similar times. It was also found that when β-galactosidase activity (per ml of culture) was examined, it was also less tightly confined within the cell cycle than *cdc22*$^{+}$ mRNA (Kersey 1995). It was thus concluded that *lacZ* was expressed similarly, though not identically to *cdc22*$^{+}$, possibly because different transcriptional termination of the two genes results in the *lacZ* transcript being more

stable than *cdc22*$^{+}$ transcript. We therefore decided to repeat this experiment using the *cdc25-22* mutant as an alternative method for synchronising fission yeast cell cultures.

pPJK7 was provided to us integrated in single copy in *S. pombe* wild-type cells, which we crossed into a *cdc25-22* background (Materials and methods, pages 62-63). pPJK7 *cdc25-22* cells were synchronised using transient temperature shifts as described in previous experiments, and the cell cycle expression of the *lacZ* transcript detected using Northern blot analysis (Figure 26). As expected, and in agreement with Dr. Kersey's results, the *lacZ* gene was periodically expressed during the cell cycle, coincident to *cdc22*$^{+}$. Two control lanes were included, containing RNA from asynchronous *cdc10-C4* cells containing (i) the pSPΔ178 plasmid with a triple MCB, known to confer *lacZ* transcription and (ii) pSPΔ178 empty vector, known not to confer *lacZ* transcription. The use of a different plasmid resulted in a difference in the size of transcript seen in the control lanes. Three identical experiments showed that although the *lacZ* transcript persisted after the first cell cycle peak, it coincided tightly with the second peak of *cdc22*$^{+}$ mRNA. This suggested that *lacZ* expression was more similar to *cdc22*$^{+}$ expression in cells synchronised using the *cdc25-22* mutant, than in wild-type cells synchronised by elutriation. We consequently decided to use *cdc25-22* for further cell cycle experiments with this construct.

A


Septation Index (%)
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80
60
40
20
0
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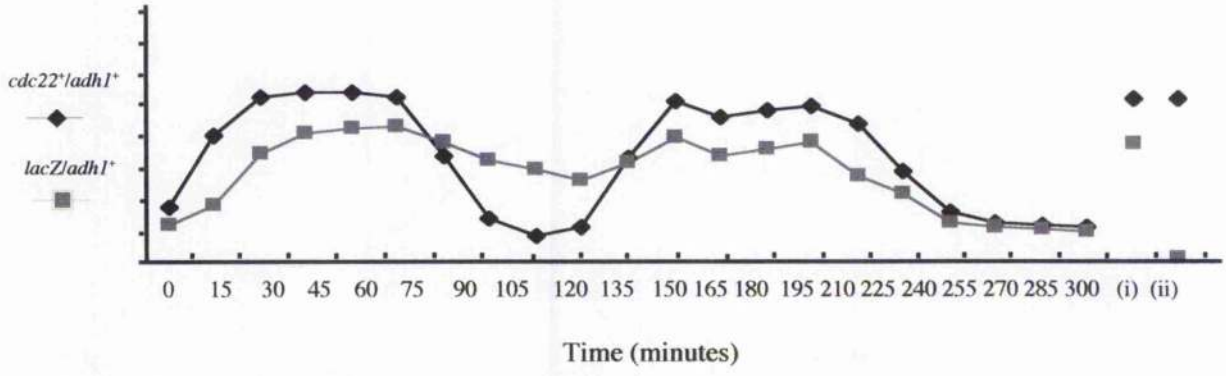
Time (minutes)

B


cdc22+/adh1+
lacZ/adh1+
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 (i) (ii)
Time (minutes)

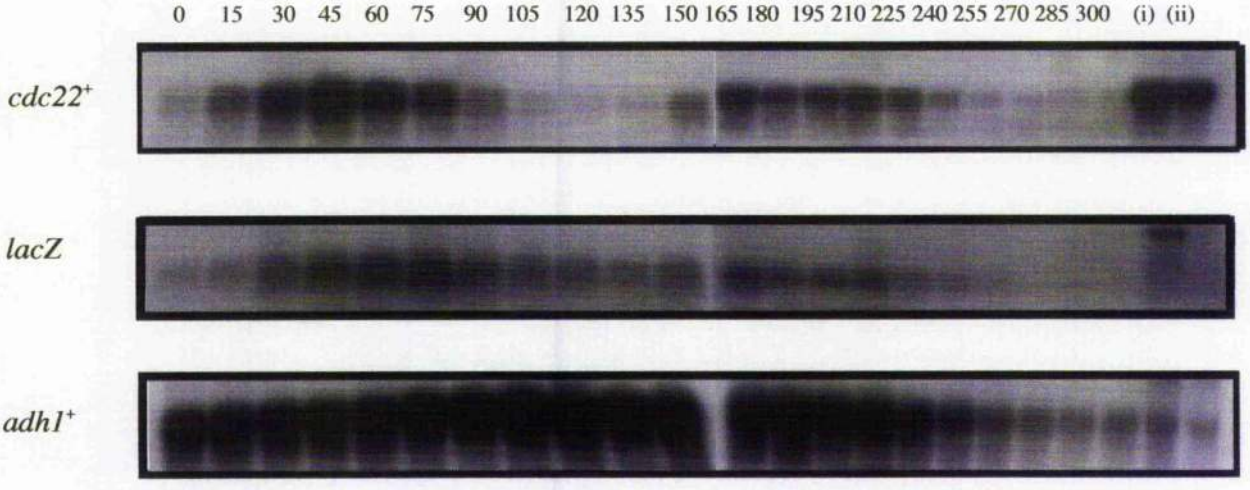

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 (i) (ii)
cdc22+
lacZ
adh1+

Figure 26. *cdc22*$^{+}$ promoter confers G1/S transcription to *lacZ*.

(A) An exponentially growing culture of pPJK7 *cdc25-22* cells (GG 470) was grown in minimal media and arrested at G2 by incubation at 36°C for 4 hours. Cells were shifted to 25°C to enter a synchronous cell cycle, with samples collected for RNA extraction every 15 minutes, and cells examined microscopically for the appearance of septa at the times indicated. (B) RNA was subjected to Northern blot analysis and the blot hybridised with *cdc22*$^{+}$, *lacZ* and *adh1*$^{+}$ probes, which were amplified by PCR using genomic DNA (oligo's described in Appendix B). Two control lanes were included containing RNA from asynchronous *cdc10-C4* cells containing the plasmid (i) pSPΔ178.3M (GG 257) and (ii) pSPΔ178 empty vector (GG 258). Quantification of each transcript against *adh1*$^{+}$, using NIH image software is shown. Data presented are representative of three identical experiments.

II *Intact MCBs in MCB1 are essential for cell cycle regulated transcription of cdc22*$^{+}$

Previous findings presented in this thesis have shown that both the MCB1 and MCB2 clusters in the *cdc22*$^{+}$ promoter can bind to DSC1 *in vitro* (Figures 21 and 22), and that MCB2 can confer G1/S expression to *lacZ* more efficiently than MCB1 *in vivo* (Figures 16 and 18). These results suggest that both MCB clusters control transcription of *cdc22*$^{+}$. At least for MCB2 this particularly interesting as this cluster lies within the transcribed region, and so MCB2 may be the first example of a downstream activating sequence in fission yeast.

To investigate the role of MCB1 and MCB2 further we next sought to study the contribution of each cluster to G1/S transcription by examining the effect of mutating MCB motifs on *lacZ* transcription. This was achieved by using another integration vector provided by Dr. Fantes's laboratory, called pPJK10 (Kersey 1995). This vector is the same as pPJK7 but differs in that the core CGCG of each MCB motif, thought to be essential for function (McIntosh 1993), has been mutated to CTAG in MCB1 (Figure 27) (Appendix A.3). We called this new mutated MCB1 cluster "MCB1m".

In the studies carried out by Dr Kersey, pPJK10, like pPJK7, had been integrated into wild-type fission yeast cells, which were size selected by elutriation (Kersey 1995). In subsequent synchronously dividing cells, it was observed that the *lacZ* transcript levels varied, with a slight peak at the same time as *cdc22*$^{+}$ transcript. However, *lacZ* mRNA was clearly present during more of the cell cycle than mRNA produced by the endogenous *cdc22*$^{+}$ gene. It was also found that the β-galactosidase activity increased for a short period immediately after synchronisation (Kersey 1995). Given that MCB1 had a strong binding affinity for DSC1 than MCB2 (Figures 24 and 25) and that this cluster can confer cell cycle transcription to *lacZ* (Figure 16), we found it surprising that mutating the MCBs in MCB1 did not have a stronger effect on periodic gene expression. We therefore decided to repeat these experiments using the *cdc25-22* mutant.

The *ura4*$^+$ gene within the vector contains a single cleavage restriction site for *BlnI*/*Avr*II, that permitted the linearisation of the vector (Appendix A.3) to integrate it in single copy into the *ura4* locus of *cdc25-22 ura4-294* cells (GG 469) (Materials and methods, pages 57-58). To confirm that pPJK10 had integrated at the correct site and in single copy, PCR was performed using genomic DNA from potential positive colonies. The oligonucleotide primers used for amplification were specific for the flanking region of *ura4*$^+$ in the chromosome and to the bacterial *lacZ* gene in the vector (Figure 29). An amplified product was detected in a potential positive clone containing the integration vector (Figure 28, lane 2), that did not appear in the control reaction (Figure 28, lane 5), which used wild-type chromosomal DNA as a template. This result confirmed that the integration of pPJK10 had occurred correctly.

pPJK10 *cdc25-22* cells were prepared to undergo synchronous mitosis by transient temperature shifts, and Northern blot analysis of RNA was completed to detect *lacZ* mRNA through the cell cycle (Figure 30). Two control lanes were included, containing RNA from asynchronous *cdc10-C4* cells containing (i) the pSPΔ178 plasmid with a triple MCB, known to confer *lacZ* transcription and (ii) pSPΔ178 empty vector, known not to confer *lacZ* transcription. The use of a different plasmid resulted in the difference in the size of *lacZ* transcript seen in the two control lanes.

In contrast to the results obtained by Dr. Kersey it was observed that, in three identical experiments, the *lacZ* transcript profile did not coincide with the *cdc22*$^+$ transcript profile. Instead, it was seen that mutating the MCB motifs in MCB1 resulted in constitutive *lacZ* transcription at moderate levels throughout the cell cycle. This result strongly suggested that intact MCB motifs in MCB1 are essential for the correct cell cycle regulation of *cdc22*$^+$, and consequently are essential for stimulating cell cycle transcription. It further suggested that MCB1 has a negative role in the context of the complete promoter, whereby it represses transcription of *cdc22*$^+$ outside the G1/S boundary.

-536 GTAGTTCAATCTCATAGAGCAGGTTGGTAGTCGGGTTGG

ACCTAGTGTTTAATTTATGTAAACAGTCTAGTCTAGTTG MCB1m

CAATTGAGACTAGTAAATAAATATTTAATTTATTACATT

CAGTCGTAAACAGAG - 405

Figure 27. Nucleotide sequence of "MCB1m", containing mutated MCB motifs in MCB1.

MCB motifs are shown in purple and mutated base changes in red. Numbers are relative to the ATG.

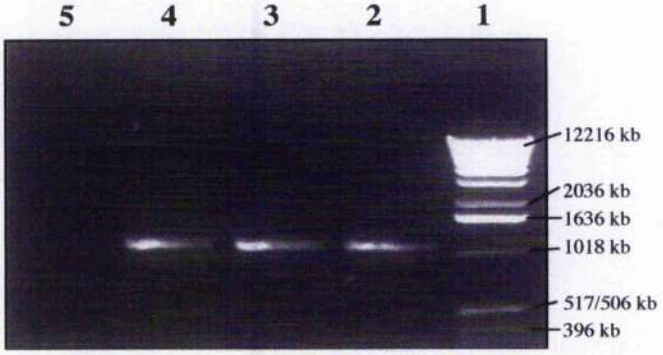


Figure 28. Agarose gel of ethidium stained DNA from PCR amplifications showing successful integration of *cdc22*$^{+}$ promoter-*lacZ* vectors into the genomic *ura4*$^{+}$ locus of fission yeast.

Lane 1: DNA molecular weight marker, lane 2: pPJK10 *cdc25-22* (GG 471), lane3: pZM1 *cdc25-22* (GG 591), lane 4: pZM2 *cdc25-22* (GG 592), and lane 5: wild type genomic DNA (GG 217) containing no vector. In each case a successful amplification, using oligonucleotides specific for the flanking region of *ura4*$^{+}$ and to the bacterial *lacZ* gene (Figure 29), resulted in the production of a ~1.3 kb DNA fragment.

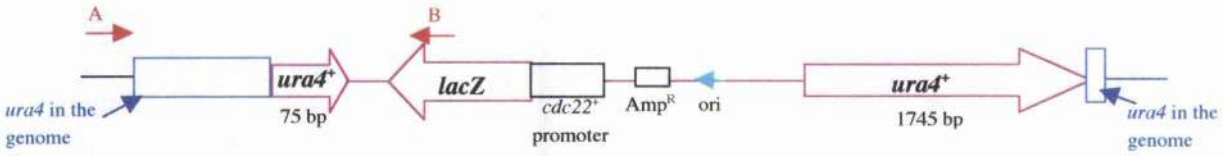
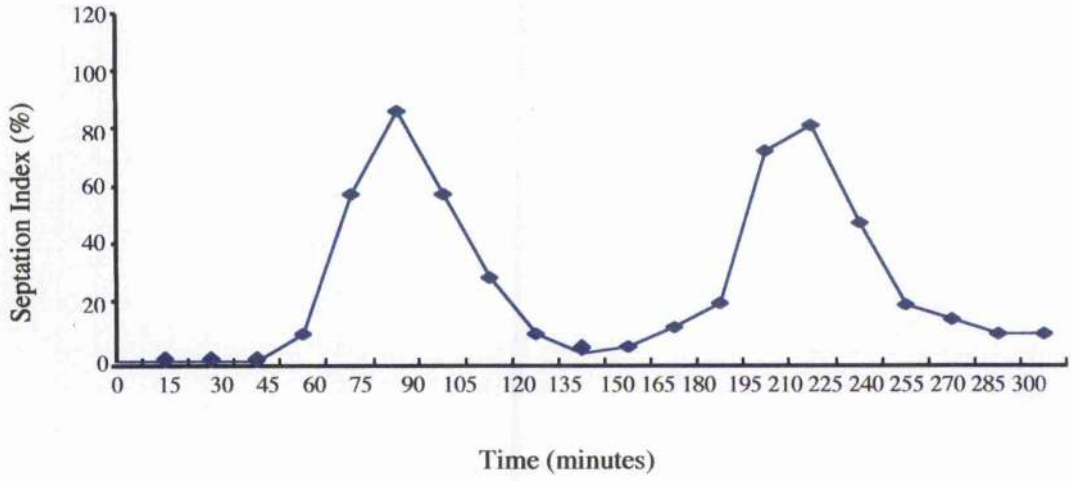


Figure 29. Schematic representation of pPJK7/10 and pZM1/2 plasmids integrated into the fission yeast chromosome.

Plasmids (Appendix A) were cut at a single restriction site in *ura4*$^{+}$ using the restriction enzyme *Bln*I at position 5275, to permit integration into fission yeast *ura4* locus. Oligonucleotide A (GO 521) bound to a sequence 24 base pairs upstream from the ATG of the *ura4*$^{+}$ ORF in genomic DNA. Oligonucleotide B (GO 522) was specific for the *lacZ* gene at position 4768 on the plasmid map. Oligonucleotides are described in Appendix B. Amplification with these oligonucleotides, demonstrating a successful integration of the vector in to the *ura4* locus, gave a DNA product of ~1.3 kb (Figure 28).

A


Septation Index (%)
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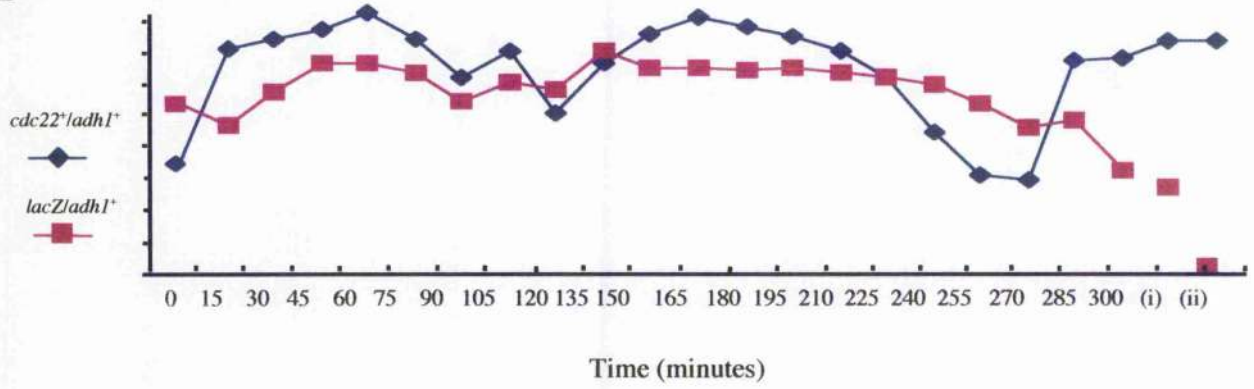
100
80
60
40
20
0
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300
Time (minutes)

B


cdc22+/adh1+
lacZ/adh1+
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 (i) (ii)
Time (minutes)

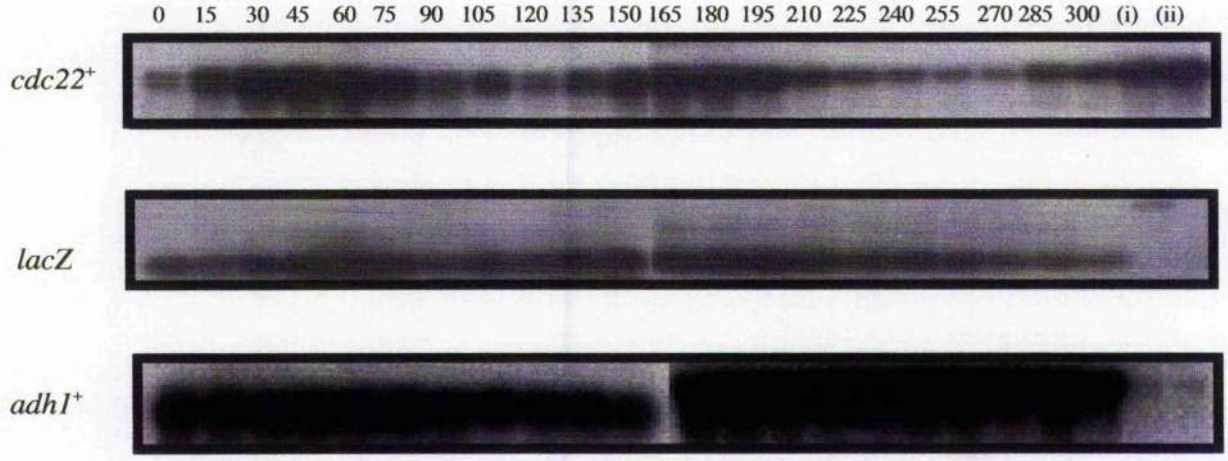

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 225 240 255 270 285 300 (i) (ii)
cdc22+
lacZ
adh1+

Figure 30. MCB1 is required for cell cycle specific expression of *cdc22*$^{+}$.

(A) An exponentially growing culture of pPJK10 *cdc25-22* cells (GG 471) was grown in minimal media and arrested at G2 by incubation at 36°C for 4 hours. Cells were shifted to 25°C to enter a synchronous cell cycle, with samples collected for RNA extraction every 15 minutes, and cells examined microscopically for the appearance of septa at the times indicated. (B) The RNA was subjected to Northern blot analysis and the blot was hybridised with *cdc22*$^{+}$, *lacZ* and *adh1*$^{+}$ probes, which were amplified by PCR using genomic DNA (oligo's described in Appendix B). Two control lanes were included containing RNA from asynchronous *cdc10-C4* cells containing the plasmid (i) pSPΔ178.3M (GG 257) and (ii) pSPΔ178 empty vector (GG 258). Quantification of each transcript against *adh1*$^{+}$, using NIH image software is shown. Data presented are representative of three identical experiments.

III *Intact MCBs in MCB2 are essential for basal transcription of cdc22*$^{+}$

We next sought to determine the role of MCB2 in regulating *cdc22*$^{+}$ expression. We used site directed mutagenesis to mutate the MCB motifs in MCB2 in pPJK7 (Materials and methods, pages 65-66), to make a new construct pZM1 (GB 245; Appendix A.4). We named this mutated MCB2 cluster "MCB2m" (Figure 31). The oligonucleotides used for this mutagenesis are listed in Appendix B, and the mutations created in MCB2 DNA were confirmed by sequencing. As before, this plasmid was integrated in single copy into the *cdc25-22 ura4-294* background (Materials and methods, pages 57-58) and its single copy integration into the *ura4*$^{+}$ locus confirmed by PCR (Figure 28, lane 2).

pZM1 *cdc25-22* cells were synchronised for division by transient temperature shifts, and the transcript profile of *lacZ* was examined by Northern blot analysis (Figure 32). Interestingly, three identical experiments showed that no *lacZ* transcript was seen during the cell cycle, indicating that MCB2 is required for expression of *cdc22*$^{+}$ and so has an essential role in conferring transcription of this gene. This observation is consistent with previous results shown in this thesis, where MCB2 was found to bind to DSC1 *in vitro* (Figure 22), and conferred transcriptional activation at G1/S to *lacZ* (Figure 18). Together, these data confirm that MCB2 has the properties of a DAS, and has the potential to function as a downstream activation sequence controlling *cdc22*$^{+}$ cell cycle specific expression.

-141 CATTGATCAACATGACTTAAAGTTCGGATGACTAGACTAG

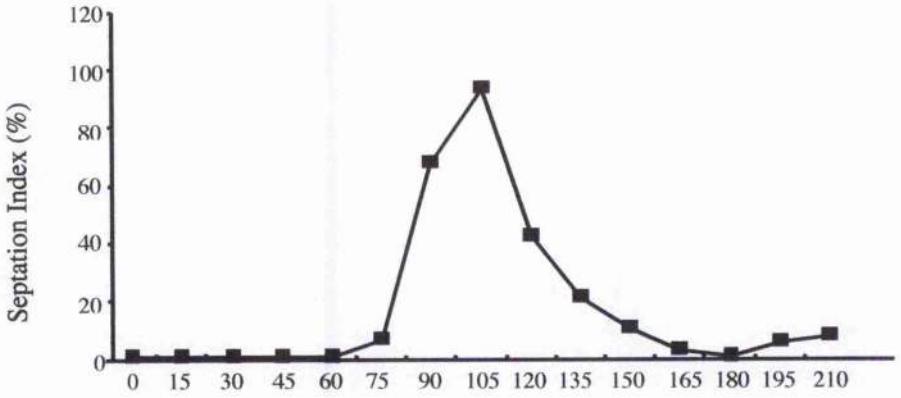
GCATCACGTTATATTAGCGTGACTAGTCTGAACTAGTTTT

TCATTTACTATAAATATTCCCGGTATTTACCACC -30

Figure 31. Nucleotide sequence of "MCB2m", containing mutated MCB motifs in MCB2.

MCB motifs are shown in purple and mutated base changes in red. Numbers are relative to the ATG.

A


Septation Index (%)
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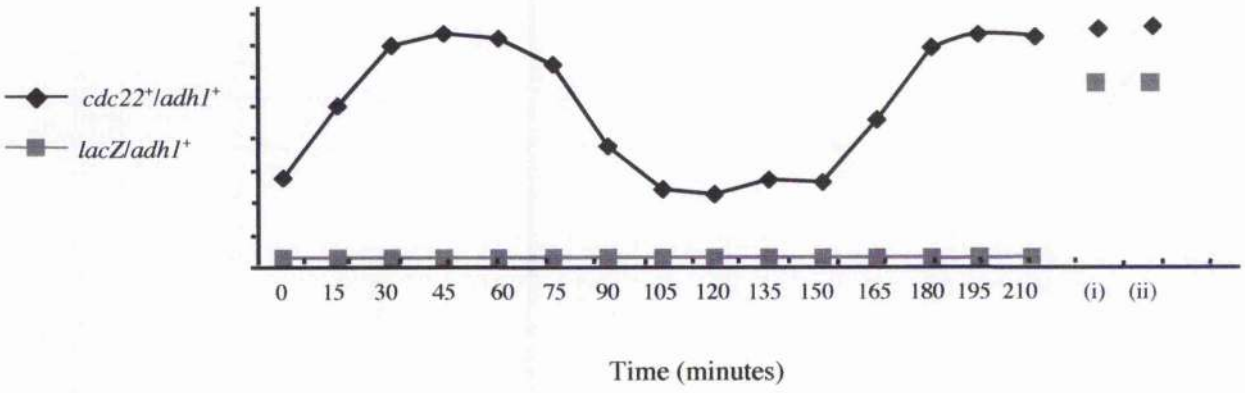
100
80
60
40
20
0
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210
Time (minutes)

B


cdc22+/adh1+
lacZ/adh1+
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 (i) (ii)
Time (minutes)

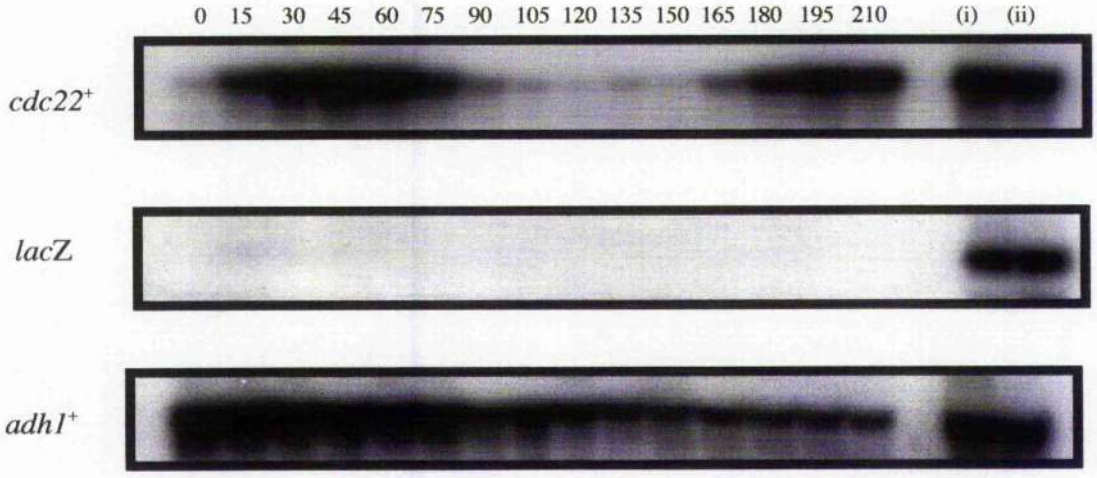

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 (i) (ii)
cdc22+
lacZ
adh1+

Figure 32. MCB2 is essential for basal transcription of *cdc22*$^{+}$

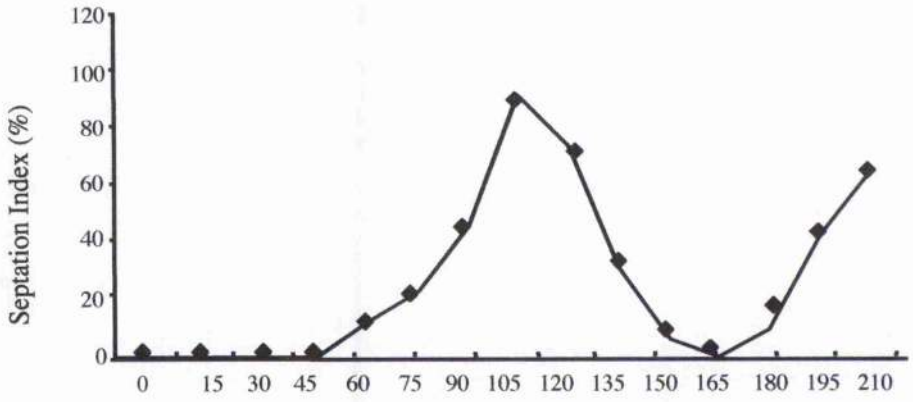
(A) An exponentially growing culture of pZM1 *cdc25-22* cells (GG 591) was grown in minimal media and arrested at G2 by incubation at 36°C for 4 hours. Cells were shifted to 25°C to enter a synchronous cell cycle, with samples collected for RNA extraction every 15 minutes, and cells examined microscopically for the appearance of septa at the times indicated. (B) The RNA was subjected to Northern blot analysis and the blot was hybridised with *cdc22*$^{+}$, *lacZ* and *adh1*$^{+}$ probes, which were amplified by PCR using genomic DNA (oligo's described in Appendix B). Two control lanes were included containing RNA from cells containing pPJK7 (GG 470) at a peak cell cycle stage. Quantification of each transcript against *adh1*$^{+}$, using NIH image software is shown. Data presented are representative of three identical experiments.

IV *Intact MCBs in MCB1 and MCB2 are essential for basal transcription*

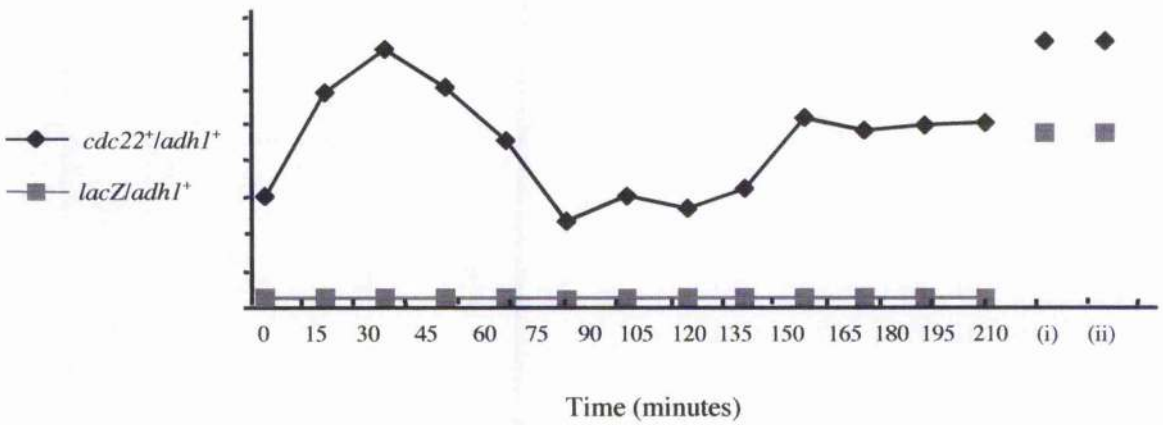
To confirm the previous experiments showing the requirement of MCB1 and MCB2 in controlling of *cdc22*$^{+}$ expression, we examined the effect of mutating the MCB motifs in both MCB1 and MCB2 on G1/S transcription of *lacZ*. This was achieved by using site directed mutagenesis to mutate the MCB2 motifs in pPJK10, to create pZM2 (GB 246; Materials and methods, pages 65-66 and Appendix A.5). pZM2 contains mutated MCB motif in both clusters, leaving only MCBX intact. The oligonucleotides used in the mutagenesis are described in Appendix B, and the mutations in both MCB clusters were confirmed by sequencing. As before, this construct was integrated in single copy into *cdc25-22 ura4-294* cells (Materials and methods, pages 57-58) at the *ura4*$^{+}$ locus, and PCR used to confirm its correct integration (Figure 28, lane 3).

A culture of synchronous pZM2 *cdc25-22* cells was prepared by transient temperature shifts and Northern blot analysis of RNA revealed that *lacZ* transcript was absent from all stages of the cell cycle (Figure 33). This result is consistent with our previous finding that both intact MCB clusters are required for controlling *cdc22*$^{+}$ G1/S specific expression. Furthermore, this result confirms that MCBX has no role in the regulation of G1/S transcription of *cdc22*$^{+}$.

A


Septation Index (%)
120
100
80
60
40
20
0
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210
Time (minutes)

B


cdc22+/adh1+
lacZ/adh1+
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 (i) (ii)
Time (minutes)

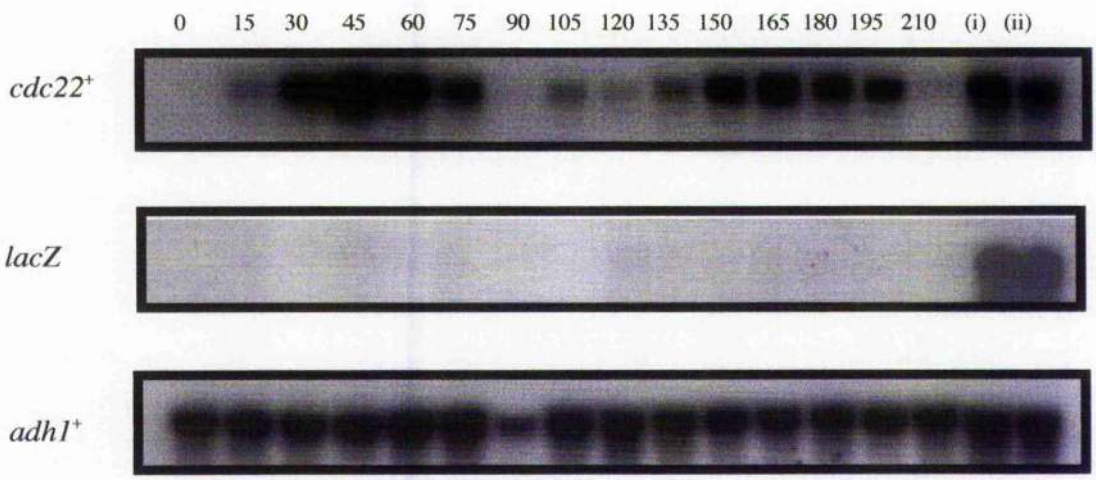

0 15 30 45 60 75 90 105 120 135 150 165 180 195 210 (i) (ii)
cdc22+
lacZ
adh1+

Figure 33. MCB1 and MCB2 are both required for transcription of *cdc22*$^{+}$

(A) An exponentially growing culture of pZM2 *cdc25-22* cells (GG 592) was grown in minimal media and arrested at G2 by incubation at 36°C for 4 hours. Cells were shifted to 25°C to enter a synchronous cell cycle, with samples collected for RNA extraction every 15 minutes, and cells examined microscopically for the appearance of septa at the times indicated. (B) RNA was subjected to Northern blot analysis and the blot hybridised with *cdc22*$^{+}$, *lacZ* and *adh1*$^{+}$ probes, which were amplified by PCR using genomic DNA (oligo's described in Appendix B). Two control lanes were included containing RNA from cells containing pPJK7 (GG 470) known to peak during the cell cycle. Quantification of each transcript against *adh1*$^{+}$, using NIH image software is shown. Data presented are representative of three identical experiments.

V *MCB1m and MCB2m do not bind to DSC1*

To further examine the importance of intact MCBs in the MCB1 and MCB2 clusters in cell cycle regulated transcription, we examined the ability of mutated MCB clusters (MCB1m and MCB2m) to separately bind to DSC1 *in vitro*. Initially, gel retardation studies were performed using MCB1m DNA as labelled substrate (Figure 34, lane 3) which revealed no band corresponding to DSC1. However, a single prominent retarded band that contained DSC1 was seen, as expected, when wild-type non-mutated MCB1 DNA was used as labelled substrate (Figure 34, lane 2, large arrow). This band disappeared when the same DNA was added (Figure 34, lane 4) as unlabelled competitor, but not when MCB1m and MCB2m were added as unlabelled competitors (lanes 5 and 6).

Gel retardation analysis using MCB2 as labelled substrate also revealed DSC1 binding (Figure 35, lane 2). This disappeared when the same DNA was added (lanes 4) as unlabelled competitor, but not when MCB2m and MCB1m were added as unlabelled competitors (Figure 35, lanes 5 and 6). Furthermore, using MCB2m as labelled substrate revealed no band corresponding to DSC1 (lane 3).

These results demonstrate that MCB1m and MCB2m have no binding activity with DSC1 *in vitro*, and so confirm that intact MCB motifs are required for binding to DSC1.

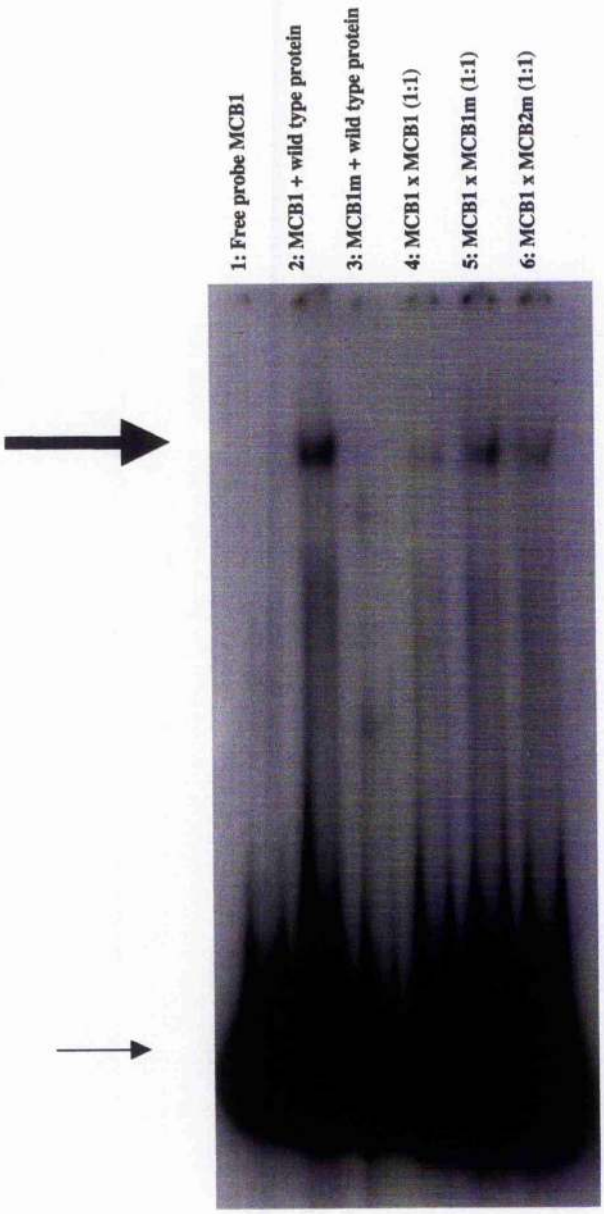


Figure 34. MCB1m does not bind to DSC1 *in vitro*.

Gel retardation assay using MCB1 and MCB1m DNA promoter fragments as labelled probes with protein extracts from wild-type (GG 217) cells. In alternate lanes equal amounts (1:1) of excess unlabelled competitor MCB DNAs, MCB1/MCB1m/MCB2m were added to the reaction mixtures prior to electrophoresis. Large arrow indicates DSC1, small arrow free probe. Data presented are representative of three identical experiments.

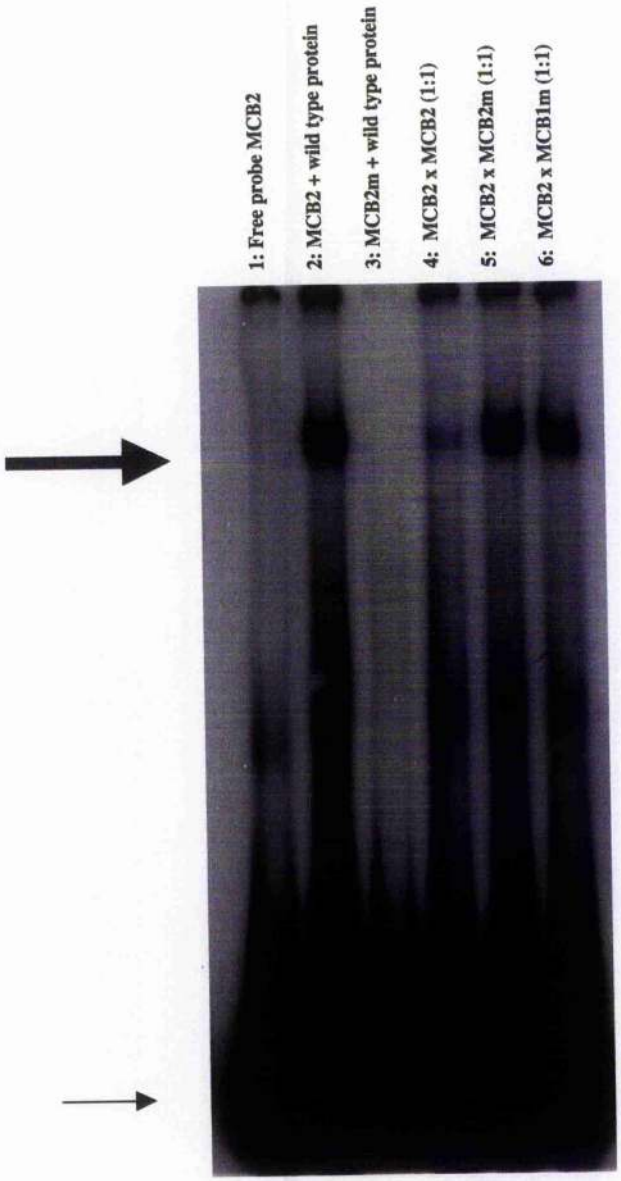


Figure 35. MCB2m does not bind to DSC1 *in vitro*.

Gel retardation assay using MCB2 and MCB2m DNA promoter fragments as labelled probes with protein extracts from wild-type (GG 217) cells. In alternate lanes equal amounts (1:1) of unlabelled competitor MCB DNAs, MCB2/MCB1m/MCB2m were added to the reaction mixtures prior to electrophoresis. Large arrow indicates DSC1, small arrow free probe. Data presented are representative of three identical experiments.

VI *MCB1m and MCB2m do not bind to DSC1 or DSC1 mutants*

To confirm that MCB1m and MCB2m do not bind to DSC1, gel retardation analysis with various DSC1 mutant strains was completed. As before, protein extracts were obtained from *res1Δ*, *res2Δ*, *rep2Δ* and *cdc10-129* cells at restrictive and permissive temperatures.

When MCB1m was used as labelled substrate no band of low mobility, corresponding to DSC1 was detected in protein extracts from the wild-type or mutant cells (Figure 36). Similarly, when MCB2m was used as a labelled substrate no band corresponding to DSC1 appears in protein extracts from the different cells (Figure 37). These data further confirm that MCB1m and MCB2m do not bind DSC1 *in vitro*, and that intact MCB motifs are necessary for this binding.

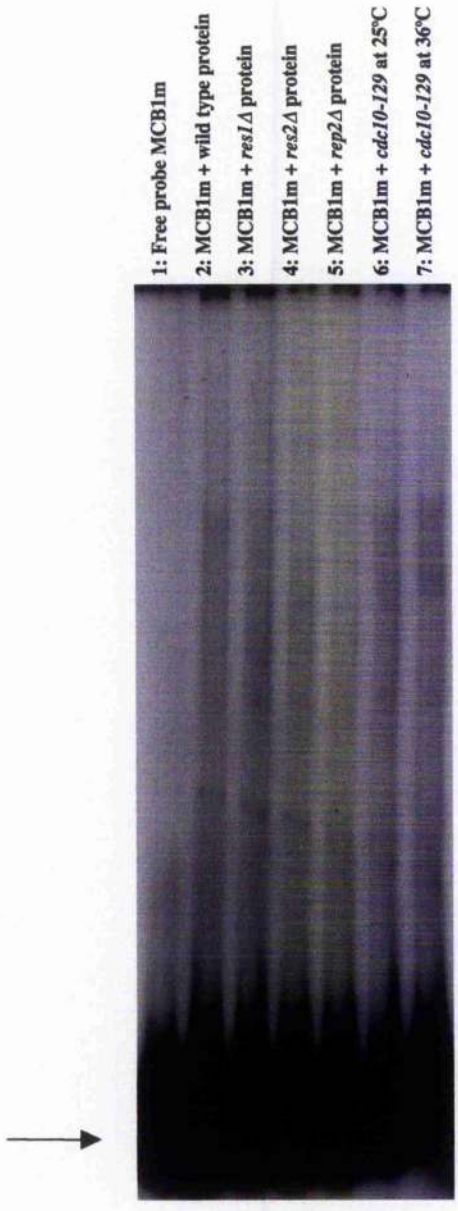


Figure 36. MCB1m does not bind to DSC1 *in vitro* in DSC1 mutant cells.

Gel retardation assay using MCB1m DNA promoter fragment as labelled probe with protein extracts from various mutants. In lanes 2-7 protein extracts were prepared from wild-type (GG 217), *res1Δ* (GG 155), *res2Δ* (GG 156), *rep2Δ* (GG 158), and *cdc10-129* (GG 28) cells grown at 25°C and 36°C, respectively. Small arrow indicates free probe. Data presented are representative of three identical experiments.

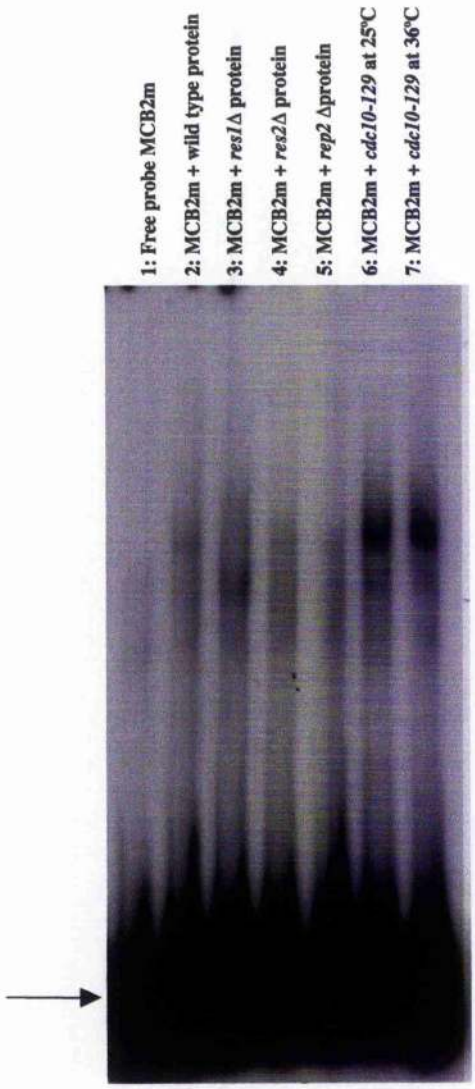


Figure 37. MCB2m does not bind to DSC1 *in vitro* in DSC1 mutant cells. Gel retardation assay using MCB2m DNA promoter fragment as labelled probe with protein extracts from various mutants. In lanes 2-7 protein extracts were prepared from wild-type (GG 217), *res1Δ* (GG 155), *res2Δ* (GG 156), *rep2Δ* (GG 158), and *cdc10-129* (GG 28) cells grown at 25°C and 36°C, respectively. Small arrow indicates free probe. Data presented are representative of three identical experiments.

3C *Genomic MCBs are not palindromes in the genome*

An interesting conclusion from some of our previous experiments (Figures 16-19) is that MCB1 and MCB2 only confer cell cycle regulation to *lacZ* in the correct orientation. This finding suggests that MCB motifs in the *cdc22*$^{+}$ promoter are orientation dependent in their ability to regulate gene expression. This observation is the first evidence that promoter elements in fission yeast may not be palindromic in their ability to control transcription. A possible explanation for this property may lie in the compact nature of the fission yeast genome, where genes are present in close proximity (Wood et al. 2002). Non-palindromic promoter elements may serve to ensure the specific control of genes.

To test this hypothesis, we studied MCB motifs present in the promoters of other fission yeast genes in the fission yeast genome (Figure 38A). We searched the *S. pombe* database (Wood et al. 2002) to identify pairs of genes, where one of the genes was MCB-regulated, and the other gene was transcribed in the opposite orientation, a short distance upstream from the MCB motifs. We then assayed transcription of both genes to determine if they were both under the control of DSC1-MCB system. We did this by using a cell division cycle fission yeast mutant, *cdc10-C4*, which is mutated in a component of DSC1. At low temperatures (16-24°C) this mutation results in deregulated expression of all known MCB regulated genes throughout the cell cycle, which in asynchronous cells manifests as over-expression relative to wild-type (McInerny et al. 1995; Ng et al. 2001).

Upon searching the *S. pombe* database we identified 5 pairs of genes, which are listed in Table 2.

MCB regulated gene	*MCB motif 3' from ATG*	*Upstream gene*
cdt1$^+$ (cell division cycle protein)	-70 CGCGACACGATAGCTACGCGACGCGTCAACAAACGCG -34	SPBC428.17c (hypothetical nuclear protein)
cdt2$^+$ (hypothetical WD-repeat protein)	-98 ACGCGTGAACGCGACGCGT -120 -377 ACGCGT -382..... 440 TCGCG -444	*psc3*$^+$ (Psc3 protein)
rad21$^+$ (double-strand-break repair protein)	-232 ACGCGCAGCGCGT -220	SPCC338.18 (hypothetical 13.3 kDa protein)
ste6$^+$ (ste6 protein)	-200 ACGCGACGCG -213	SPCC1450.16c (hypothetical protein)
mik1$^+$ (mitosis inhibitor protein kinase)	-370 CGCGTTATCCCAAATGAAAAACGCGAACGCGTCATCACGCGT ACGCGT -328	*rad11*$^+$ (replication factor-a protein 1)

Table 2. Position of genomic MCB motifs in promoters of genes.

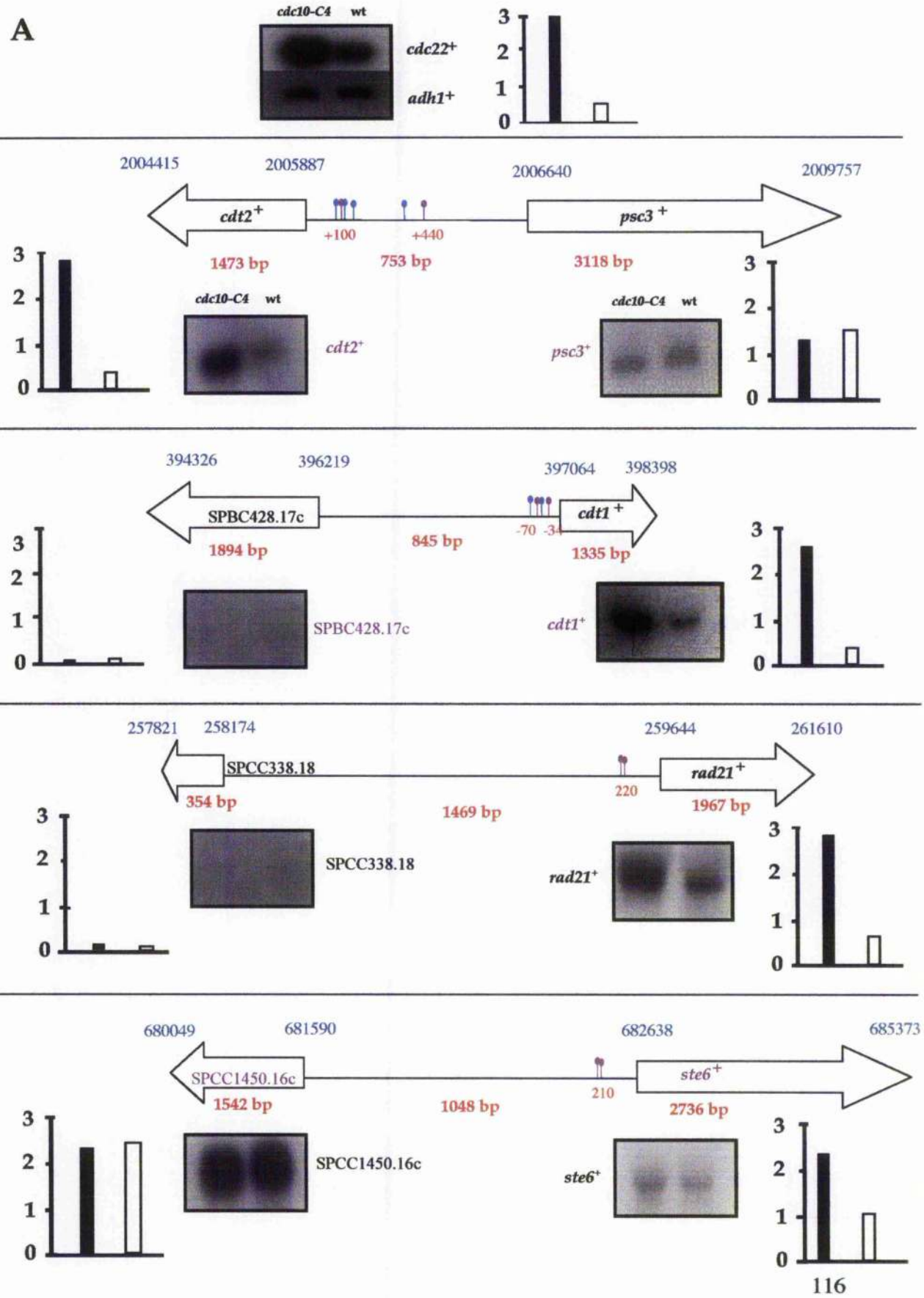
Pairs of genes were identified from the fission yeast data base (Wood et al. 2002), where one is known to be under DSC1-MCB control, with the other gene transcribed in the opposite orientation, a short distance upstream from the MCB motifs. Protein functions are indicated in brackets. MCB motifs upstream to each gene relative to the ATG are indicated; underlined motifs are exact *Mlu*I matches and remaining motifs are MCB motifs containing 5/6 base pair matches containing the core CGCG, thought to be essential for function.

In the case of four known MCB regulated genes *ste6*$^+$, *rad21*$^+$, *cdt1*$^+$ and *cdt2*$^+$ (Nishitani et al. 2000; Papadaki et al. 2002; Pati et al 2002; Yoshida et al 2003) genes SPAC17H9.19c, SPCC338.18c, SPCC1450.16c and *psc3*$^+$ were identified upstream to each MCB cluster in the opposite orientation, respectively (Figure 38A). Oligonucleotides were designed for each gene (Appendix B) and amplification by PCR produced DNA fragments of ~1 kb that were used as probes. Transcripts were detected by Northern blot analysis in asynchronous cells wild-type (GG 217) and *cdc10-C4* cells (GG 108) at 25°C. The Northern blot was also hybridised with probes for *cdc22*$^+$ and *adh1*$^+$ as controls, and the quantification of each gene's transcript against *adh1*$^+$ transcript, using NIH image software was completed (Figure 38A).

The transcript levels of MCB-regulated genes in *cdc10-C4* were present at a similar level to *cdc22*$^+$ (Figure 38A) significantly higher than in wild-type cells, confirming that they were all under DSC1-MCB control. In contrast, the transcripts of genes in the opposite orientation to the MCB motifs were either not expressed at all (SPBC428.17c and SPCC338.18c), or occurred at similar levels to that seen in wild-type cells (SPCC1450.16c and *psc3*$^+$) in *cdc10-C4*, indicating that these genes are not under DSC1-MCB control (Figure 38A).

We confirmed and extended these results for one of the pair of genes, *cdt2*$^+$ and *psc3*$^+$. Northern blot analysis of RNA obtained from *cdc25-22* cells, undergoing a synchronous mitotic cell cycle, revealed cell cycle regulation of *cdt2*$^+$ transcript coincident to *cdc22*$^+$ profile (Figure 38B). Subsequent hybridisation with a *psc3*$^+$ probe revealed two transcripts, one of which was the remaining *cdt2*$^+$ transcript (~1.5 kb), and the other the *psc3*$^+$ transcript (~3 kb) (Figure 38B). *psc3*$^+$ transcript was present throughout the cell cycle. This result confirmed that *cdt2*$^+$ was under DSC1-MCB controls, whilst *psc3*$^+$ was not.

One exception to the situation concerning the pairs of genes we have described is *mik1*$^+$ and *rad11*$^+$, which are two adjacent gene both known to be MCB-regulated and transcribed at G1/S (Table 2) (Parker et al. 1997; Ng et al. 2001). In this particular case, with 5 MCB motifs present between the two genes they are able to confer G1/S specific expression in both orientations. Possible explanations for this difference are described in the Discussion (pages 130-131).


A
cdc10-C4 wt
cdc22+
adh1+
2004415
2005887
2006640
2009757
cdt2+
psc3+
+100
+440
1473 bp
753 bp
3118 bp
cdc10-C4 wt
cdt2+
cdc10-C4 wt
psc3+
394326
396219
397064
398398
SPBC428.17c
cdt1+
-70
-34
1894 bp
845 bp
1335 bp
SPBC428.17c
cdt1+
257821
258174
259644
261610
SPCC338.18
rad21+
220
354 bp
1469 bp
1967 bp
SPCC338.18
rad21+
680049
681590
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210
1542 bp
1048 bp
2736 bp
SPCC1450.16c
ste6+

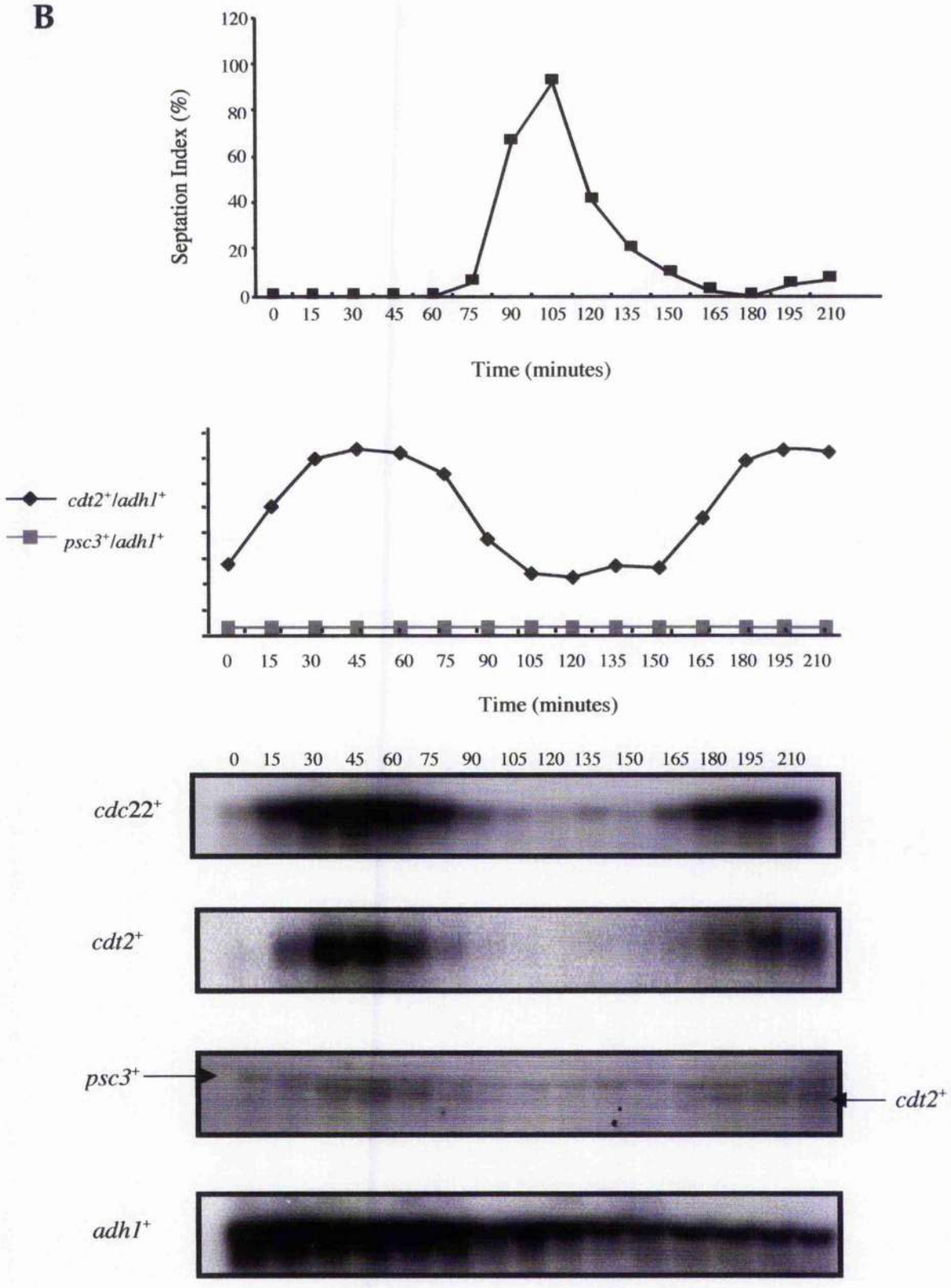
B
Septation Index (%)
120
100
80
60
40
20
0
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210
Time (minutes)
cdt2+/adh1+
psc3+/adh1+
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210
Time (minutes)
0 15 30 45 60 75 90 105 120 135 150 165 180 195 210
cdc22+
cdt2+
psc3+
cdt2+
adh1+

Figure 38. MCB motifs are orientation specific within the fission yeast genome.

(A) Schematic diagram showing position and orientation of pairs of genes, with MCB motifs; purple indicates MCB motifs with *Mlu*I exact matches and blue indicates 5/6 matches. Base pair distances indicated; sizes of intergenic regions, ORFs and MCBs relative to the ATG of the MCB gene are indicated in red and numbers in blue indicate chromosomal location of genes. RNA was prepared from wild-type (GG 217) and *cdc10-C4* (GG 108) cells, and subjected to Northern blot analysis. The Northern blot was hybridised with probes for the indicated genes, and with probes for *cdc22*$^{+}$ and *adh1*$^{+}$ as controls, which were amplified by PCR using genomic DNA (oligo's described in Appendix B). Quantification of each gene's transcript against *adh1*$^{+}$ transcript, using NIH image software, is shown, with *cdc10-C4* (dark bar) and wild-type (open bar). (B) An exponentially growing culture of *cdc*25-22 (GG 193) cells was grown in minimal media and arrested at G2 by incubation at 36°C for 4 hours. Cells were shifted back to 25°C and samples collected at 15 minute intervals RNA extraction, and cells examined microscopically for the appearance of septa. The RNA was subjected to Northern blot analysis and the blot was hybridised with *cdc22*$^{+}$, *cdt2*$^{+}$, *psc3*$^{+}$ and *adh1*$^{+}$ probes. Quantification of *cdt2*$^{+}$/*adh1*$^{+}$ and *psc3*$^{+}$/*adh1*$^{+}$ transcripts using NIH image software is shown. Data presented are representative of three identical experiments.

3D *MCB1 has a slower "on-off" rate than MCB2*

Previous experiments have also demonstrated that MCB2 has a significant role in regulating *cdc22*$^{+}$ transcription (Figures 18, 22 and 32), which is intriguing as the MCB2 cluster lies within the transcribed region of this gene (Figure 14). We were interested in investigating how it is possible for MCB2 to be bound to DSC1 to activate gene transcription, but also allow RNA pol II to bind whilst transcribing the gene. Thus, we examined the "on-off" binding properties of DSC1 bound to either MCB1 or MCB2 using competitive gel retardation analysis, to suggest a mechanism how this may occur.

A gel retardation assay performed using MCB1 as a labelled substrate, revealed a single prominent band that is DSC1 (Figure 39, lane 2, large arrow). When cold competitor MCB1 DNA was sequentially added in separate reactions, this band partially disappeared by 5 minutes (Figure 39, lane 5), and was almost entirely gone by 20 minutes (Figure 39, lane 7). Thus it took 5-10 minutes for DSC1 to "come off" MCB1.

In contrast, when MCB2 was used as a labelled substrate (Figure 39, lanes 8-14), DSC1 was seen to "come off" almost immediately after the cold competitor was added, with DSC1 entirely absent by 5 minutes.

This experiment demonstrates that MCB2 has a significantly higher "on-off" rate than MCB1. This result is, therefore, consistent with the suggestion that DSC1 might temporarily dissociate from MCB2, but remain bound to MCB1, to allow RNA polymerase II to pass during transcription.

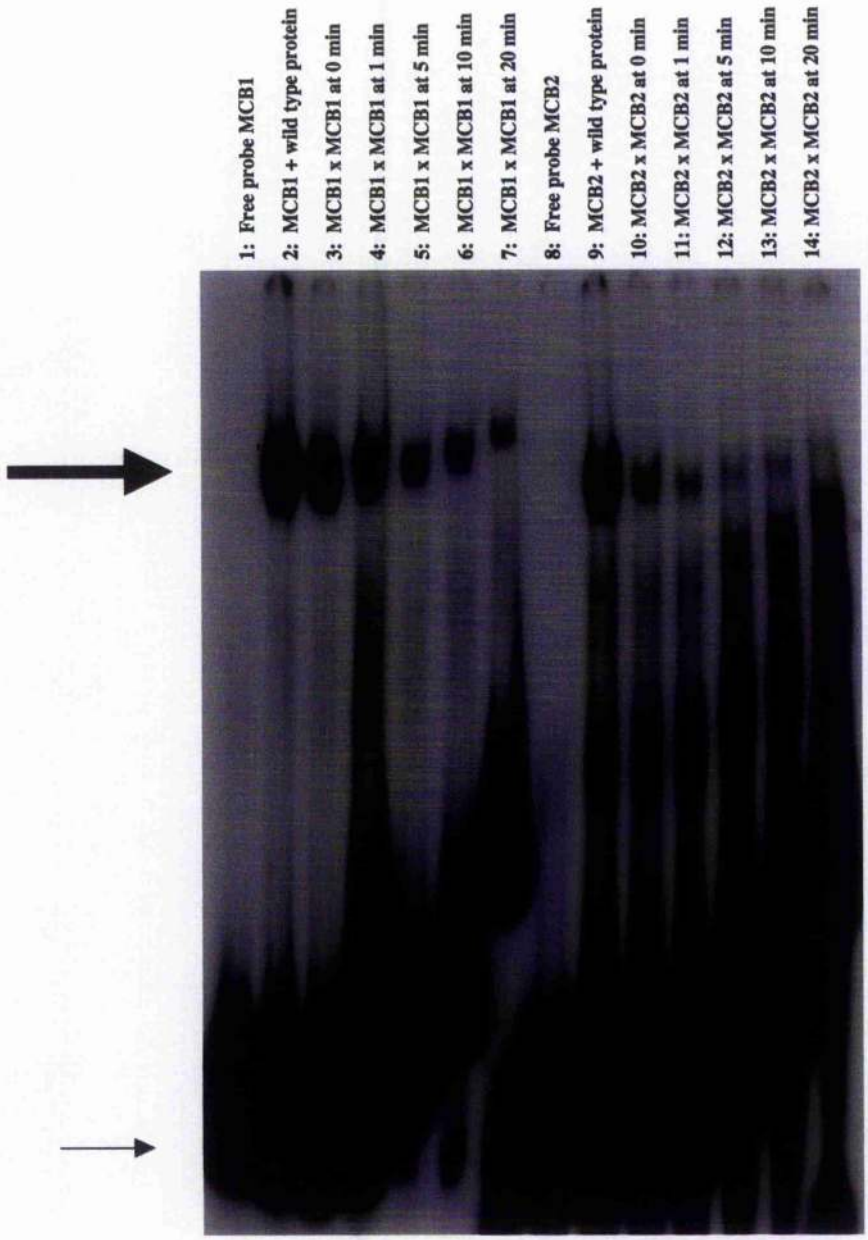

Figure 39. MCB1 "on-off" rate is slower than MCB2.

Gel retardation assay using MCB1 and MCB2 DNA promoter fragments as labelled probes with protein extracts from wild-type (GG 217) cells. Large arrow indicates DSC1; small arrow free probe. In alternate lanes equal amounts of excess unlabelled competitor MCB1/MCB2 DNA was added to protein extracts from wild type cells at 0 mins, 1 min, 5 min, 10 min and 20 minute time intervals, to the reaction mixture prior to electrophoresis. Data presented are representative of three identical experiments.

Chapter 4: Discussion

1 *Introduction*

The restriction point ("R" point) in the G1 phase of the mammalian cell cycle is an essential control point responsible for regulating cell duplication (Pardee 1989). This is the point between early and late G1 that represents an irreversible commitment of the cell to undergo cell division. An understanding of the R point in the cell cycle is of crucial significance, as its de-regulation is thought to contribute to cancerous division of cells *in vivo* (Blagosklonny and Pardee 2002). To study the R point it is important to use the simplest possible experimental system that can be easily manipulated in the laboratory. Yeasts have been found to be good model organisms as they have been used with great success over the past few years to elucidate eukaryotic cell cycle control mechanisms (Qin and Li 2003). They have proved to be a particularly powerful tool in understanding the role of genes that are critical for controlling cell division in all eukaryotes.

Progression through the cell division cycle in yeasts is dependent on passage through a critical point at G1 called START, that is analogous to the R point in mammalian cells. In mammalian cells, E2F is the transcription factor essential for regulating cell cycle progression by influencing the expression of proteins required for the G1/S transition phase and DNA synthesis. The E2F family of proteins are considered critical for the passage of cells through the restriction point in G1 and into S phase. In fission yeast passage through START is controlled in part by the transcription factor complex DSC1, functionally similar to E2F, which regulates the expression of genes essential for DNA synthesis (Lowndes et al. 1992; Connolly and Beach 1994; Hoffmann and Beach 1994; Baum et al. 1997; Nishitani et al. 2000; Ng et al. 2001). In this thesis I describe experiments that analyse DSC1 function in fission yeast, to establish a better understanding of cell cycle control mechanisms that may be applicable to mammalian and other eukaryotic systems.

The DSC1 transcription factor complex in fission yeast is essential for the periodic expression of genes during the G1/S transition (White et al. 2001). It functions by binding MCB UAS motifs present in the promoters of a group of genes expressed at this cell cycle time. The conserved core of this element consists of one or more hexameric sequence that coincides with the *Mlu*I restriction site (ACGCGT), and is referred to as the *Mlu*I cell cycle box, or MCB motif. Each of the MCB-regulated genes contains one or more MCB motifs in its promoter, which bind specifically to DSC1 to form the transcription control system (Lowndes et al. 1992; Ng et al. 2001). The MCB motif was initially identified in budding yeast, where it is also responsible for co-ordinating transcriptional regulation of genes essential for DNA synthesis at the G1/S boundary (McIntosh et al. 1991). Significantly, the most similar sequence currently found in mammalian genes is the E2F transcription factor site, TTTGCGC or CGCGCAAAA, found upstream of several genes that are associated with or involved in DNA replication (Kel et al. 2001). This suggests that similar types of control mechanisms regulating the cell cycle at G1/S may be present amongst all eukaryotes.

We analysed the control of expression of *cdc22*$^{+}$, the first fission yeast G1/S transcribed gene to be discovered. *cdc22*$^{+}$ has been studied in the past and found to contain an interesting array of MCB motifs in its promoter (Lowndes et al. 1992; Fernandez-Sarabia et al. 1993). The promoter region contains two clusters of MCB motifs, in this thesis named "MCB1" and "MCB2", both of which bind DSC1 (Figures 13 and 14) (Lowndes et al. 1992). Within MCB1 and MCB2, there are three *Mlu*I motifs that are identical to the *Mlu*I recognition sequence, while the remaining five all contain the central CGCG core thought to be essential for function (McIntosh 1993). There is also a single core *MluI* site in between the two MCB clusters, named "MCBX".

In previous experiments, the mapping of transcriptional start of *cdc*22^{+}, using primer extension and Northern blot analysis revealed that the start point of the gene lies between the two MCB clusters (Figures 13 and 14) (Maqbool et al. 2003). Furthermore, *in vitro* DNAseI footprint analysis showed there are protected regions over MCB2, the downstream cluster of MCBs (Maqbool et al. 2003). These results imply that either one

or both of the MCB clusters is essential for the binding of DSC1 and activating transcription of *cdc22*$^{+}$. The possibility that MCB2 may participate in the initiation of the transcription of *cdc22*$^{+}$ was particularly interesting because in fission yeast there has been no evidence so far of a UAS downstream to the transcriptional start for a RNA polymerase II regulated gene. This is particularly interesting in light of recent findings in human cells, which have revealed that the E2F binding sites in the promoters of cell cycle regulated genes occur in close proximity to the transcriptional start site, between positions –400 and +100 (Kel et al. 2001).

II *Summary of main conclusions*

The main findings of my research can be summarised as follows:

- The two MCB motif clusters in the *cdc22*$^{+}$ promoter, MCB1 and MCB2, both contribute to its cell cycle regulation (Figures 16 and 18).
- MCB1 and MCB2 are not palindromic in function (Figures 17 and 19).
- Both MCB1 and MCB2 bind to DSC1 *in vitro* (Figures 21 and 22).
- MCBX does not bind to DSC1 *in vitro* (Figure 20).
- MCB1 has a stronger binding affinity for DSC1 than MCB2 *in vitro* (Figures 24 and 25).
- Intact MCB motifs in MCB1 are necessary for the correct cell cycle regulation of *cdc22*$^{+}$ (Figure 30).
- Intact MCB motifs in MCB2 are essential for basal transcription of *cdc22*$^{+}$ (Figure 32).
- MCBX has no role in the regulation of G1/S transcription of *cdc22*$^{+}$ (Figure 33).
- Mutated MCB1m and MCB2m do not bind DSC1 *in vitro* (Figures 34 and 35).
- MCB motifs in the promoters of genes in their native genomic locations are only able to function in one orientation in fission yeast (Figure 38).
- MCB2 has a significantly higher "on-off" binding rate for DSC1 compared to MCB1 (Figure 39).

III *Both MCB1 and MCB2, in the correct orientation, confer cell cycle regulation*

Initial studies in budding yeast identified the conserved MCB DNA sequence, ACGCGTNA, in the promoters of cell cycle regulated genes needed for DNA synthesis (McIntosh et al 1991). Using a high copy reporter plasmid, it was demonstrated that this sequence was able to direct periodic transcription of a reporter gene at the G1/S boundary, and that it was orientation independent. It was also noted that deletions or point mutations within the MCB motif severely inhibited expression of G1/S expressed genes (Gordon and Campbell 1991).

Following these studies, Lowndes et al. (1992) investigated the ability of MCB motifs to confer cell cycle regulation in fission yeast. A synthetic DNA construct containing three adjacent *Mlu*I sequences was placed in a UAS reporter plasmid, pSPΔ178, and shown to confer G1/S transcription to the heterologous *lacZ* gene in fission yeast (Lowndes et al 1992). This result suggested that MCB motifs present in the *cdc22*$^{+}$ promoter (and in the promoters of other fission yeast G1/S expressed genes) regulate cell cycle transcription at the start of S phase.

To prove that natural MCB motifs from a native fission yeast promoter can also confer G1/S transcription to *lacZ* we initially examined the ability of the two MCB clusters from the *cdc22*$^{+}$ promoter to regulate G1/S transcription. Using the same UAS reporter construct pSPΔ178 (Lowndes et al. 1992) we showed that both MCB clusters could independently confer cell cycle transcription to the *lacZ* gene, coincident to endogenous *cdc22*$^{+}$ expression, in the correct orientation. MCB2 drove two peaks of *lacZ* expression over two cell cycles, whereas MCB1 gave only one prominent peak of transcription during the first cell cycle, with a weaker second peak (Figures 16-19).

These experiments allow us to conclude that each MCB cluster is capable of contributing to *cdc22*$^{+}$ cell cycle expression in the correct orientation, as each conferred G1/S transcription in the correct orientation only. At least for MCB2 this result was

surprising, as this cluster lies downstream to transcriptional start of the native *cdc22*$^{+}$ promoter. These data implicate MCB2 as a downstream UAS, here termed a DAS, for a downstream activating sequence, the first example of such a promoter element in fission yeast.

DAS sequences have already been described in cell cycle regulated genes in human systems, where E2F transcription factor binding sites are found in close proximity, both upstream and downstream to the transcriptional start site. This sequence is recognised as the most similar sequence to MCB motifs in mammalian systems and also has the ability to control expression of genes when present in either orientation (Kel et al. 2001), similar to MCB motifs in budding yeast (McIntosh 1993). However, contrary to this, our results show that both MCB1 and MCB2 do not have palindromic activity as they are unable to confer G1/S transcription to *lacZ* in the opposite orientation (Figures 17 and 19). It is possible that this occurs because the genes present in the fission yeast genome are tightly packed, with some genes present close together in opposite orientations. Having a non-palindromic MCB cluster, therefore, ensures that only the genes required specifically at the start of S-phase are expressed at this cell cycle time.

Previous experiments using *in vitro* gel retardation analysis have also implicated the transcription complex DSC1 in regulating G1/S transcription, by its binding to both MCB clusters, MCB1 or MCB2 (Lowndes et al. 1992). In this thesis these results were confirmed and extended by *in vitro* gel retardation analysis using MCB1, MCB2 and MCBX DNA instead as labelled substrates. These studies revealed that MCB1 and MCB2 can independently bind to DSC1, but MCBX has no binding activity (Figures 20-25). We also found that DSC1 has a stronger *in vitro* binding affinity for MCB1 than MCB2 (Figures 24 and 25) as assayed by *in vitro* gel retardation analysis.

These gel retardation results by themselves suggest that MCB1 has a more important role in regulating *cdc22*$^{+}$ expression, if *in vitro* binding affinity reflects *in vivo* activity. This is surprising as MCB2 conferred G1/S expression to *lacZ* more efficiently

than MCB1 *in vivo* (Figures 16 and 18). This contradiction might be reconciled by the observation that MCB2 has a higher "on-off" rate than MCB1 (Figure 39).

IV *Intact MCBs are essential for cdc22$^+$ transcription*

Our experiments using the UAS reporter plasmid pSPΔ178 allowed two novel observations about the regulation of *cdc22*$^+$ expression by the MCB clusters in its promoter. First, that both clusters can confer G1/S transcription to *lacZ*, and so both are likely to contribute to regulating endogenous *cdc22*$^+$. Second, that both clusters are orientation dependent in their regulation, as they do not show UAS activity when present in the opposite orientation.

These experiments have two potential flaws that might be significant for our conclusions. Firstly, a serious limitation when using pSPΔ178 is the fact that transcriptional start is artificially manipulated using this construct. A major conclusion we wish to make from these studies is that the MCB2 cluster, which lies downstream of transcriptional start of endogenous *cdc22*$^+$, contributes to the cell cycle expression of the gene. We cannot be sure of this conclusion using pSPΔ178 alone, as the transcriptional start site in this construct containing MCB2 are likely to have been altered. Transcription would be expected to start downstream of the MCB motifs, at the cytochrome *c* (*CYC1*) minimal promoter locus where the TATA box sequence resides.

The second limitation is that pSPΔ178 is a multi-copy plasmid, which has two inherent, and potentially serious, implications:

a) pSPΔ178 containing MCB clusters and present in high copy, results in an unnaturally high number of MCB motifs in cells containing the construct. This might result in binding to and titrating out of DSC1 function, causing transcription to be altered in the cellular context.

b) pSPΔ178, being a circular extra chromosomal plasmid, does not form genomic chromatin DNA structure, with associated proteins and ancillary factors. This chromatin structure may be important for regulation of expression of MCB-controlled genes.

To overcome these potential problems we used another approach to address the role of the MCB motifs in the *cdc22*$^{+}$ promoter. We examined the effect of mutating MCB motifs on G1/S transcription, by initially using an integration vector, pPJK7, containing the entire *cdc22*$^{+}$ promoter linked in frame to the *lacZ* gene. This vector was provided to us by Dr. Kersey in Dr Fantes's laboratory, at the University of Edinburgh (Kersey 1995). pPJK7 overcomes both of the limitations described for pSPΔ178, as it is used in single copy integrated into the fission yeast chromosome.

A series of experiments with pPJK7 to study the regulation of *cdc22*$^{+}$ expression, where elutriation of wild-type cells was used to synchronise cells containing pPJK7 were initially performed by Dr Kersey (1995). When *cdc22*$^{+}$ and *lacZ* transcript levels were monitored in such cells, it was observed that *lacZ* was expressed similarly, though not identically, to *cdc22*$^{+}$. *lacZ* mRNA was present through a higher proportion of the cell cycle than the *cdc22*$^{+}$ mRNA, although the two transcripts appeared to peak at similar times. It was also found that β-galactosidase activity (per ml of culture) was less tightly confined within the cell cycle than *cdc22*$^{+}$ expression (Kersey 1995). A possible explanation given for this difference was that alternative transcriptional termination of the two genes resulted in the *lacZ* transcript being more stable than *cdc22*$^{+}$ transcript (Kersey 1995).

We tested this hypothesis using the *cdc25-22* mutant as an alternative method for synchronising fission yeast cells. pPJK7 was integrated in single copy into the *cdc25-22* mutant and, in three identical experiments, it was found that *lacZ* expression profile was more coincident to *cdc22*$^{+}$, than in wild-type cells synchronised by elutriation (Figure 26). We consequently decided to use *cdc25-22* for further cell cycle experiments to analyse the effect of mutating the MCB motifs within each of the MCB clusters on cell cycle transcription.

The fact that that both MCB1 and MCB2 can bind to DSC1 *in vitro* (Figure 20), and that MCB2 can confer G1/S expression to *lacZ* more efficiently than MCB1 (Figures 16 and 18) *in vivo*, prompted us to examine the contribution of each MCB cluster to G1/S transcription more closely. To do this, we examined the effect of mutating MCB motifs in each cluster on *lacZ* transcription. This was achieved by initially using another vector provided by Dr. Fantes's lab, pPJK10. This vector is the same as the pPJK7 vector but differs in that the core CGCG of each MCB motif in MCB1 has been mutated to CTAG (Figure 27). We also used site directed mutagenesis to introduce mutations into the core of MCB2, within pPJK7 and pPJK10, to make two new constructs: pZM1, containing MCB2 mutated only, and pZM2 containing both MCB1 and MCB2 mutated. Such constructs allowed the effect of mutating either or both MCB clusters to be assayed. Again, in light of our concerns about using pSPΔ178, these constructs were present in single copy in the genomic context.

Using this alternative approach, it was demonstrated that loss of MCB motifs in either MCB cluster resulted in cell cycle transcription of *lacZ* being altered.

Mutating MCBs in MCB1 resulted in constitutive *lacZ* transcription at moderate levels throughout the cell cycle (Figure 30), suggesting that MCB1 is required for stimulating cell cycle specific transcription, and may have a negative role in the context of the native promoter. This result was similar, though not identical, to previous results obtained using the same MCB1 mutated construct in wild type cells synchronised by elutriation (Kersey 1995). In the latter case it was observed that *lacZ* transcript was weakly coincident to the *cdc22*$^{+}$ profile, suggesting some periodic expression. However, in these experiments *lacZ* mRNA was present during more of the cell cycle than that of the endogenous *cdc22*$^{+}$ gene, and β-galactosidase activity increased immediately after synchronisation. We believe, as we suggested with pPJK7, the slight differences between our results were due to variations in synchronisation efficiencies in the two experiments.

Using the construct pZM1, in three identical experiments, we found that mutating MCB2 resulted in no *lacZ* transcription at any time during the cell cycle (Figure 32). This result strongly suggests that MCB2 has an essential role in conferring basal

transcription of *cdc22*$^+$. This observation also confirms that MCB2 does have the properties of a DAS, as it functions as a downstream activation sequence. Furthermore, mutating both MCB1 and MCB2, leaving only MCBX, also resulted in no *lacZ* transcription (Figure 33), confirming that intact MCBs in both MCB1 and MCB2 are essential for conferring cell cycle transcription and regulation, and that MCBX by itself has no role in regulating G1/S specific expression.

One possible problem that the integration approach has not entirely eliminated is that of genomic location and structure. It is possible that the endogenous native location of the *cdc22*$^+$ promoter is important for its control of gene expression. This may be due to the natural position of histones and other chromatin protein, which might bind this region of DNA (Beaujean 2002). For technical reasons the integration vector had to be inserted at the *ura4*$^+$ locus. Ideally, the MCB motifs should be mutated in their natural position at the *cdc22*$^+$ locus. Such experiments are difficult, especially as *cdc22*$^+$ is an essential gene. However, it is technically feasible using a method previously described (McInerny et al. 1997) to integrate mutated MCBs into the natural *cdc22*$^+$ promoter locus, and determine their effect on transcriptional activation of the gene.

In brief, the method exploits two rounds of homologous replacements in a diploid fission yeast strain, first to remove one copy of the *cdc22*$^+$ promoter, the second to replace it with mutated MCB DNA. Subsequent sporulation permits the requirement of either MCB clusters to be determined on cell viability. Given that we found that mutated MCB1 results in the constitutive expression of *cdc22*$^+$ (Figure 30), we would predict such mutations would confer constitutive *cdc22*$^+$ transcription and so permit cell growth. In contrast, as MCB2 was found to be essential for the basal transcription of *cdc22*$^+$ (Figure 32), we would predict that mutations introduced into the MCB2 native region would result in no *cdc22*$^+$ expression, and consequent cell death. In either case such experiments would allow us to confirm (or refute) our proposed functions for the MCB clusters in the *cdc22*$^+$ promoter.

V *MCB clusters in fission yeast are not palindromes*

An important part of our studies was to establish whether specific findings we have made about the regulation of *cdc22*$^{+}$ expression, is generally applicable in fission yeast to other MCB-regulated genes.

One major and surprising conclusion from our experiments was that MCB clusters are orientation-specific in their ability to confer G1/S transcription (Figures 17 and 19). This is contrary to the MCB motifs found in budding yeast and the E2F binding sites recognised in mammalian cells, which are orientation independent (McIntosh et al. 1991, Kel et al. 2001). To establish if MCB motifs present in the promoters of other fission yeast genes are also orientation specific, we extended our initial investigation to the whole of the fission yeast genome (Figure 38). There are eleven known mitotic MCB regulated genes: *cdc22*$^{+}$, *cdc18*$^{+}$, *cig2*$^{+}$, *cdt1*$^{+}$, *cdt2*$^{+}$, *mik1*$^{+}$, *rad11*$^{+}$, *rad21*$^{+}$, *ste6*$^{+}$, *ste9*$^{+}$ and *suc22*$^{+}$ (large transcript) (Harris et al. 1996; Mondesert et al. 1996; Parker et al. 1997; Liu et al 2000b; Nishitani et al. 2000; Ng et al. 2001; Blanco et al. 2000; Papadaki et al. 2002; Pati et al. 2002; Maqbool et al. 2003; Yoshida et al. 2003). We therefore searched the *S. pombe* database to identify pairs of genes, where one of the genes was MCB-regulated, and the other gene was transcribed in the opposite orientation, a short distance upstream from the MCB motifs. We identified four examples of such genes: *cdt2*$^{+}$, *cdt1*$^{+}$, *rad21*$^{+}$ *and ste6*$^{+}$; with *psc3*$^{+}$, SPBC428.17c, SPCC338.18 and SPCC1450.16c upstream to the MCB motifs in the opposite orientation, respectively (Figure 38A, Table 2) (Wood et al. 2002). Transcription of these genes was assayed in wild type and *cdc10-C4* mutant cells, the latter which are mutated in a component of DSC1 and result in the constitutive expression of MCB-regulated genes at low temperature. It was demonstrated that MCB motifs in the promoters of these genes, like *cdc22*$^{+}$, only stimulate transcription of genes in one orientation (Figure 38), and so are non-palindromic in function.

During our database search we also identified *mik1*$^{+}$ and *rad11*$^{+}$ as a pair of genes that are upstream to one another with MCBs in the promoter region. The MCBs in this

case appear to confer transcription of the two genes as both are periodically expressed at G1/S (Parker et al. 1997; Ng et al. 2001). A closer look at the MCB motifs in the promoter region of these genes shows it contains five MCB motifs: three exact matches to the *Mlu*I sequence and two motifs that contain the core CGCG, thought to be essential for function (McIntosh et al. 1993). We propose that only one or two of these motifs are specific for UAS activity to either gene, and within this context are still orientation dependent. This hypothesis is supported by the observation that *ste6*$^{+}$ and *rad21*$^{+}$ have only two MCB motifs in their promoter region (Figure 38A), and so this number of motifs are sufficient to confer G1/S UAS activity in fission yeast. A more detailed analysis of these motifs is needed to confirm this hypothesis. It would be interesting to identify which of the motifs is indeed able to confer UAS activity, and whether transcriptional activity of either gene is altered when the core of one or more of the motifs is mutated.

A closer look at the MCB motifs in the promoter of *cdc22*$^{+}$ shows that only three motifs within the two MCB clusters are palindromes in their DNA sequence - ACGCGT. The other 5 motifs are not palindromes, having only a 5/6 match. Fission yeast MCBs do not fit a consensus as defined in budding yeast, and instead have only the central CGCG conserved, with either an A or a T on each side. Sometimes these MCBs occur in tandem. In either case such MCBs are not palindromes. It is possible that the non-palindromic MCBs are responsible for the orientation specificity conferred by MCB1 or MCB2 in *cdc22*$^{+}$. This may also be true for the other genes *cdt1*$^{+}$, *cdt2*$^{+}$, *rad21*$^{+}$ and *ste6*$^{+}$ as all these genes also contain one or more non-palindromic MCB motifs.

VI *MCB2 has a higher "on-off" rate*

Another interesting finding in this thesis is the observation that the MCB2 cluster occurs within the transcribed region of *cdc22*$^{+}$, and has been demonstrated to have a role in regulating the gene's expression (Figures 18 and 33). This suggests that a mechanism

must exist which facilitates the binding of DSC1 to MCB2, while at the same time also allowing RNA polymerase II to pass when transcribing the gene.

By examining the "on-off" rates of DSC1 bound to MCB1 and MCB2 we examined how this mechanism might function. Using competitive gel retardation analysis we were able to show that MCB2 had a significantly higher "on-off" rate than MCB1. DSC1 came 'off' the MCB2 DNA in 1-2 minutes, whereas MCB1 took 5-10 minutes (Figure 39). This higher "on-off" rate for MCB2 suggested that DSC1 might temporarily dissociate from MCB2 once it has activated transcription, to allow RNA polymerase II to pass. The significantly higher "on-off" rate of MCB2 may also account for our previous observation that MCB1 shows a stronger binding affinity for DSC1 (Figures 20 and 21).

Recently it was found that Res2p specifically interacts with the C-terminal domain of RNA polymerase II in yeast (Aranda and Proudfoot 2001), suggesting that Res2p may have a dual function both as a component of DSC1, and as a separate mRNA termination factor. It may be possible, therefore, that DSC1 mediates mRNA termination when bound to DNA at MCB sequences positioned far away from the site of RNAP II binding (Birse et al. 1997). Our results implicate binding of DSC1 to MCB1 in the promoter of *cdc22*$^{+}$ in regulating this termination mechanism.

VII *DSC1-MCB interactions*

The MCB clusters in the *cdt1*$^{+}$, *cdt2*$^{+}$ and *cdc18*$^{+}$ promoters are similar to *cdc22*$^{+}$, as they also contain a complicated array of multiple MCB motifs. It is therefore possible that the same mechanisms for regulating gene expression occur at G1/S. Other genes, such as *rad21*$^{+}$ and *ste6*$^{+}$, which have much simpler MCB motif arrays in their promoters must have differences in regulating gene expression at G1/S. Similar studies of the promoters of these genes as described in this thesis with *cdc22*$^{+}$ would provide important information in this regard.

The presence of highly related sequences similar to MCBs present in budding yeast, fungi and mammalian systems suggests that similar types of control may also be present amongst all eukaryotes (McIntosh et al. 1993). In mammalian systems it is found that the E2F transcription factor site is one of the most commonly known sequences similar to MCBs (Kel et al. 2002). Although E2F was thought to be a functional homologue of DSC1 (La Thangue et al. 2001), more recently a new mammalian protein has been found to complement the fission yeast DSC1 called HBP2 (Sanchez-Diaz et al. 2001). The *HBP2* gene has been isolated by functional complementation of cells unable to undergo DNA replication in fission yeast, and is found to activate transcription at G1/S in *S. pombe*. The protein itself is a member of the sequence specific high mobility group (HMG) box protein, whereby HBP1 is known to repress the expression of a set of genes activated by E2F, contributing to cell-cycle exit (Shih et al. 1998).

VIII *Model*

Bringing all of the observations presented in this thesis together I suggest the following model to explain DSC1-MCB regulation of *cdc22*$^{+}$ transcription (Figure 40). Given that both MCB clusters bind to DSC1 *in vitro* (Figures 21 and 22), and that both clusters confer cell cycle regulation of *cdc22*$^{+}$ in one orientation *in vivo* (Figures 16-19), we propose that cell cycle transcription of *cdc22*$^{+}$ is stimulated when DSC1 binds to MCB1 and MCB2 in an orientation-dependent manner (Figure 40a). Furthermore, binding of DSC1 to MCB1 and MCB2 positions RNA polymerase II on the promoter sequence and initiates transcription of the gene (Figure 40b). MCB1 has an ancillary role and assists MCB2 to cause transcription to occur in a cell cycle dependent manner: it was observed that intact MCB motifs in MCB1 are necessary for the correct cell cycle regulation of *cdc22*$^{+}$, and that MCB2 is essential for basal transcription (Figure 30 and 32). Possibly, MCB1 has a negative function, preventing *cdc22*$^{+}$ expression outside the G1/S boundary. As the 'on-off' binding rate for DSC1 was significantly higher for MCB2 compared to MCB1 (Figure 39), we suggest that DSC1 binds to and dissociates from MCB2 to allow

RNA polymerae II to pass (Figure 40 c), to permit RNA polymerase II to transcribe the *cdc22*$^{+}$ mRNA (Figure 40d).

In budding yeast, cell cycle regulated genes commonly contain an MCB motif upstream to the transcriptional start site and there is no evidence of a downstream activation sequence (McIntosh 1993). In human systems however, a recent study on the distribution of the potential E2F sites in cell cycle regulated genes suggests that they localise close to the transcription start site, between positions –400 and +100 (Kel et al 2001). Thus, as transcriptional mechanisms involving passage through Start are known to be conserved from yeast to mammals (Qin and Li 2003), it is possible that MCBs and the way they function may be an integral component of a transcription system conserved amongst eukaryotic organisms.

However, one major difference observed in our findings between the MCBs in different eukaryotes is that MCB elements in fission yeast are not palindromic. The fission yeast genome consists of tightly packed genes, which occur close together and in some cases in opposite orientations (Wood et al. 2002). It is possible therefore that the non-palindromic activity of the MCB cluster in fission yeast ensures that only the genes required specifically at the start of S-phase are expressed exclusively at this cell cycle time.

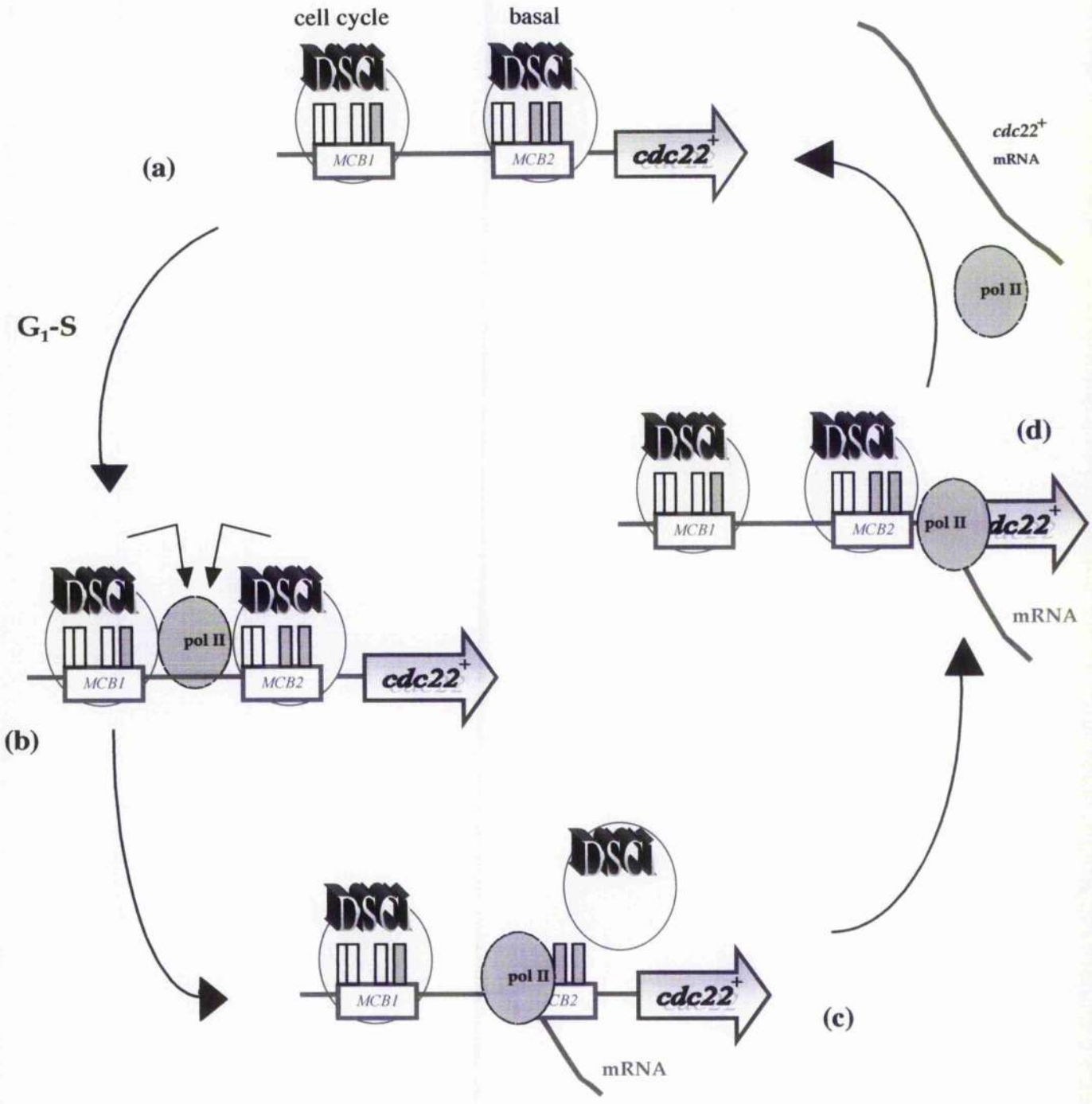

Figure 40. Schematic model of DSC1-MCB interaction in the regulation of *cdc22*$^+$ transcription.

(a) In late G1, at the start of S-phase, DSC1 binds to the two MCB motifs clusters MCB1 and MCB2 to stimulate *cdc22*$^+$ transcription. MCB1 controls cell cycle transcription, possibly through a negative function, and MCB2 controls basal transcription of *cdc22*$^+$. **(b)** RNA polymerase II initiates transcription between the two MCB clusters. **(c)** DSC1 temporarily dissociates from MCB2 allowing RNA polymerase II to pass and complete transcription of *cdc22*$^+$ **(d)**.

IX *Future aims*

Define more precisely the MCB DNA sequence in fission yeast

The importance of the MCB motif as a component of the system regulating cell cycle-dependent transcription was initially identified in budding yeast. MCB sequences were characterised in this organism using site-directed mutagenesis in combination with deletion analysis and sub-cloning experiments. These experiments allowed the consensus sequence ACGCGTNA to be proposed (McIntosh et al. 1991).

In fission yeast, experiments with a synthetic DNA fragment containing three adjacent *Mlu*I sequences implicated MCB motifs as having UAS activity (Lowndes et al. 1992). This synthetic MCB linked to the heterologous *lacZ* gene in a reporter construct conferred G1/S transcription in wild-type fission yeast cells. Consequently, it was suggested that MCB motifs, found in the promoter of the *cdc22*$^{+}$ gene, regulated its G1/S expression. However, a limitation in these findings is that the synthetic MCB is very different to the MCB motifs that occur in their native context in the *cdc22*$^{+}$ ORF. *cdc22*$^{+}$ contains two clusters of MCB motifs within which there are eight MCB motifs, three of these are exact matches to the *Mlu*I restriction sequence, ACGCGT, whilst the remaining five, contain the central core CGCG, thought to be essential for function (McIntosh 1993). There is also a core MCB motif between the two clusters, MCBX.

In the research presented in this thesis I have shown that intact MCB motifs are essential for G1/S expression in fission yeast, as their mutation from ACGCGT to ACTAGT, resulted in loss of activity. Although this shows that the core GC of the MCBs in each cluster is essential for UAS function, we have not yet characterised the complete sequence in the *cdc22*$^{+}$ promoter that has G1/S activity. Thus, it would be interesting to define the actual MCB sequence necessary for G1/S activity within the *cdc22*$^{+}$ promoter, and determine if all the MCB motifs within an MCB element are essential. This could be done using the integration vector pPJK7 where the effect of mutating individual bases within each MCB motif on *lacZ* transcription examined. This would allow us to identify any functional differences which may exist between the

palindromic MCB motif sequences and the non-palindromic sequences. It may be possible that only some of the MCB motifs in each cluster possess G1/S activity, as not all the motifs are exact *Mlu*I sequences and like MCBX, they may not bind to DSC1 or contribute to cell cycle transcription. Previous *in vitro* studies have indicated that DSC1 specifically binds to the two MCB motifs in MCB2 that are palindromic in their sequence (Maqbool et al. 2003). Alternatively, the non-palindromic MCB motifs may be able to confer UAS activity, as in the case of the *ste6*$^{+}$/*rad21*$^{+}$ genes, where two non-palindromic MCB motifs solely confer transcriptional activation of these genes (Papadaki et al. 2002; Pati et al. 2002).

It would also be interesting to examine the effect of altering the spatial relationship between the two MCB clusters within the *cdc22*$^{+}$ promoter, and determine if this has an overall effect on the G1/S activity. It is possible that the complex organisation of the *cdc22*$^{+}$ promoter is related to the location of the MCB2 cluster within the transcribed gene. In this regard it would be useful to examine the effect on transcriptional activation of placing MCB2 upstream of the transcription start site, replacing MCB1 with MCB2 sequence and/or *vice versa*. Such experiments could also provide a test of the idea in our proposed model (Figure 40) that the two MCBs serve to position the RNA polymerase II.

Other MCB-regulated genes

MCB-regulated genes in fission yeast vary considerably in the arrangement or number of MCB motifs present in their promoters (Table 2, Figure 38). Some genes, like *cdc22*$^{+}$, possess a complicated array of motifs, whereas others have only one or two MCB motifs in their promoters, such as *ste6*$^{+}$ or *rad21*$^{+}$ (Papadaki et al. 2002; Pati et al. 2002). It would be interesting to analyse the role of MCB motifs in these genes with simpler MCB arrays. As before, the MCB clusters from the different genes would be cloned into pSPΔ178 in the forward and reverse orientations and *lacZ* transcript detected in synchronized cells. This would also establish if other MCB motifs in fission yeast are also orientation specific.

In other MCB-regulated genes that have more complicated arrays of MCB motifs, like *cdc18*$^{+}$ and *cdt2*$^{+}$, it would be useful to perform primer extension (Liu et al. 2000b; Yoshida et al. 2003). This would determine the transcriptional start of these genes and indicate if any of the MCB motifs occur within the transcribed regions. Such downstream MCB motifs might have DAS activity, as shown in this thesis with *cdc22*$^{+}$.

It would also be informative to make new integration vectors, similar to pPJK7 but instead containing the promoters of other MCB-regulated genes. In this way it would be possible to mutate the MCBs, and detect *lacZ* transcript to determine if there are differences in the way the genes with similar complicated MCB motifs function.

More recently it has been observed that the arrangement of origin of replication initiation (ORI) sequences in *S. pombe* could be similar to those found in animal cells, and a number of fission yeast ORIs have been mapped in close proximity to gene promoters (Gomez and Antequera 1999). It has been suggested ORIs may contribute to transcriptional activation of genes and transcription factors could contribute to recruiting the replication machinery onto DNA. It would therefore be pertinent to establish if ORIs occur in the intergenic regions of MCB regulated genes, using the 'sucrose gradient' approach, which allows the isolation of short DNA replication intermediates (Gomez and Antequera 1999). Although it has been suggested that replication initiation is not effected when the promoters within close proximity to ORIs are altered (Gomez and Antequera 1999), it might still be informative to establish if this is the case when MCB motifs are mutated.

In vivo footprint analysis

In vivo footprint analysis would be the preferred method to confirm which region of the *cdc22*$^{+}$ promoter DSC1 binds. *In vitro* footprint analysis has revealed that DSC1 binds to MCB2 (Maqbool et al. 2003), whereas findings presented in this thesis, using gel retardation studies, showed that both MCB1 and MCB2 are able to bind to DSC1 *in vitro*. This discrepancy may be partly due to the fact that we are working with fragments of promoter DNA, which have been taken out of their native chromosome context. Thus,

performing *in vivo* footprint analysis will give a clearer indication of DSC1-MCB interactions in the promoters of genes in their native context within the fission yeast genome, and possibly reconcile this anomaly.

Chromosome immuno-precipitation (ChIp) analysis

In recent studies, ChIp and genomic micro-array hybridisations have shown that SBF, a transcription factor homologous to DSC1 in budding yeast, binds the promoters of several other transcription factors, which play a role in regulating the periodic expression of genes during the G1/S (Horak et al. 2002). Such studies have also facilitated the definition more precisely of genomic binding sites of the MBF/SBF transcription factors in budding yeast *in vivo* (Iyer et al. 2001).

Other ChiP experiments have shown that DSC1 binds constitutively to the *cdc18*$^{+}$ promoter (Wuarin et al. 2002). It would be interesting to further understand the events of the G1/S transition in the fission yeast, to carry out similar experiments with other genes, including *cdc22*$^{+}$, in this organism.

ChIp analysis and micro-array hybridisation studies could also be used to identify the direct binding targets of DSC1 across the fission yeast genome and so elucidate the importance of the DSC1 components to transcription of the cell cycle genes on a genome-wide basis.

Computer-assisted identification of MCB regulated genes

In a recent study in mammalian systems it has been shown that E2F binding sites in the promoters of cell cycle regulated genes occur in close proximity to the transcription start site (Kel et al. 2001). A method was developed by which it was possible to identify composite substructures (modules) in regulatory regions of genes consisting of a binding site for a key transcription factor and additional contextual motifs; potential targets for other transcription factors that may synergistically regulate gene transcription. Applying this method to cell cycle-related promoters, a programme was created for context-specific identification of binding sites for transcription factors of the E2F family. In addition a ChiP assay experimentally verified the binding of E2F *in vivo* to the promoters predicted by the computer-assisted methods validating its use. It was consequently found that,

although the expression of most E2F dependent genes peaks at G1/S boundary, E2F factors are also involved in regulating genes that control other phases of the cell cycle. Thus, such studies in fission yeast would complement the ChIp assay and micro-array hybridisation studies and indicate if DSC1 is capable of regulating genes that control other phases of the cell cycle. Such analysis might suggest that similar types of cell cycle control mechanisms exist amongst all eukaryotes.

Nascent-chain transcription assay

Regulation of transcription initiation is by far the most widespread form of gene control in eukaryotes. This has often been demonstrated by direct measurement of the transcription rates of multiple genes. To understand more about MCB-gene control in fission yeast it would thus be beneficial to measure the transcription rates of these genes. This can be achieved by use of a 'nascent-chain' (run-on) assay (Turner et al. 2000). In this method, labelled RNA is initially prepared in isolated nuclei by allowing extension of already initiated RNA chains. The reactions are then run for a brief period, enabling RNA polymerase II to add a few hundred nucleotides to nascent RNA chains. By hybridising the labelled RNA transcribed to a cloned DNA from a specific gene, the fraction of the total RNA copied from a particular gene (i.e. its relative transcription rate) can be determined.

Induction of MCB genes

In previous studies hydoxyurea (HU), an RNR inhibitor, has been shown to induce MCB-containing genes in asynchronous yeast (Harris et al. 1996). This observation suggested that DNA replication was not necessary for MCB gene induction. However, HU also triggers the replication-arrest checkpoint (Allen et al. 1994) and induction of MCB genes due to triggering of this checkpoint may mask whether replication is needed for MCB gene induction at G1/S during a normal cell cycle. In a recent study in budding yeast this issue has been addressed by using a temperature sensitive *dbf4* mutant (Horak et al. 2002), which in the presence of HU or at the non-permissive temperature blocks DNA chain elongation/initiation. The findings of this study resulted in a proposed model, whereby it was suggested that MCB gene induction serves to increase the levels of

enzymes such as RNR, thymidylate synthase and thymidylate kinase, which in turn support a higher rate of DNA precursor synthesis. Furthermore, it was found that both MCB gene induction and dNTP accumulation occur in the absence of DNA chain initiation/elongation, suggesting that a mechanism exists to pre-emptively rather than reflexively increase the rate of DNA precursor synthesis to ensure that the replication machinery has an adequate supply of dNTPs. It would therefore be of benefit to conduct a similar study in fission yeast in order to establish if DNA replication may contribute to MCB gene induction at G1/S similar to budding yeast. It may be possible that a similar mechanism exists amongst other eukaryotes.

X *Why are some genes expressed only at G1/S?*

Recent use of micro-array analysis has allowed the characterisation of gene expression through the budding yeast cell cycle. Analysis of mRNA levels of all genes in synchronous cell cultures have, surprisingly, revealed that more than 10% of genes in the budding yeast genome are cell cycle regulated (Cho et al. 1998; Spellman et al. 1998). Due to the amount of conservation already shown to occur between budding and fission yeast in cell cycle control mechanisms, it is likely that a similar number of genes are also cell cycle regulated in this organism.

Why are so many of these genes cell cycle regulated?

- Some genes are cell cycle regulated because they are only needed once per cell cycle, as there is a particular demand for their activities during a specific cell cycle phase. For example, *cdc22*$^{+}$, which encodes the large sub-unit of RNR, is expressed at G1/S as it is essential for DNA precursor metabolism required at the the onset of S-phase (Fernandez-Sarabia et al. 1993). Such expression patterns also facilitates cellular economy. Expression of these genes throughout the cell cycle may not harm the cell, but might waste cellular resources.

- Other genes are rate-limiting and are necessary to control regulation of the cell cycle during the specific phases. For example, cyclins are needed at particular phases to bind CDKs, to allow cell cycle progression (Coqueret 2002). Expression of such genes, therefore, throughout the cell cycle would interfere with cellular mechanisms and disturb the normal progression of the cell cycle.

- Some genes, although not rate-limiting, can harm the normal cell cycle if they are expressed throughout division. Thus, cell cycle regulation of these genes ensures other components of the cell cycle machinery are not disrupted. For example constitutive expression of *mikl*$^{+}$, a newly discovered MCB regulated gene, inhibits cell cycle entry into mitosis, which manifests as cells abnormally long at division (Ng et al. 2001).

XI *Fission yeast: a good model organism?*

The increasing number of human gene homologues emerging in yeast provides encouragement for the further development of fission yeast as a host in which to elucidate cell cycle and other molecular mechanisms within all eukaryotes.

For example, recent findings confirm that *S. pombe* is a useful model for studying the molecular mechanism of Vpr-induced G2/M arrest (Elder et al. 2002). It was found that Vpr, one of the HIV-1 accessory proteins thought to play an important role in viral replication and pathogenesis, also causes cell cycle arrest in *S. pombe*. The toxicity of Vpr has been documented for mammalian cells and other systems including bacteria and the budding yeast and is thought to affect the viability of *S. pombe* by deteriorating a basic biological function highly conserved in various species (Masuda et al. 2002).

Fission yeast has also been established as an experimental model for the study of anti-sense RNA-mediated gene suppression (Clarke et al. 2000). Anti-sense RNA is used to regulate gene expression, and is established as a basis for the development of gene

therapeutics and as a tool for elucidating gene function (Raponi et al. 2000). It has been recently shown that the efficacy of anti-sense RNA in fission yeast cells is comparable to that seen in mammalian cells and this validates *S. pombe* as a model for the development of strategies to suppress target genes in human cells (Arndt et al. 2000).

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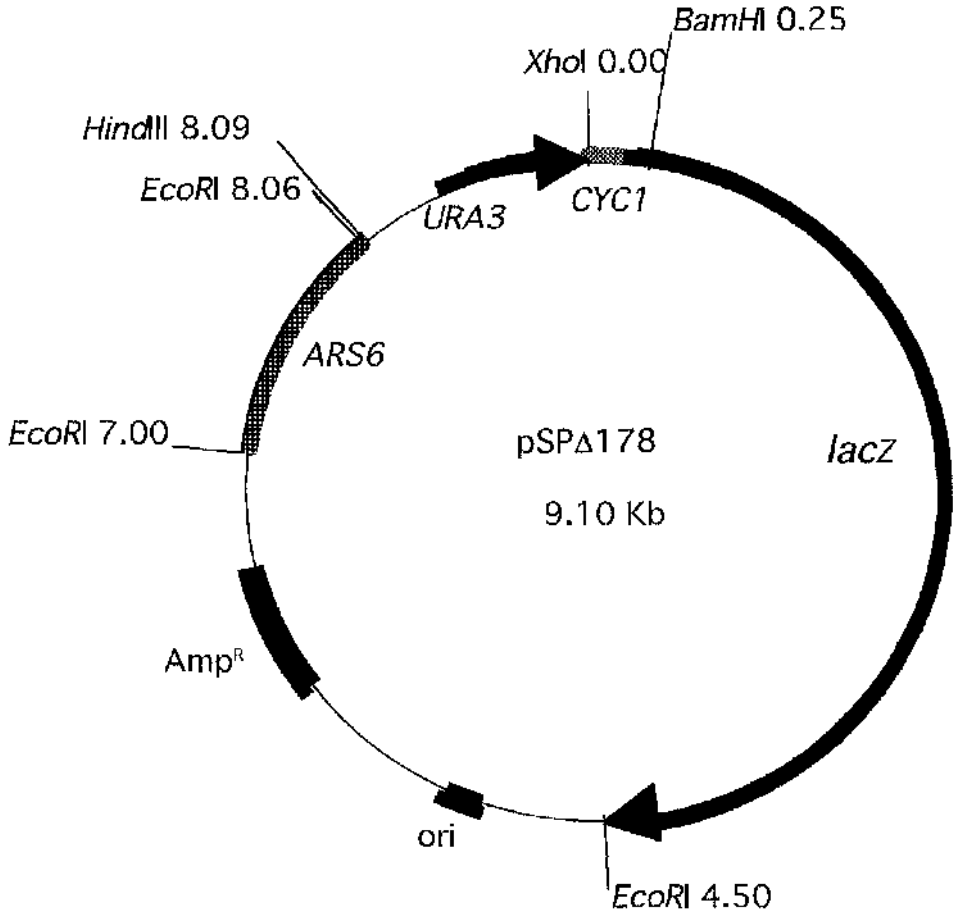
Appendix A

Appendix A.1 Restriction Map of pSPΔ178

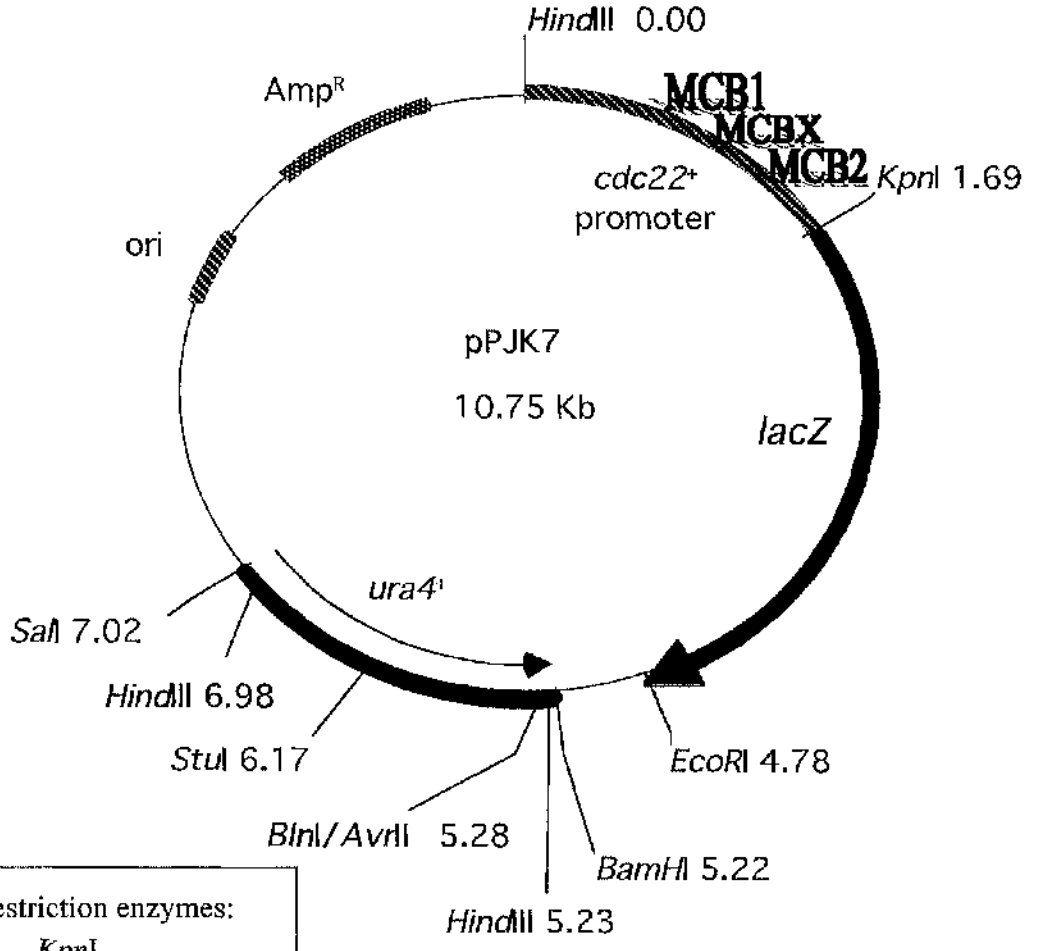
Restriction map of fission yeast UAS reporter construct, pSPΔ178. Potential UAS is inserted into *Xho*I restriction site and UAS activity is assayed by *lacZ* transcription.



Single cut restriction enzymes: *Xho*I, *Bam*HI *Hind*III produces a fragment of ~ 9.1 kb	A double restriction digest with *Bam*HI and *Hind*III produces two fragments at: ~ 7.5 kb 1.5 kb	*EcoR*I digestion produces fragments at: ~ 3 kb, 4.5 kb 1.05 kb

Appendix A.2 Restriction Map of pPJK7

Restriction map of fission yeast integration vector, pPJK7. Entire *cdc22*$^{+}$ promoter region fused in frame with *lacZ*. Plasmid contains wild type promoter of *cdc22*$^{+}$ with MCB1, MCB2 and MCBX. Plasmid was cut at a single restriction site in *ura4*$^{+}$ using the restriction enzyme *Bln*I/*Avr*II to permit integration into fission yeast *ura4* locus.



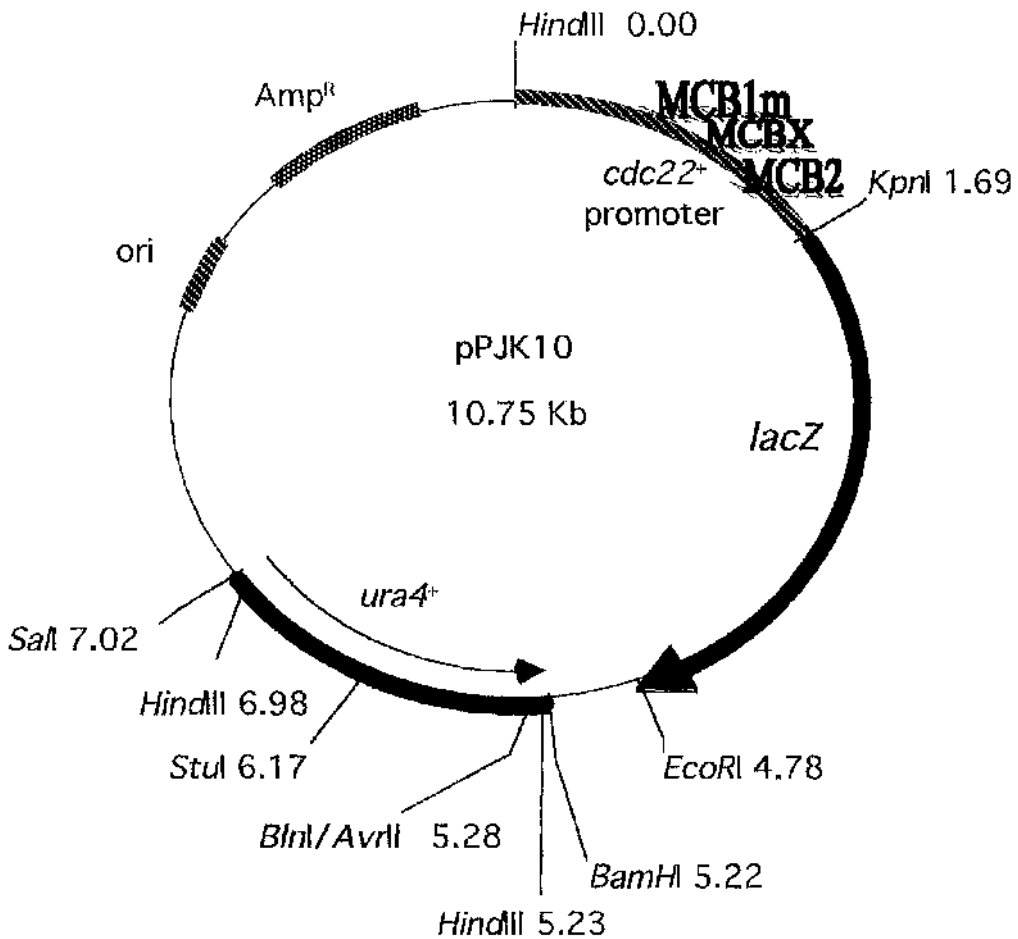
Single cut restriction enzymes:
*Kpn*I,
*Eco*RI,
*Bam*HI,
*Stu*I,
*Bln*I/*Avr*II,
*Sal*I
produces a fragment of ~ 10.75 Kb

A double restriction digest with *Kpn*I and *Eco*RI produces two fragments at:
~ 7.7 kb
3.1 kb

*Hind*III digestion produces fragments at:
~ 5.2 kb,
1.8 kb
3.8 kb

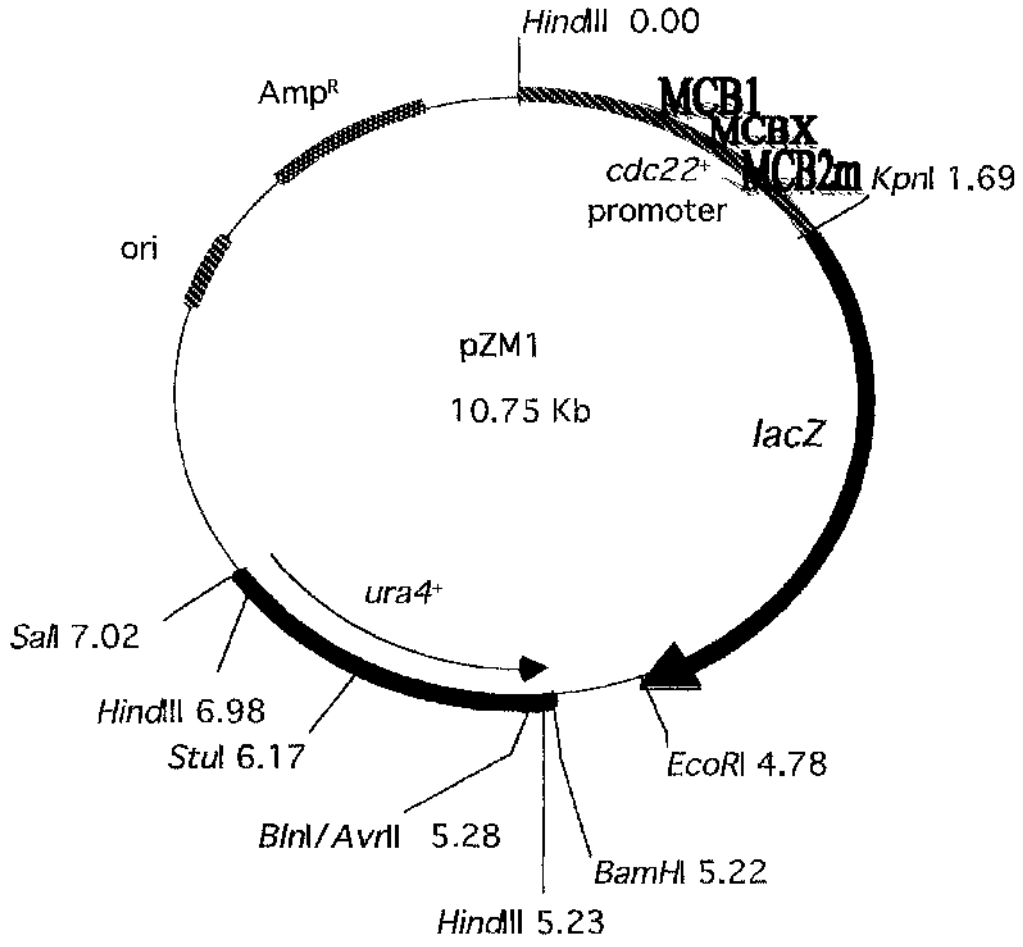
Appendix A.3 Restriction Map of pPJK10

Restriction map of fission yeast integration vector, pPJK10. Entire *cdc22*$^{+}$ promoter region fused in frame with *lacZ*. Similar to pPJK7 but plasmid contains MCB1 mutated, MCB1m.



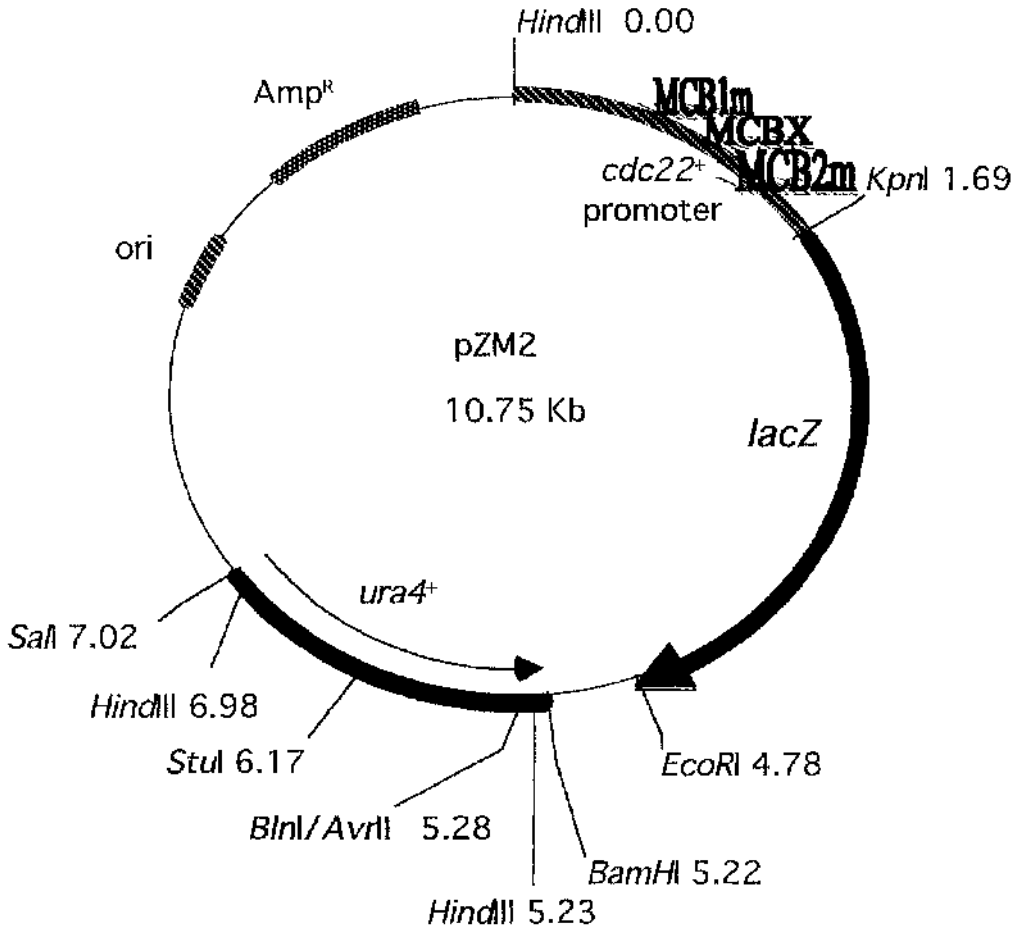
Appendix A.4 Restriction Map of pZM1

Restriction map of fission yeast integration vector, pZM1. Entire *cdc22*$^{+}$ promoter region fused in frame with *lacZ*. Similar to pPJK7 but plasmid contains MCB2 mutated, MCB2m.



Appendix A.5 Restriction Map of pZM2

Restriction map of fission yeast integration vector, pZM2. Entire *cdc22*$^{+}$ promoter region fused in` frame with *lacZ*. Similar to pPJK7 but plasmid contains MCB1mutated, MCB1m and MCB2 mutated. MCB2m.



Appendix B

- Oligonucleotide primer list

Glasgow collection number	Sequence
GO 36	GCGC CTCGAG GTA GTT CAA TCT CAT AGA *cdc22*$^+$ clone 5' MCB1 region with *Xho*I site
GO 37	GCGC CTCGAG CTC TGT TTA CGA CTG AAT G *cdc22*$^+$ clone 3' MCB1 region with *Xho*I site
GO 39	GCGC CTCGAG CAT TCA GTC GTA AAC AGA G *cdc22*$^+$ Clone 3' MCBX region with *Xho*I site
GO 40	CGCG CTCGAG CCG CTA AAA TAA GTC CGA *cdc22*$^+$ Clone 5' MCBX region with *Xho*I site
GO 42	GCGC CTCGAG GGT GGT AAA TAC CGG GAA *cdc22*$^+$ Clone 3' MCB2 region with *Xho*I site
GO 44	GCGC CTCGAG CAT TGA TCA ACA TGA CTT AAA G *cdc22*$^+$ Clone 5' MCB2 region with *Xho*I site
GO 46	CAC GCC TGG CGG ATC TG 3' to amplify insert from pSPΔ178 *Xho*I site
GO 47	CTA AAC TCA CAA ATT AGA GC 5' to amplify insert from pSPΔ178 *Xho*I site
GO 365	CTT CTC CCG CAG CAC CTT CC 5' to amplify *cdt2*$^+$
GO 366	CCA TCC CGA GAA CAA CTT ACC 3' to amplify *cdt2*$^+$
GO 367	GGA GAC AGA GAA TCT TCC CC 3' to amplify *psc3*$^+$
GO 368	CAT GGC AC GAC CCA AGA C 5' to amplify *psc3*$^+$
GO 369	GGG CAG AAG CAC ATA ACG 3' to amplify gene upstream to *rad21*$^+$, Spcc338.18c
GO 370	CTG CGC AAA ACA GTT CCC G 5' to amplify gene upstream to *rad21*$^+$, Spcc338.18c
GO 373	ACT CCC GAG GAC GTC TTC AT 3' to amplify gene upstream to *cdt1*$^+$, Spb428.17c
GO 374	CTT CCA CTA CCT ATC GGG 5' to amplify gene upstream to *cdt1*$^+$, Spb428.17c
GO 375	TAC AAG TCG CGG CAT CCC AA 3' to amplify *ste9*$^+$
GO 376	ACC TCG CAC AAA CGA GGG AG 5' to amplify *ste9*$^+$

Appendix B

- Oligonucleotide primer list continued

GO 377	GAG GAT CTA CTG CG GTC G 3' to amplify gene upstream of *ste9*$^{+}$, Spac144.14c
GO 378	TGT AGG CGT GAT GCT TTC 5' to amplify gene upstream of *ste9*$^{+}$, Spac144.14c
GO 379	CAT CTG GTG ATG CCT GAG GA 3' to amplify *spk1*$^{+}$ gene
GO 380	GTA TGT GTG CCG CGC TTC AC 5' to amplify *spk1*$^{+}$ gene
GO 381	CTC GGC CTG GAA GTT TAA TG 3' to amplify gene upstream to *spk1*$^{+}$, Spac31g5.10
GO 382	CTC ATC CGG AAG CCA AAC TC 5' to amplify gene upstream to *spk1*$^{+}$, Spac31g5.10
GO 383	GAG GTT TCA AAC GAC CGC 3' to amplify *ste6*$^{+}$
GO 384	CAT GCG CTA TCA AAG AGC CC 5' to amplify *ste6*$^{+}$
GO 385	CGG CAC TCC AGA TCA GCA C 3' to amplify gene upstream to *ste6*$^{+}$, Spcc1450.16c
GO 386	ACA AGG CGT CGG GGC GTT G 5' to amplify gene upstream to *ste6*$^{-}$, Spcc1450.16c
GO 499	GTT CGG ATG ACT AGA CTA GGC ATC GT "D" 3' to amplify MCB2(a) mutated in *cdc22*^{ts} for site directed mutagenesis
GO 500	ATT AGC GTG ACT AGT CTG AAC TAG TTT TTC ATT TAC "F" 3' to amplify MCB2(b) mutated in *cdc22*$^{+}$ for site directed mutagenesis
GO 501	ACG TGA TGC CTA GTC TAG TCA TCC GAA C "C" 5' to amplify MCB2(a) mutated in *cdc22*$^{-}$ for site directed mutagenesis
GO 502	GTA AAT GAA AAA TA GTT CAG ACT AGT CAC GCT AAT "E" 5' to amplify MCB2(b) mutated in *cdc22*$^{+}$ for site directed mutagenesis
GO 503	AAG CTT TCT CAC AGC ACG TAA TTG C "A" *cdc22*$^{+}$ clone 3' MCB2 region with *Hind*III site
GO 504	GGT ACC ACG AGC AGT GAT TTT GTC "B" *cdc22*$^{+}$ clone 5' MCB2 region with *Kpn*I site
GO 521	GCT TCG TCG GCA TCT CTG C 5' specific to *ura4*$^{-}$ promoter region in chromosome (to check integration vector is inserted into the *ura4*$^{+}$ locus, with GO 521)
GO 522	TTC GCG CGT CCC GCA GCG C 3' specific to *lacZ* in integration vectors, pPJK7, pPJK10, PZM1 and PZM2 (to check integration vector is inserted into the *ura4*$^{+}$ locus with GO 522)

Appendix C

- Bacterial strain list

Glasgow collection number	Genotype
GB10	*E. coli* DH5α containing pSPΔ178 plasmid
GB 57	*E. coli* DH5α containing pSPΔ178 plasmid with MCB2 forward orientation insert
GB 58	*E. coli* DH5α containing pSPΔ178 plasmid with MCB2 reverse orientation insert
GB 73	*E. coli* DH5α containing pSPΔ178 plasmid with MCB1 forward orientation insert
GB 74	*E. coli* DH5α containing pSPΔ178 plasmid with MCB1 reverse orientation insert
GB 75	*E. coli* DH5α containing a TA cloning vector with MCB1 insert
GB 76	*E. coli* DH5α containing a TA cloning vector with MCB2 insert
GB 150	*E. coli* DH5α containing pPJK7 integration plasmid containing wilt type *cdc22*$^{+}$ promoter fused inframe with *lacZ*.
GB 154	*E. coli* DH5α containing pPJK10 integration plasmid, similar to pPJK7 with MCB1 cluster mutated (MCB1m)
GB 245	*E. coli* DH5α containing pZM1 integration plasmid, similar to pPJK7 with MCB2 cluster mutated (MCB2m) using primers: GO 499, GO 500, GO501, GO 502, GO 503 and GO 504
GB 246	*E. coli* DH5α containing pZM2 integration plasmid, similar to pPJK7 with MCB1 and MCB2 clusters mutated (MCB1m and MCB2m) using primers: GO 499, GO 500, GO 501, GO 502, GO 503 and GO 504

Appendix D

- Fission yeast strain list

Glasgow collection number	Genotype		Plasmid
GG 28	h^+	*cdc10-129*	
GG 75	h^+	*leu1-32 ura4-294*	
GG 108	h^+	*cdc10-C4 ura4-D18*	
GG 155	h^-	*res1::ura4$^+$ ade6-M216 ura4-D18 leu1-32*	
GG 156	h^-	*res2::ura4$^+$ ura4-D18 leu1-32*	
GG 158	h^-	rep2::*ura4$^+$ ura4-D18 leu1-32*	
GG 193	h^-	*cdc25-22 leu1-32 ura4-D18*	
GG 217	h^+	*972* ("wild-type")	
GG 251	h^-	*cdc25-22 leu1-32 ura4-D18*	pSPΔ178.MCB1 'forward'
GG 252	h^-	*cdc25-22 leu1-32 ura4-D18*	pSPΔ178.MCB2 'forward'
GG 253	h^-	*cdc25-22 leu1-32 ura4-D18*	pSPΔ178.MCB2 'reverse'
GG 257	h^+	*cdc10-C4 ura4-D18*	pSPΔ178.3MCB
GG 258	h^+	*cdc10-C4 ura4-D18*	pSPΔ178
GG 265	h^-	*cdc25-22 leu1-32 ura4-D18*	pSPΔ178.MCB1 'reverse'
GG 469	h^-	*cdc25-22 leu1-32 ura4-294*	
GG 470	h^+	*cdc25-22 ura4$^+$::ura4-294 leu1-32*	pJK7 (wild type MCBs)
GG 471	h^+	*cdc25-22 ura4$^+$::ura4-294 leu1-32*	pJK10 (MCB1m)
GG 591	h^+	*cdc25-22 ura4$^+$::ura4-294 leu1-32*	PZM1 (MCB2m)
GG 592	h^+	*cdc25-22 ura4$^+$::ura4-294 leu1-32*	PZM2 (MCB1m and MCB2m)