Control of S-phase genes in fission yeast

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This thesis is dedicated to my parents, with love and gratitude

'what can be said at all can be said clearly, and what we cannot talk about we must pass over in silence'

'Back off - I'm a scientist!'

Declaration

I declare that this thesis was composed by myself and that the research presented is my own. Due acknowledgement is made within the text for the assistance of others.

Christopher McInerny

August 1992

Acknowledgements

Working for this thesis over the last two and half years have been a very happy period of my life. Not only do I feel that I have learnt a great deal, but I've been lucky enough to see how exciting science can be, and how much I enjoy doing it. This happiness is due to a number of people.

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Abbreviations

A_x	Absorbance (turbidity) at x nm wavelength
AMV	avian myoblastosis virus
ATP	adenosine triphosphate
BSA	bovine serum albumin
CCB	cell cycle box
cdc, CDC	cell division cycle
cDNA	complementary DNA
cps	counts per second
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide
dNTPs	all four deoxy triphosphates
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EEO	electroendosmosis
EMM	Edinburgh minimal medium
Exo III	exonuclease III
Fig.	Figure
GABP	guanosine adenine binding protein
Hepes	N-(Hydroxyethyl]piperazine-N'-[2-ethanosulphonic acid]
IPTG	isopropylthio-β-D-galactoside
kb	kilobase
kDa	kilodalton
MCB	*Mlu*I cell cycle box
min	minutes
MOPS	3-(N-morpholino)propane-sulphonic acid
MPF	maturation promoting factor
mRNA	messenger RNA
MW	molecular weight
NETO	new end take off
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulphonylfluoride
PNK	polynucleotide kinase
RNA	ribonucleic acid
rpm	revolutions per minute
RNR	ribonucleotide reductase
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
ts	temperature sensitive
UAS	upstream activating sequence
UV	ultraviolet light
UWGCG	University of Wisconsin GCG sequence analysis program
v/v	volume/volume
w/v	weight/volume
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YE	yeast extract medium

Abstract

The work described in this thesis uses the fission yeast *Schizosaccharomyces pombe* as model system to analyse the molecular processes that control passage through the G_1 and S-phases of the cell cycle. In particular, the work analyses the control of expression of *cdc22*$^+$, a gene whose transcript varies in abundance during the cell cycle with a maximum at the G_1-S phase boundary.

Chapter 3 describes the sequencing of *cdc22*$^+$ and shows it encodes the large subunit of ribonucleotide reductase, an enzyme required for DNA precursor metabolism.

In the next Chapter, *cis*-acting elements, resembling MCBs previously identified in budding yeast, are shown to be present 5' to the *cdc22*$^+$ open reading frame. MCBs can confer cell cycle expression on a heterologous gene in fission yeast, implicating them in controlling periodic expression of *cdc22*$^+$. A *trans*-acting complex that specifically binds MCBs is identified, and called DSP1- for DNA synthesis control in *S. pombe*. DSP1 is related to DSC1, an MCB binding activity identified in budding yeast.

Experiments in Chapter 5 demonstrate that the gene product of the *cdc10*$^+$ START gene, p87^{cdc10}, is a component of DSP1. Furthermore, *cdc22*$^+$ is shown to be constitutively over-expressed in a *cdc10* mutant, *cdc10-C4*. Over-expression of *cdc22*$^+$ in *cdc10-C4* is recessive to wild-type. It is proposed that DSP1 containing p87$^{cdc10\text{-}C4}$ is hyperactive and deregulated as a transcription complex.

In summary, p87^{cdc10} is part of a transcription complex that controls expression of *cdc22*$^+$, a gene required for DNA synthesis. Thus this work demonstrates a molecular link between START and S-phase.

Chapter 1: Introduction

1 a Introduction

The cell cycle can be broadly defined as the ordered biological processes that occur when a cell duplicates its cell contents and divides, forming two daughter cells.

Over the past few years there have been considerable advances in our understanding of the molecular mechanisms that control progression through the eukaryotic cell cycle. This rapid progress is due to the summation of two areas of scientific analysis: genetics in yeasts (particularly the fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae*); and biochemistry in metazoan cells such as *Xenopus* oocytes and sea urchin eggs. Using these systems, proteins have been identified that are required for cell cycle progress. Functions for the proteins have been found, and molecular mechanisms suggested which control various cell cycle stages. These cell cycle mechamisms are of wide interest because homologues of the proteins are present all eukaryotic cells.

This introduction will discuss current understanding of the mechanisms that control the cell cycle. A vast number of genes and proteins from a wide range of organisms are now implicated in controlling all stages of the cell cycle: to discuss them all would be beyond the scope of this introduction. I will concentrate on the principal organisms used as cell cycle models systems, and discuss only those genes and proteins for whom cell cycle functions have been found. The molecular processes that control mitosis and cytokinesis will not be mentioned.

First, general cell cycle concepts are outlined, followed by a discussion of the main model organisms used to study cell cycle mechanisms. Next there is a detailed description of the molecular mechanisms that control the two most important cell cycle control stages, START and the G_2-M transition. Finally, the role of transcriptional regulation in cell cycle control is discussed.

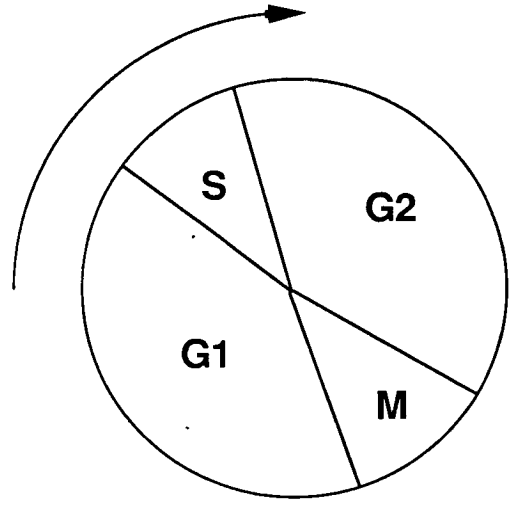


Figure 1 A **The four successive phases of a typical eukaryotic cell cycle.**

The cell cycle is split into four phases: G1, S, G2, and M. S-phase is the period during which DNA synthesis occurs when the chromosomes are duplicated. During M-phase mitosis occurs resulting in the separation of duplicated chromosomes. G_1 and G_2 are 'gaps ' between S and M phase and contain the important cell cycle control stages of START and the G_2-M transition.

1b Cell cycle concepts

i Phases

The eukaryotic cell cycle has been traditionally divided into four distinct phases: G_1, S, G_2, and M (see Fig. 1A). S-phase represents DNA synthesis, the period during which the DNA of the cell is replicated. M-phase represents mitosis, where the mitotic spindle is formed and the the sister chromatids are separated. G_1 and G_2 were originally designated as 'gaps' between these two phases, but are now known to contain the two important control points of the cell cycle, the restriction point or START, and the G_2-M transition.

ii Control points

In higher eukaryotic cells and in budding yeast, the principal control point of the cell cycle occurs during G_1. Cells which have been propagated in tissue culture, if starved of serum, cease to proliferate and enter a quiescent state from G_1, called G_0 (Baserga, 1985). G_1 has been split into two parts, depending on the serum sensitivity of the cell (Zetterberg & Larsson, 1985). Cells in early G_1 are serum-sensitive and so if starved of serum enter G_0; in later G_1, cells becomes serum-insensitive and continue to enter S-phase, even in absence of serum. The transition point between these two parts of G_1 has been called the 'restriction point' (Pardee, 1974).

A stage analogous to the restriction point in G_1 has been identified in budding yeast, and is called 'START' (Hartwell, 1974). START is the stage at which cells become committed to either the vegetative or sexual life cycles. Cells arrest at START if deprived of essential nutrients, or when exposed to mating pheromone from cells of the opposite mating type. Additionally, START is the arrest point of *CDC28*.

Fission yeast is different from higher eukaryotes and budding yeast in that START is not rate-limiting for cell cycle progress during exponential growth, this role being taken over by the control over entry into mitosis (Nurse, 1975). START is less understood in fission yeast: the only evidence for its existence coming from the ability of cells at different stages during G_1 to conjugate: START is the arrest point of the *cdc2* and *cdc10* mutants (Nurse & Bissett, 1981).

Budding yeast cells enter an analogous stage to G_0 - stationary phase - when nutritionally arrested, before the completion of START. Fission yeast cells can enter stationary phase from either G_1 or G_2 (Costello *et al.*, 1986).

iii Coordination of growth and division

When cells grow faster than they divide, they become larger; and when they grow slower than they divide, they get smaller. In a normal population of growing cells cell size at division remains constant, so coordination between growth and division must be occurring. The model most attractive to explain this coordination is that cell are able to gauge their size by means of some internal 'sizer', and are are only able to pass certain cell cycle stages having obtained a critical size (Nurse & Fantes, 1981). Examples of such cell cycle stages are START in budding yeast (Johnston *et al.*, 1977), and the G_2-M transition in fission yeast (Nurse, 1975).

1 c How is the cell cycle studied?

i Yeasts

The seminal breakthrough in yeast cell cycle studies was the identification, by mutation, of genes required for cell cycle progress (Hartwell *et al.*, 1974; Nurse *et al.*, 1976). Such *c*ell *d*ivision *c*ycle genes (*cdc* or *CDC* for fission or budding yeast, respectively) encode mutants in proteins essential for cell cycle progress; thus, the cells block at the particular cell cycle stage at which the gene product is required. Obviously, cells with mutations in such essential genes would be inviable: this problem is overcome by using conditional mutants which only display their mutant phenotype under certain conditions.

Temperature-sensitive (ts) *cdc* mutants, at the permissive temperature, progress through the cell cycle apparently normally: on shifting to the restrictive temperature cells accumulate which cannot traverse the specific cell cycle stage that the gene product is required at. This is because the change in the gene product only disrupts its function at a certain temperature. In fission yeast the cdc phenotype is manifested usually as abnormally long cells which cannot divide, but continue to

grow. In budding yeast CDC arrested cells have a range of phenotypes from an unbudded cell, to a mother cell with a single daughter bud. Temperature-sensitive *cdc* mutants have been selected at both high or low temperatures: this reflects the respective mutant protein's inability to function at the restrictive temperature.

Techniques have been developed which permit the cloning of *cdc*$^+$ genes by mutant rescue (Nasmyth & Reed, 1980). Shuttle vectors which can be maintained in both bacteria and yeast have been constructed (Wright *et al.*, 1986). Gene libraries, made from yeast chromosomal DNA, are ligated into the shuttle vectors, and the recombinant DNA transformed into yeast *cdc* strains at the permissive temperature (Beach & Nurse, 1981). On incubating the cells at the restrictive temperature, those cells transformed with a vector containing the equivalent *cdc*$^+$ gene, or other genes that can complement the cdc lesion, survive. Once the gene has been cloned, this allows sequencing and subsequent genetic characterization. Knowing the sequence of a gene permits further manipulations, such as over-expression of the gene and its deletion from the chromosome.

Other genes that interact with *cdc*$^+$ genes can be isolated by two methods. The first method exploits the fact that mutants can be complemented by other genes when present in multiple copies. Such multicopy suppressors may be cloned when attempting to screen for the *cdc*$^+$ gene using a wild-type genomic library, as the shuttle vector carrying the complementing gene is maintained in fission yeast at high copy number. Examples of multicopy suppressors include the *CLN*s and *suc22*$^+$, which complement *cdc28*^{ts} and *cdc22*^{ts} mutants, respectively (Hadwiger *et al.*, 1988; Gordon & Fantes, 1986). The second method uses the fact that mutants can sometimes be complemented by compensatory mutations in other genes. Such extragenic suppressors can be cloned by making a genomic DNA library from cells carrying the extragenic suppressor, and using this to complement the original *cdc* mutation. An example of an extragenic suppressor is *sdc1*$^+$ which, when mutated, suppresses *cdc10* mutants (Caligiuri & Beach, 1992).

Cloning of a *cdc*$^+$ gene allows its gene product to be analysed biochemically. The gene product can be expressed in bacterial systems for the production of specific antisera, which permits the identification of native protein from yeast cell extracts. A number of proteins required for

cell cycle progress have been found to be putative protein kinases or phosphoproteins from sequence data: specific antisera permits formal characterization of such functions. Additionally, antisera allow sub-cellular localization of the gene product by indirect immunofluorescence, which may be informative about potential functions.

A wide range of genes required for a variety of cell cycle processes have been isolated by such genetic approaches (Review: Fantes, 1989), including START (*cdc2*$^+$, *cdc10*$^+$ and *CDC28*), S-phase (*cdc22*$^+$, *cdc17*$^+$, *CDC9*), mitotic onset (*cdc2*$^+$, *cdc13*$^+$, *cdc25*$^+$, *CDC28*), mitosis (*cut7*$^+$, *nuc1*$^+$), and cytokinesis (*cdc11*$^+$ and *cdc16*$^+$). The functions of the products some of these genes are discussed in Section 1e.

ii *Xenopus* oocytes and sea urchin eggs

In contrast to yeasts, cell cycle studies in higher eukaryotic cells began with biochemical analysis. Identification of proteins involved in cell cycle progress was permitted by the exploitation of several experimental systems which provide populations of synchronously dividing cells. Two examples of such systems are *Xenopus* oocytes and sea urchin eggs.

Fully mature *Xenopus* oocytes are physiologically arrested in prophase I of meiosis, but can be artificially stimulated to resume cell division by the addition of progesterone (Review: Masui & Clark, 1979). The same response can be observed if the cytoplasm of mature oocytes is transferred to immature oocytes, as the recipients enter metaphase and undergo oocyte maturation (Masui & Markert, 1971). The component transferred between the two oocytes which is responsible for inducing metaphase, was named maturation promoting factor (MPF; Masui & Markert, 1971). MPF has been purified (Lohka *et al.*, 1988), and shown to consist of a homologue of p34^{cdc2} (Gautier, *et al.*, 1988; Dunphy *et al.*, 1988) and a mitotic cyclin (Gautier *et al.*, 1990).

Sea urchin eggs can be stimulated to divide synchronously *en masse* after addition of sperm. Such large numbers of dividing cells allowed the discovery of a group of polypeptides that oscillate in level during the cell cycle, with steady-state synthesis and accumulation until metaphase when they are rapidly and specifically degraded as cells enter anaphase (Evans *et al.*, 1983). These polypeptides were christened 'cyclins', and are required

for mitotic entry (Swenson *et al.*, 1986; Murray *et al.*, 1988). Cyclins have homologues in both fission and budding yeast. Fission yeast cyclin homologues so far isolated are *cdc13*$^+$(Hagan *et al.*, 1989), *puc1*$^+$ (Forsburg & Nurse, 1991) and *cig1*$^+$ (Bueno *et al.*, 1991). Budding yeast cyclins include *CLN*s (Hadwiger *et al.*, 1988), *CLB*s (Surana *et al.*, 1991) and *SCB1* (Ghiara, *et al.*, 1991).

1 d Fission yeast

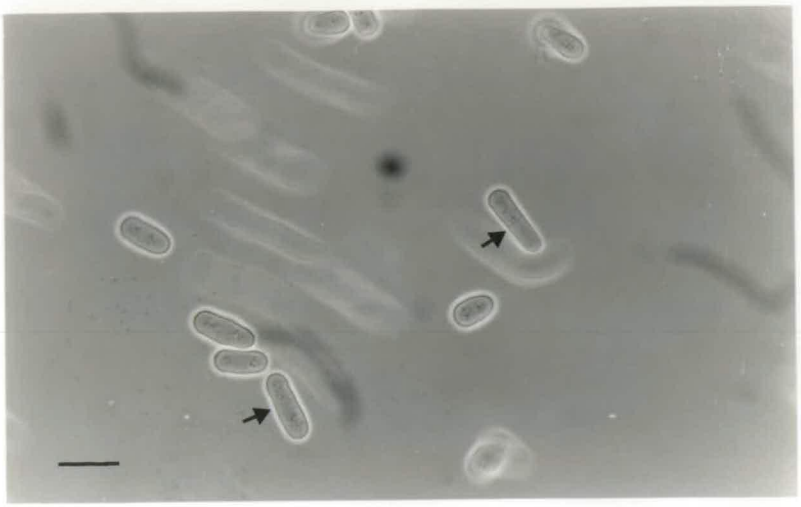
i Classification, cell biology and cell cycle

The fission yeast *Schizosaccharomyces pombe* Lindner is a unicellular ascomycete yeast, whose basic growth properties have been well defined (Mitchison & Nurse, 1985; Tanaka & Kanbe, 1986).

Fission yeast is a rod with hemispherical ends, and grows by length extension alone (Figs. 1B and 1C). During interphase cells grow only at the tips, and increase in length for approximately three quarters of the cell cycle. Cell size can therefore be used as a marker of a cell's position in the cell cycle. Cells begin the cell cycle ~7 μm in length, and first grow at one end, until they reach a length of ~9.5 μm, when then initiate growing at both ends. This transition has been termed new end take off (NETO; Mitchison & Nurse, 1985). Cells grow ~1.7 times faster during bipolar growth than monopolar growth, until they reach ~14 μm, when they enter the "constant volume" stage. The cells then stop growing, undergo mitosis and cytokinesis, and lay down a medial septum, which is cleaved to form two daughter cells.

The cytoskeleton of fission yeast has studied by indirect immunofluoresence, and provides additional markers for cell cycle stages (Review: Robinow & Hyams, 1989). In interphase cells an array of cytoplasmic microtubules is present which run longitudinally from one pole to another (Hagan & Hyams, 1988). At the beginning of mitosis these disappear and are replaced by an intranuclear mitotic spindle. During anaphase A this spindle separates the sister chromosomes, and in anaphase B it elongates, and moves the duplicated nuclei to the ends of the cells. As the nuclei reach the ends of the cells, two microtubule

B



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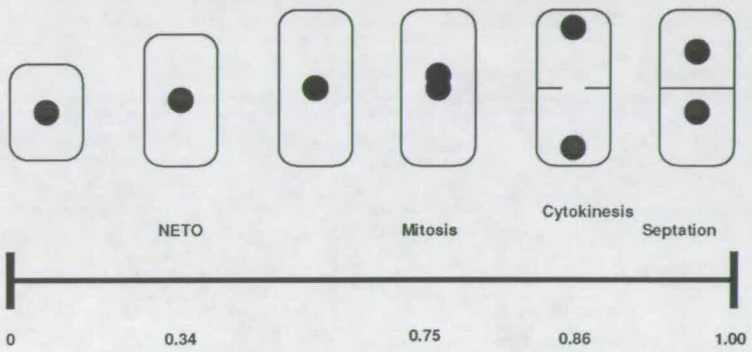


Figure 1 **B** **Phase contrast image of living fission yeast cells.**

Arrows indicate a mitotic cell and a cell undergoing septation. Bar - 5μm.

C **Schematic diagram summarizing the principal events of the fission yeast cell cycle.**

The numbers indicate that stages at which events occur in a wild type cell cycle of abitrary total length 1. In the first 0.34 of the cell cycle cells grow at one end, until new end take off (NETO), when bipolar growth commences. At a stage 0.75 through the cell cycle cells enter the constant volume stage, and proceed into mitosis. From 0.86 to the end of the cell cycle, cytokinesis and septation occur to form two daughter cells.

organising centres are formed at the equator, which, coincidentally with the breakdown of the mitotic spindle and start of the next interphase, seed new cytoplasmic microtubules in the two daughter cells.

Actin microfilaments occur as two forms in fission yeast: dots and filaments (Marks & Hyams 1985). Dots are only found in growing regions of the cell. Before NETO, when cells are only growing at one end, dots are only found at one end; after NETO, dots are found at both ends. Filaments have been observed during the two major transitions of cell growth: the initiation of single end growth following septation, and the switch to bipolar growth at NETO. A microfilament ring is formed at the equator during anaphase A, predicting the site of septation.

Fission yeast has a typical higher eukaryotic cell cycle at a cytological level, as each of the described phases are discrete and consecutive, the only major difference being that the nuclear membrane remains intact during mitosis (Review: Nurse, 1985). In particular, DNA is fully duplicated before a mitotic spindle is formed and mitosis occurs. This is in contrast to budding yeast, the organism traditionally used as a cell cycle model system, where the mitotic spindle is formed before DNA synthesis occurs, though mitosis does not occur until the DNA is replicated. Furthermore, in a recent reappraisal of the phylogeny of fission yeast has argued that it is more closely related to metazoans than budding yeast (Sipiczki, 1989). Fission yeast should, therefore, be a good model for studying eukaryotic cell cycle processes.

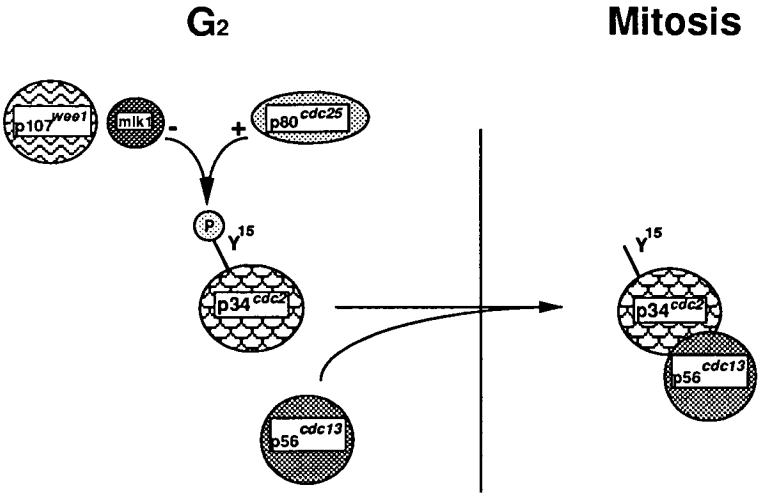


Figure 1 D **Outline model for the control of mitotic onset in fission yeast.**

Entry into mitosis, due to activation of p34^{cdc2}, occurs by a combination of dephosphorylation of tyrosine (Y) at residue 15, and complexing to p56^{cdc13}. Dephosphorylation is promoted by a combination of p56^{cdc13} and p80^{cdc25}, which together form a phosphatase. Inhibition of p34^{cdc2} activation is mediated by the action of the p107^{wee1} and mik1 protein kinases.

1 e Molecular mechanisms of cell cycle control

i Mitotic control

The fission yeast *cdc2*$^+$ gene product is extremely important in the control of mitotic onset (Review: Nurse, 1990). At the restrictive temperature *cdc2* mutants arrest at two cell cycle stages: one arrest point being at the G_2-M boundary, and the other in G_1 at START (Nurse & Bissett, 1981).

The *cdc2*$^+$ gene encodes a 34 kDa protein, p34^{cdc2}, which is a serine/threonine protein kinase (Simanis & Nurse, 1986). *cdc2*$^+$ has a pivotal role at the G_2-M boundary, as the regulation of p34^{cdc2} activity determines the precise timing of entry into mitosis (Fig. 1D).

p34^{cdc2} activation is determined by its phosphorylation state. A tyrosine residue at position 15 must be dephosphorylated for p34^{cdc2} activation, and consequent entry into mitosis (Gould & Nurse, 1989). Additionally, at this stage, p34^{cdc2} is complexed with a mitotic cyclin, encoded by the *cdc13*$^+$ gene (Hagan *et al.*, 1988; Booher & Beach, 1988).

Two classes of gene products act antagonistically to regulate the phosphorylation state of p34^{cdc2} at tyrosine 15: p80^{cdc25} dephosphorylates and so activates p34^{cdc2} ; p107^{wee1} and mik1 phosphorylate, and so inactivate p34^{cdc2} (Fig. 1D).

If *cdc25*$^+$ is over-produced cells enter mitosis prematurely, which is reflected in a smaller size at division (Russell & Nurse, 1986). Thus, *cdc25*$^+$ is a dose dependent inducer of mitosis. A number of observations suggest that *cdc25*$^+$ induces mitosis by dephosphorylation of the tyrosine residue 15 of p34^{cdc2}. *cdc25*$^+$ is dispensable if p34^{cdc2} is permanently dephosphorylated, by mutating the tyrosine to phenylalanine (Gould & Nurse, 1989). A mammalian T-cell phosphatase will compensate for loss of *cdc25*$^+$ (Gould *et al.*, 1990); and p80^{cdc25} produced in bacteria will activate p34^{cdc2} by dephosphorylating tyrosine 15 *in vitro* (Kumagai & Dunphy, 1991). Although the predicted *cdc25*$^+$ gene product is not obviously similar to phosphatases, some homologies have been noticed (Moreno & Nurse, 1991). Recently, it has been shown that a mitotic cyclin promotes the phosphatase activity of p80^{cdc25} (Galaktionov & Beach, 1991). As *cdc25*$^+$ transcript varies in amount during the cell cycle with a maximum

at the G_2-M boundary (Moreno *et al.*, 1991), it is proposed that it is a combination of a mitotic cyclin and $p80^{cdc25}$ that forms the phosphatase that activates $p34^{cdc2}$.

Inactivation of $p34^{cdc2}$ is under complex regulation. If the tyrosine residue at position 15 remains permanently phosphorylated, cells cannot enter mitosis (Gould & Nurse, 1989). Two genes encoding protein kinases are thought to be important in this regulation: *wee1*+and *mik1*+. *wee1*+ is a dose dependent inhibitor of mitosis (Russell & Nurse, 1987), and itsgene product, $p107^{wee1}$, is a protein kinase that phosphorylates serine and tyrosine residues (Featherstone & Russell, 1991). If $p34^{cdc2}$ and $p107^{wee1}$ are expressed in insect cells, $p34^{cdc2}$ is maximally phosphorylated in the presence of $p107^{wee1}$ (Parker *et al.*, 1991). This observation argues that $p107^{wee1}$ directly phosphorylates $p34^{cdc2}$. In contrast, in fission yeast in the absence of *wee1*+, $p34^{cdc2}$ is still phosphorylated. This contradiction is explained by the discovery of a *wee1*+ homologue, encoded by *mik1*+ (Lundgren *et al.*, 1991), whose function is redundant with *wee1*+. In cells carrying mutants of both *wee1* and *mik1* $p34^{cdc2}$ becomes rapidly tyrosine dephosphorylated, suggesting that either one or other of the genes is required for $p34^{cdc2}$ phosphorylation (Lundgren *et al.*, 1991).

ii Dependency of mitosis on S-phase

If cells are arrested in S-phase, by drugs such as hydroxyurea, mitosis does not occur: there is a dependency between the completion of S-phase and the initiation of the next mitosis.

In fission yeast this dependency can be obviated if $p80^{cdc25}$ is over-produced, or a mutant form of *cdc2*+, which does not require $p80^{cdc25}$ for activation, is present (Enoch & Nurse, 1989). In the presence of hydroxyurea such cells attempt premature mitosis, with consequent rapid loss of viability. Thus, $p80^{cdc25}$ is implicated in dependency of mitosis on the previous S-phase.

A corollary observation to this experiment supports this interpretation: cell cycle arrest is observed in cells which lack either *wee1*+ or *mik1*+ when exposed to hydroxyurea. If both of these genes are lost, in the presence of hydroxyurea, cells enter mitosis unregulated (Lundgren *et al.*, 1991). Such mitotic catastrophe is lethal and reflects a complete loss of dependency.

iii Dependency of S-phase on mitosis

Whereas cells have to finish S-phase before they can initiate the next mitosis, DNA synthesis can commence when only early stages of mitosis have been completed. Indeed, certain mutants which block in mid-mitosis can initiate the next round of S-phase (Uzawa *et al.*, 1990).

This dependency has been shown to involve $p34^{cdc2}$ (Broek *et al.*, 1991). If certain mutants of *cdc2+* are shifted to the restrictive temperature for a brief period, they undergo a second round of DNA synthesis without an intervening mitosis, thus forming diploid cells. This abnormal DNA duplication correlates with the disappearance of $p34^{cdc2}$. It has been proposed that $p34^{cdc2}$ carries the cell memory of cell cycle stages, and in these mutant cells such memory is lost, as $p34^{cdc2}$ is destroyed. On re-synthesis $p34^{cdc2}$ is made as default in a G_1 form, resulting in an S-phase prior to mitosis.

iv S-phase control

Budding yeast

In budding yeast a considerable amount is known about the molecular processes that mediate passage through G_1 and into S-phase. The two cell cycle control stages about which most is known are START and the DNA synthesis genes.

START is the major rate-limiting cell cycle stage during normal vegetative growth in budding yeast. *CDC28* (the budding yeast *cdc2+* homologue) is required at START, as mutants of this gene arrest at this stage (Hartwell, 1974). *CDC28* encodes a protein kinase, $p34^{CDC28}$, whose activity is crucial for cell cycle advancement (Review: Reed, 1991).

Two G_1 cyclins have been identified by multicopy suppressor analysis that interact with *CDC28*, *CLN1* and *CLN2*, as they can rescue the conditional G_1 arrest of *CDC28* (Hadwiger *et al.*, 1988). The *CLN1* and *CLN2* products are similar to each other, and are related to previously identified mitotic cyclins. Another gene isolated independently shows similarities to these two genes, and has been renamed *CLN3*. *CLN3* was first isolated as *WHI1*, in a screen for small sized mutants (Sudbery *et al.*, 1980), and second as *DAF1*, in a screen for genes conferring resistance to

mating factor-induced cell cycle arrest (Cross, 1988). The three *CLNs* are functionally redundant, as one will complement deletions of the other two; deletion all three genes, however, results in G_1 arrest (Richardson *et al.*, 1989; Cross, 1990).

CLN1 and *CLN2* transcripts levels vary during the cell cycle with a maximum during early G_1, decreasing before S-phase (Wittenberg *et al.*, 1990). The *CLN2* product, CLN2, has been shown, at least *in vitro*, to stimulate the protein kinase activity of $p34^{CDC28}$, suggesting that it may interact in cells with $p34^{CDC28}$ to activate it (Wittenberg *et al.*, 1990). Consistent with this hypothesis are the observed low levels of both *CLN1* and *CLN2* transcript, and protein kinase activity of $p34^{CDC28}$, in G_1 arrested cells (Mendenhall *et al.*, 1987). In contrast, *CLN3* transcript levels are invariant during the cell cycle (Wittenberg *et al.*, 1990). To accommodate this observation with the known redundancy of the three genes, it has been suggested CLN3 activity is modified post-translationally (Reed, 1991).

START transition only occurs when conditions, such as cell size and nutrients, and mating pheromone are permitting (Section 1b.ii). The *CLNs* are thought to mediate the integration of these conditions on START transition. A dominant mutant of *CLN2* in which the gene product is stabilized, is unable to arrest at START in response to nutrient limitation (Hadwiger *et al.*, 1988). Similarly, the *CLN3-2* allele (*DAF1*) confers α-factor resistance, allowing cells to traverse START in the presence of mating pheromone; furthermore, over-expression of *CLN* genes results in cells prematurely dividing at reduced size (Cross, 1988).

Two independent lines of research have indicated that a positive feedback loop operates in G_1, involving $p34^{CDC28}$, CLNs, Swi4, and Swi6 (Fig. 1E). CLNs bind to $p34^{CDC28}$ to activate it, and so stimulate transcription of *CLN1* and *CLN2*: thus, they can induce their own production (Dirick & Nasmyth, 1991; Cross & Tinkelenberg, 1991). *SWI4* and *SWI6* have also been shown to positively regulate *CLN1*, *CLN2*, and CLN3 (Nasmyth & Dirick, 1991), and another putative G_1 cyclin *HCS26* by binding CCB (cell cycle box) elements in their promoters (Ogas *et al.*, 1991). Thus, it has been proposed that G_1 cyclin expression is stimulated by Swi4 and Swi6, which in turn are activated by $p34^{CDC28}$ complexed with CLNs. Although both Swi4 and Swi6 are excellent candidates for direct phosphorylation by $p34^{CDC28}$, this has not yet been proven.

Such a positive feedback model could account for the START

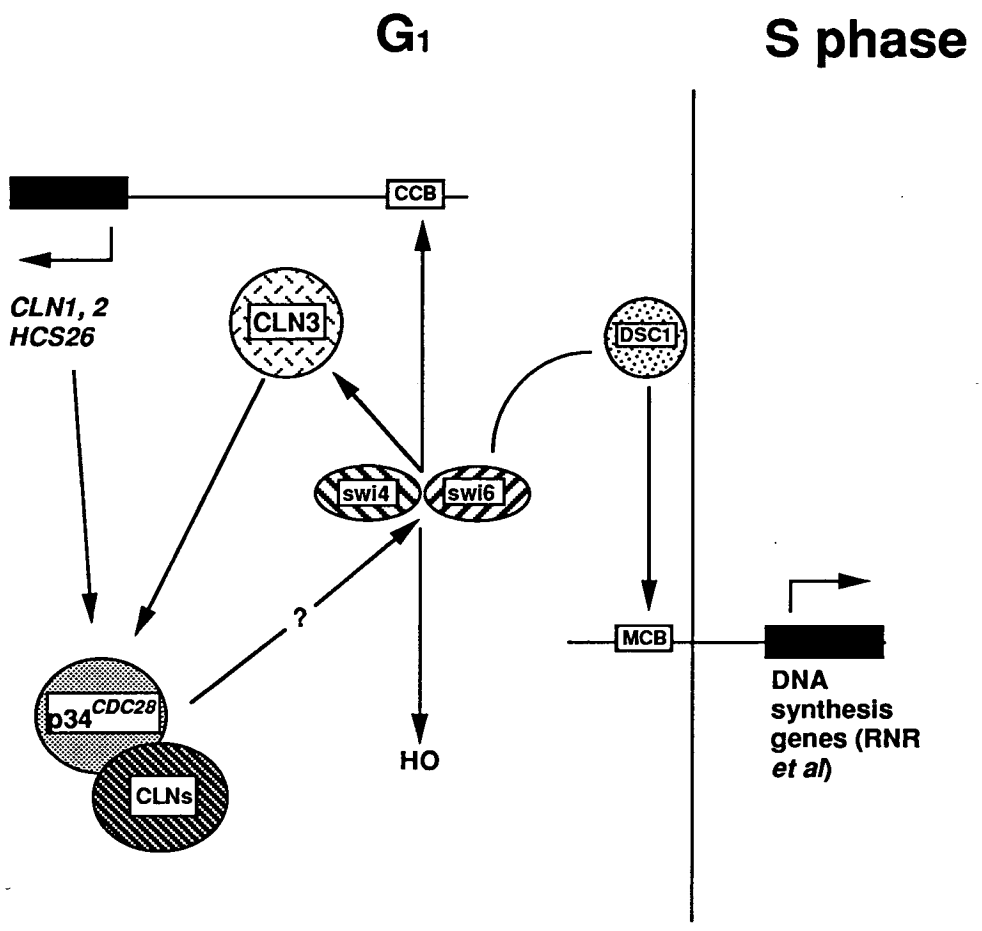


Figure 1 E **Proposed regulatory network controlling START transition and entry into S-phase in budding yeast.**

To pass START cells must have active $p34^{CDC28}$, in which form it is complexed with G_1 cyclins, CLNs. *CLN1, 2* and *HCS26* (*CLN4*) transcript are positively regulated by Swi4 and Swi6, which bind to cell cycle box (CCB) motifs in their promoter regions. Additionally, Swi4, 6 post-translationally activate CLN3. These cyclins bind to $p34^{CDC28}$, activate it, and stimulate the production of more CLNs, possibly by direct phosphorylation of Swi4, 6. Such a positive feedback loop would only have two stable states: one in which $p34^{CDC28}$ is inactive, and the other where it is maximally active. Such a transition from low to high kinase state could be stimulated by factors such as nutrients, or inhibited by pheromones, affecting any part of the network, and might account for the biochemical basis of START.

Swi6 is also a component of a transcription complex, DSC1, which binds *Mlu*I cell cycle boxes (MCBs) in the promoter regions of DNA synthesis genes, to stimulate their expression at the G_1-S boundary.

Swi4 and Swi6 activate expression of the HO endonuclease which mediates mating type switching by binding CCBs in the *HO* promoter (Section 1f.ii).

transition. With such regulation, $p34^{CDC28}$ is either inactive, or fully active. Stimulation by nutrients or cell size, or inhibition by pheromones, could affect any of the proteins in the network, and so trigger a cascade which would result in the activation of $p34^{CDC28}$, completion of START, and entry into S-phase.

In budding yeast a group of genes required for DNA synthesis have been identified whose transcripts are periodically expressed during the cell cycle, with a maximum at the G_1-S phase boundary (Table 1A, Johnston 1990).

Gene	Gene product	Reference
CDC21	Thymidylate synthase	Storms *et al.*, 1984
CDC9	DNA ligase	White *et al.*, 1986
CDC8	Thymidylate kinase	White *et al.*, 1987
POL1	DNA polymerase I	Johnston *et al.*, 1987
POL2	DNA polymerase II	Araki *et al.*, 1992
DPB2	DNA polymerase II subunit B	Araki *et al.*, 1991a
DPC2	DNA polymerase II subunit C	Araki *et al.*, 1991b
POL3 (CDC2)	DNA polymerase III	Bauer & Burgess, 1990
POL30	PCNA	Bauer & Burgess, 1990
PRI1	DNA primase I	Johnston *et al.*, 1990
PRI2	DNA primase II	Foiani *et al.*, 1989
RNR1	Large subunit of RNR	Elledge & Davis, 1990

Table 1 A **Budding yeast DNA synthesis genes that are periodically expressed at the G_1-S phase boundary.**

The DNA synthesis genes appear to be coordinately expressed, in that the pattern of abundance of their transcripts during the cell cycle is very similar (White *et al.*, 1987). Furthermore, differences in relative transcript levels cannot be distinguished in a variety of cell cycle arrest conditions (Johnston *et al.*, 1987).

A common *cis*-acting sequence in the promoter region of these genes has been discovered, and been called the MCB (for *Mlu*I cell cycle box, Verma *et al.*, 1992), since they are either identical or closely related to

the site recognized by the restriction endonuclease *Mlu*I. MCBs consist of the nucleotides ACGCGT, or a $^{5}/_{6}$ match, with the core CGCG intact; a few MCBs have an adenine at position 8 (McIntosh *et al.*, 1991; Gordon & Campbell, 1991). MCBs are strongly implicated in the periodic control of gene expression as they can confer cell cycle expression on a reporter gene at the G_1-S phase boundary (Lowndes *et al.*, 1991).

A *trans*-acting activity has been identified in budding yeast protein extracts by bandshift analysis which specifically binds MCBs, and called DSC1 (for DNA synthesis control; Lowndes *et al.*, 1991). Originally, DSC1 was reported to bind MCBs coincidentally with DNA synthesis gene expression at the G_1-S phase boundary (Lowndes *et al.*, 1991), suggesting a mechanism by which it stimulates expression of the DNA synthesis genes. Subsequently, however, DSC1 has been shown to bind MCBs invariantly in cell extracts after α-factor block and release (Dirick *et al.*, 1992). Swi6 has recently been shown to be a component of DSC1 (Lowndes *et al.*, 1992b; Dirick *et al.*, 1992).

As Swi6, in combination with Swi4, is also part of the transcription complex that controls expression of *CLN*s and the *HO* endonuclease by binding CCB elements (Ogas *et al.*, 1991; Nasmyth & Shore, 1987), binding specificity must be conferred by other parts of the complexes.

Fission yeast

The molecular processes controlling S-phase onset in fission yeast are not as well understood as those in budding yeast. Two *cdc* genes are required for START transition: *cdc2*$^{+}$ and *cdc10*$^{+}$ (Nurse & Bissett, 1981).

The potential roles of p34^{cdc2} in G_1 are at present unknown (Review: Bartlett & Nurse, 1990). This is largely due to the fact that no G_1 specific *cdc2* mutants have been isolated.

The *cdc10*$^{+}$ gene has been cloned and sequenced (Aves *et al.*, 1985), but the sequence was uninformative about its function, although similarities with cell cycle transcriptional activators, such as SWI4 and SWI6 were subsequently noticed (Breeden & Nasmyth, 1987b; Andrews & Herskowitz, 1990b). The *cdc10*$^{+}$ gene product, p87^{cdc10}, has been identified and shown to be a phosphoprotein (Simanis & Nurse, 1989). The work described in this thesis demonstrates that p87^{cdc10} is part of a transcription complex, DSP1, controlling the periodic expression of *cdc22*$^{+}$ transcript at the onset of S-phase (Lowndes *et al.*, 1992a). Two genes, which when

mutated, suppress *cdc10* mutants have been identified, *sct1* and *sdc1* (Marks *et al.*, 1992; Caligiuri & Beach, 1992). Both can suppress a deletion of *cdc10*$^+$. *sdc1*$^+$ has been cloned and sequenced, and is essential (Caligiuri & Beach, 1992). *sdc1*$^+$ encodes a predicted polypeptide with strong similarities to the cdc10 polypeptide.

Several *cdc* mutants defined two genes, *cdc22* and *cdc20*, that were required late in G_1 after START, or in early S-phase (Nasmyth & Nurse, 1981). *cdc22*$^+$ has been cloned, and an extragenic multicopy suppressor, *suc22*$^+$, also isolated (Gordon & Fantes, 1986).

cdc22$^+$ is unusual in that its transcript varies in abundance during the cell cycle with a strong maximum at the G1-S phase boundary (Gordon & Fantes, 1986). Both *cdc22*$^+$ and *suc22*$^+$ have been sequenced and shown to encode the large and small subunits of ribonucleotide reductase, respectively (This thesis, Chapter 3; M-J. Fernandez-Sarabia, P. Harris & P. Fantes, pers. comm.). *cdc22*$^+$ has a number of motifs resembling budding yeast MCBs in its promoter which are strongly implicated as *cis*-acting control elements, as an MCB trimer can confer periodic expression on a reporter gene in fission yeast (This thesis, Chapter 4). Two transcripts hybridize to *suc22*$^+$ probes: a smaller mRNA which is present constitutively; and a larger mRNA which is only observed after conditions of cell cycle arrest, or after treatment with hydroxyurea (C. Gordon, P. Harris & P. Fantes, pers. comm.). A transcript that hybridizes to *cdc22*$^+$ probes is also over-expressed after hydroxyurea treatment (P. Harris & P. Fantes, pers. comm.).

Two other fission yeast genes have been isolated that are required for DNA synthesis: DNA ligase (*cdc17*$^+$; Johnston *et al.*, 1986) and DNA polymerase I (*POL1*; Damagnez *et al.*, 1991). Both genes are constitutively expressed during the cell cycle (White *et al.*, 1987; Bouvier *et al.*, 1992), and so are unlikely to be under DSP1/MCB controls.

A putative fission yeast G_1 specific cyclin has been cloned and sequenced, and named *puc1*$^+$ (Forsburg & Nurse, 1991). *puc1*$^+$ shows similarities to previously characterised mitotic cyclins, and when over-expressed causes a delay in mitosis. However, *puc1*$^+$ has not yet conclusively been shown to have a role in G_1.

Higher eukaryotes

A limited amount is known about G_1 controls and the onset of S-phase in higher eukaryotes. A number of genes required for DNA synthesis are periodically expressed during the cell cycle cycle, with peak levels of transcript during early S-phase. These include such examples as thymidylate synthase (Pelka-Fleischer *et al.*, 1989), proliferating-cell nuclear antigen (PCNA; Morris & Matthews, 1989) and both subunits of ribonucleotide reductase (Engstrom *et al.*, 1985; Bjorklund *et al.*, 1990). For each of the three genes studied it has been suggested that their expression may control the onset of S-phase. Indeed, in the case of PCNA, injection of antisense oligodeoxynucleotide does inhibit DNA synthesis (Liu *et al.*, 1989), which is consistent with such a proposal. However, there is a large pool of PCNA protein present throughout the cell cycle, only a small proportion of which associates with the nucleus during S-phase (Morris & Matthews, 1989), suggesting that PCNA is likely to exert only a coarse control on DNA synthesis.

Dihydrofolate reductase (DHFR) is also periodically expressed during the cell cycle, with a transcript peak in late G_1 (Farham & Schimke, 1985). DHFR has a sequence in its promoter, TTTCGCGC, which is functionally important (Blake & Azizkan, 1989), and shows some similarity to the MCB sequence (5 out of 6 nucleotides). This sequence is bound by the transcription factor E2F which is active during G_1 (Muridj *et al.*, 1990). E2F may, therefore, be nearest metazoan relative to DSC1/DSP1.

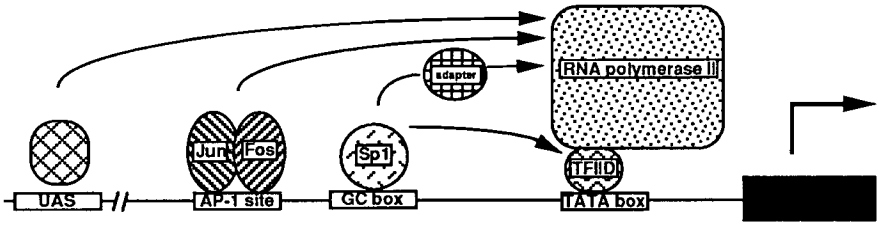


Figure 1 F Schematic diagram of eukaryotic promoter structure.

Eukaryotic transcriptional activators can be split into two classes: general and specific. The former class contains TFIID which binds the TATA box, and directs assembly of the general transcription complex (including RNA polymerase II) onto the DNA. The latter class contains proximal factors such as Sp1, and Jun and Fos which bind the GC box and AP1 site, respectively; and distal enhancer-like upstream activating sequences (UASs) which bind *trans*-acting complexes. It is a combination of the general and specific transcription activators that control expression of yeast genes.

1 f Eukaryotic transcriptional regulation

i Promoter structure and function

Studies of transcriptional promoters in metazoan cells and yeasts have revealed that genes transcribed by DNA polymerase II are controlled by a variety of *cis*-acting sequence elements 5' the transcriptional initiation site which bind *trans*-acting factors (Fig. 1F). The molecules and underlying mechanisms controlling transcription are remarkably conserved in all eukaryotes (Review: Guarente & Bermingham-McDonogh, 1992).

The *cis*-acting elements and *trans*-factors can be split into two types: *cis*-elements that interact with the general transcription apparatus, and so are common to all genes; and *cis*-elements that bind specific 5' regulatory factors to stimulate transcription of particular genes at appropriate times.

The TATA box element binds the general transcription factor TFIID and directs assembly of the general transcription complex, containing RNA polymerase II and associated factors, onto the promoter DNA (Sawadogo & Roeder, 1985). Alone, the general transcription complex is capable of inducing low level transcription. TFIID is highly conserved: budding yeast TFIID can functionally substitute *in vitro* for TFIID from HeLa cells (Buratowski *et al.*, 1989); and a functional homologue of budding yeast TFIID has been cloned from fission yeast (Fikes *et al.*, 1991). The core region of budding yeast TFIID carries out all the essential functions, such as binding to the TATA box, and interaction with other general factors (Reddy & Hahn, 1991). It is thought that the peripheral regions of TFIID interact with regulatory factors.

The efficiency and specificity of transcription is controlled by further sequence specific elements 5' to the TATA box, which bind regulatory factors. Such sequences can be near to the TATA box, and include examples such as the mammalian GC box which binds Sp1 (Pugh & Tjian, 1990) and the AP-1 site, present in mammalian cells (Bohmann *et al.*, 1987) and fission yeast (Jones *et al.*, 1988), which binds Fos and Jun transcription factors. Additionally, transcription can be affected by *cis*-acting elements that are far away from the transcriptional START site which bind

transcription complexes. In yeast, such *cis*-acting elements are called upstream activating sequences (UAS) and are related to metazoan enhancer elements (Struhl, 1982).

Upstream regulatory factors generally have two two domains: one that binds sequence specific DNA, and another which influences transcriptional activity by binding with the general transcriptional apparatus (Brent & Ptashne, 1985). In some instances it has been suggested that coactivators, in addition to the regulatory and general factors, are required to potentiate transcription.

For Sp1, the need for coactivators has been inferred from *in vitro* transcription studies. Replacement of the TFIID fraction by pure cloned TFIID restores basal transcription, but not the ability to respond to Sp1 (Pugh & Tjian, 1990). Thus, a coactivator is thought to be present in the TFIID fraction. Heat treatment of the TFIID fraction selectively inactivates TFIID, but leaves the coactivator intact, as cloned TFIID can be added back to restore both basal transcription and the ability to respond to Sp1 (Peterson *et al.*, 1990). It is thought that the amino terminus of TFIID interacts with the coactivator: if truncated TFIID is added back, only basal transcription is restored.

There is evidence for the presence of coactivators in yeast. Herpes virus regulatory factor VP16 which has lost its DNA binding domain, when added to an *in vitro* transcription system made from budding yeast extracts, inhibited activated but not basal transcription (Kelleher *et al.*, 1990). This result was interpreted as meaning that VP16 binds a coactivator and not the general transcription complex, to selectively reduce activated transcription. Recently, the potential coactivator that mediates VP16 activation has been cloned and called *ADA2* (Berger *et al.*, 1992).

Two models have been proposed to account for the role of the coactivators (Guarente & Bermingham-McDonogh, 1992). The coactivator may bind the regulatory factor to provide additional activation surface to the general transcription complex. Alternatively, the coactivator could be an adaptor that bridges the gap between the regulatory and general transcription complexes (Fig. 1F).

ii Yeast cell cycle transcriptional control

A number of yeast genes are expressed in a cell cycle manner, and are controlled at the level of transcription. The mechanisms that control periodic expression of DNA synthesis genes and *CLN*s in budding yeast have already been discussed (Section 1e.ii). Three other examples of cell cycle control of gene expression will be discussed in this section: histone genes, *HO* endonuclesase, and the chitinase gene.

Histones are present in both budding and fission yeast. The four core histones, H2A, H2B, H3, and H4 have all been identified; H1 has not been yet been found in either organism (Hereford *et al.*, 1979; Matsumoto & Yanagida, 1985).

In fission yeast nine histone genes have been identified: two genes for histone H2A, one for H2B, and three each for H3 and H4 (Matsumoto & Yanagida, 1985). Both histone H2A1 and H2B are periodically expressed during the cell cycle with a maxima during S-phase, later than the peak of *cdc22*$^+$ transcript (Matsumoto *et al.*, 1987). A common upstream sequence has been noted in the promoters of all the histone genes (the AACCCT box) that has internal nucleotide repeats. It is thought that this repeat is a *cis*-acting element that controls their coordinate regulation.

In budding yeast there are eight histone genes, with two genes for each of the four core histones, arranged as two pairs of H2A-H2B and H3-H4 (Hereford *et al.*, 1979). All eight of the genes are expressed during S-phase. Using fusions of one of the H2A genes to *lacZ*, a fragment of DNA required for periodic expression was identified, consisting of three copies of GCGAAAANTNNGAAC (Osley *et al.*, 1986). In addition there is a negative control element situated between two of the repeats, deletion of which results in dramatic over-production of the transcript and an impairment but not abolition of cell cycle regulation (Osley *et al.*, 1986; Lycan *et al.*, 1987). Three genes (*HIR1-3*) involved in the negative regulation of H2A/H2B have been isolated by mutation studies, by means of the negative control element in the promoter (Osley & Lycan, 1987).

Mating type switching in budding yeast is determined by the *HO* gene which encodes an endonuclease. *HO* transcription is confined to a short period during late G_1 (Nasmyth, 1983), and is dependent on cells passing START. In addition to cell cycle control, *HO* is also regulated by

the mating type locus (Jensen *et al.*, 1983) and is only expressed in mother cells (Review: Nasmyth & Shore, 1987). The *HO* promoter contains at least three different types of control element, one of which, the $CACGA_4$ repeat, is sufficient to give START dependent transcription when joined to the cytochrome *c* TATA box (Breeden & Nasmyth, 1987 b). The repeat (recently renamed as the cell cycle box CCB; Andrews, 1992) is present 10 times in the *HO* promoter: removal of them causes the promoter to become START independent during G_1 (Nasmyth, 1985).

At least six different genes, *SWI1-6*, are required for efficient *HO* expression (Haber & Garvik, 1977). Of these only *SWI4* and *SWI6* are required for expression specifically mediated by CCBs (Andrews & Herskowitz, 1989a). Both Swi4 and Swi6 have been shown to be part of a transcription complex that specifically binds to CCBs (Andrews & Herskowitz, 1989a). Two forms of Swi4/Swi6-DNA complexes have been identified by bandshift assays, called L and U, which are distinguished by their electrophoretic mobility (Taba *et al.*, 1991). L complexes are detectable at all cell cycle stages, whereas U complexes are only detected in cells that have just undergone START. It has been suggested that formation of the U form of the complex may trigger *HO* activation.

HO activation is inessential for cell division. As *SWI4* and *SWI6* are required for *HO* activation, it is surprising that deletion of both genes is fatal to the cell. This is explained by the discovery that *SWI4* and *SWI6* have other essential functions in cell cycle regulation, including the transcriptional control of *CLNs* and DNA synthesis genes (Section 1e.iv).

The gene encoding chitinase in budding yeast (*CTS1*) is expressed in late G_1 at the same time as *HO* (Kuranda & Robbins, 1991), and is thought to be required for separating the daughter cell from the mother cell, by dissolving the 'neck'. *SWI4* and *SWI6* have been shown recently to be required for this periodic expression (Dorhmann *et al.*, 1992). Surprisingly, CCBs have not been found in the promoter region of *CTS1*, suggesting that the Swi4/Swi6 complex must bind other *cis*-acting elements to stimulate expression of *CTS1*. Why two genes that are coordinately expressed by the same *trans*-factor should have different *cis*-acting elements is as yet unclear.

There are similarities between the transcriptional mechanisms that control periodic expression of yeast cell cycle genes. In each case, *cis*-acting elements 5' to the transcriptional start of the gene influence expression

levels by binding *trans*-acting factors. The *cis*-acting elements are often present as repeats, and are common to groups of genes that are coordinately expressed. In none of the genes studied is it understood how the *trans*-acting factor stimulates transcription at the appropriate time during the cell cycle.

1 g Universal cell cycle controls?

The exciting prospect of all cell cycle research is that many of the molecular mechanisms so far elucidated will be found to be universally conserved. The most well known example of this is $p34^{cdc2}$. Human and yeast *cdc2*$^+$ are functionally interchangeable (Lee & Nurse, 1987). A number of the other cell cycle genes initially isolated in yeasts are also being found to have homologues in higher eukaryotes, such as *cdc25*$^+$ (Sadhu *et al.*, 1990) and *wee1*$^+$ (Igarashi *et al.*, 1991). Indeed, the more we know about the cell cycle, the more universal it seems the controlling mechanisms are.

One of the great beauties of cell cycle research is the simplicity of both the core observations and models proposed to explain cell cycle events - for examples see Figures 1D and 1E. In yeasts the molecular mechanisms are simple, but in higher eukaryotes, although the underlying mechanisms may be the same, the systems have become complicated and confusing. For example, in fission yeast there is one *cdc2*$^+$ gene and one mitotic cyclin (*cdc13*$^+$), whereas in humans there are at least three genes of each (Lew *et al.*, 1991; Yue *et al.*, 1991; Meyerson *et al.*, 1992). What do these extra genes do? Are they merely redundant genetic copies? Because of the difficulty of genetics in higher eukaryotes, the most productive organisms in cell cycle research will continue to be yeasts.

1 g Aims of the work

The work described in this thesis uses fission yeast as a model system to understand the molecular mechanisms that control the onset of S-phase. This is done by studying *cdc22+*, a gene which is periodically expressed during the cell cycle at the G_1-S phase boundary. *cdc22+* is sequenced and shown to encode the large subunit of ribonucleotide reductase. The mechanisms controlling *cdc22+* expression during the cell cycle are then examined: both *cis-* and *trans-* acting components required for *cdc22+* expression are identified. The *cis*-acting elements are MCBs, and the *trans*-factor a protein complex we have called DSP1. Both MCBs and DSP1 are related to components previously isolated in budding yeast. A part of DSP1 is shown to the gene product of the *cdc10+* 'START' gene. Finally, a mutant form of *cdc10*, *cdc10-C4*, is shown to cause constitutive over-expression of *cdc22+*.

In essence, this work has demonstrated that the product of a gene required for START is part of a transcription complex which controls the expression of a gene during S-phase.

Chapter 2: Materials & Methods

2 a Commonly used reagents and buffers

Most of the methods used in this work were based on those described in Sambrook *et al.* (1989).

All chemicals used were of analytical grade, and were bought from Sigma, British Drug Houses (BDH), Gibco-BRL, Fisons, or Pharmacia.

H_2O refers to distilled autoclaved water.

Eppendorf tube refers to 1.5 ml centrifuge tube. High speed centrifugation in Eppendorf tubes refers to a Microcentaur centrifuge at 13, 000 rpm.

Tris. Cl

Tris base (tris[hydroxymethyl]aminomethane, Sigma T-1503) was dissolved in H_2O, and the pH adjusted to the required value by addition of HCl. H_2O was added to give a 1 M stock solution.

EDTA

A stock solution of 0.5 M EDTA (ethylenediaminetetramino acid, di-sodium salt, Fisons D-0452) was made by dissolving solid EDTA in H_2O, adjusting the pH to 8.0 with NaOH, and adding water to the required volume.

TE

A buffer solution, suitable as a routine DNA solvent, consisting of 10 mM Tris. Cl (pH 7.5) and 1 mM EDTA.

Phenol

Phenol (Fisons P-2360) was pre-equilibriated with 1 M Tris. Cl (pH 7.5), followed by TE buffer. To retard oxidation of the phenol solution 0.1% (v/v) hydroxyquinoline (Sigma H-5876) and 0.2% (v/v) β–mercaptoethanol were added, and it was stored at 4°C in the dark. For work with RNA, phenol pre-equilibriated with H_2O (Rathburn 3024), was used.

Chloroform

Chloroform (BDH 10077) refers to a 24:1 (v/v) mixture of chloroform and isoamyl alcohol (Sigma I-1381).

Sodium acetate

Sodium acetate was dissolved in H_2O, the pH adjusted to 5.2 with HOAc, and H_2O added to a final concentration of 3 M.

Ethidium bromide

Ethidium bromide (Sigma E-875) was dissolved as a stock solution of 10 mg ml^{-1} in H_2O, and stored at 4°C in the dark.

Loading buffer

5 x loading buffer for gel electrophoresis of nucleic acids was prepared and stored at room temperature:

0.25% (w/v) bromophenol Blue (BDH 20015)
15% (w/v) ficoll (Sigma F-2637) in H_2O.

TBE

TBE was routinely made up as a 10 x stock solution, and stored at room temperature:

	stock	1 l
0.89 M Tris base		108 g
0.89 M boric acid		55 g
20 mM EDTA	0.5 M	40 ml.

TAE

TAE was made as a stock solution of 50 x, and stored at room temperature:

	stock	1 l
2 M Tris base		242 g
HOAc		57.1 ml
50 mM EDTA	0.5 M	100 ml

DEPC H_2O

RNAase free water, suitable for dissolving RNA, was made by adding 0.1% (v/v) diethyl pyrocarbonate (Sigma D-5758) to H_2O, mixing for 5 minutes at room temperature, and autoclaving.

2b Nucleic acid manipulations

i Dissolving and storage

All DNA and RNA was dissolved in a solution of either TE or H_2O, depending on the subsequent use of the nucleic acid. Nucleic acids were routinely stored as solutions at -20°C.

ii Extraction with phenol/chloroform

Proteins were removed from solutions containing DNA and RNA, by extraction with an equal volume of a 1:1 mixture of phenol and chloroform. Traces of phenol were removed by a further extraction with chloroform.

Extractions were carried out by adding a volume of phenol/chloroform equal to that of the nucleic acid solution. The two solutions were mixed thoroughly by vortexing to form an emulsion, and separated by centrifugation, routinely 5 minutes high speed, at room temperature. The aqueous phase was then transferred to a fresh tube carefully avoiding protein at the interface of the two phases, and the nucleic acids recovered by precipitation.

iii Precipitation of nucleic acids

DNA was precipitated by the addition of a $^1/_{10}$ volume of 3 M sodium acetate (pH 5.2), followed by 2 volumes of -20°C absolute ethanol. RNA was precipitated by the addition of $^1/_{10}$ volume 3 M sodium acetate, followed by 2.5 volumes of -20°C absolute ethanol. In each case the solution was mixed thoroughly by vortexing, frozen at -20°C for 30 minutes, and the nucleic acids pelleted by centrifugation, at high speed at 4°C. Very small fragments of DNA (<200 nucleotides) were precipitated as above, with the addition of 1 µl of carrier (20 mg ml^{-1}, Boehringer Mannheim 12463420), before freezing at -20°C.

To remove trace amounts of salt remaining from the precipitation, the nucleic acid pellet was washed in 100 µl of either 70% (v/v) -20°C ethanol (for RNA, or large fragments of DNA >500 nucleotides), or 90% (v/v) -20°C ethanol (for small fragments of DNA <500 nucleotides), and

pelleted by centrifugation. The nucleic acid pellet was air dried, and dissolved in an appropriate volume of H_2O or TE buffer.

iv Quantification and quality control

DNA and RNA quality and amount was assayed by using two methods: spectrophotometry, and ethidium bromide staining after gel electrophoresis.

The first method involves taking an absorbance reading at wavelengths 260 and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An A_{260} of 1 corresponds to approximately 50 $\mu g\ ml^{-1}$ for double stranded DNA, 40 $\mu g\ ml^{-1}$ for single stranded DNA and RNA, and 20 $\mu g\ ml^{-1}$ for single stranded oligonucleotides. The ratio between the readings at 260 nm and 280 nm provides an estimate of purity of the nucleic acid. Pure preparations of DNA and RNA have ratios of 1.8 and 2.0, respectively. Any ratios less than these values indicate protein and/or phenol contamination of the sample.

The second method utilises ethidium bromide, which specifically and proportionately binds to DNA, and fluoresces under UV light. The nucleic acid sample is run in an appropriate gel containing ethidium bromide at a concentration of 0.5 $\mu g\ ml^{-1}$, in parallel with a nucleic acid sample of known amount (usually standard molecular weight markers), and visualised on a trans-illuminator. A rough estimate of DNA amount can be gauged from the relative staining intensities of the bands.

v Plasmid vectors

pUC-based plasmids

The pUC based plasmids are used for the manipulation of foreign genes in *E. coli* (Vieira & Messing, 1982). They contain the pBR322 derived ampicillin resistance gene and origin of DNA replication, and a polylinker inserted into a portion of the *lacZ* gene of *E. coli*. Non-recombinant plasmids are able to synthesize this enzyme which breaks down X-Gal (Section 2f.ii), to release a blue pigmented derivative. In recombinant plasmids the *lacZ* gene is interrupted by foreign DNA, resulting in colourless colonies. The vectors pTZ18/19 (Pharmacia) are similar to the

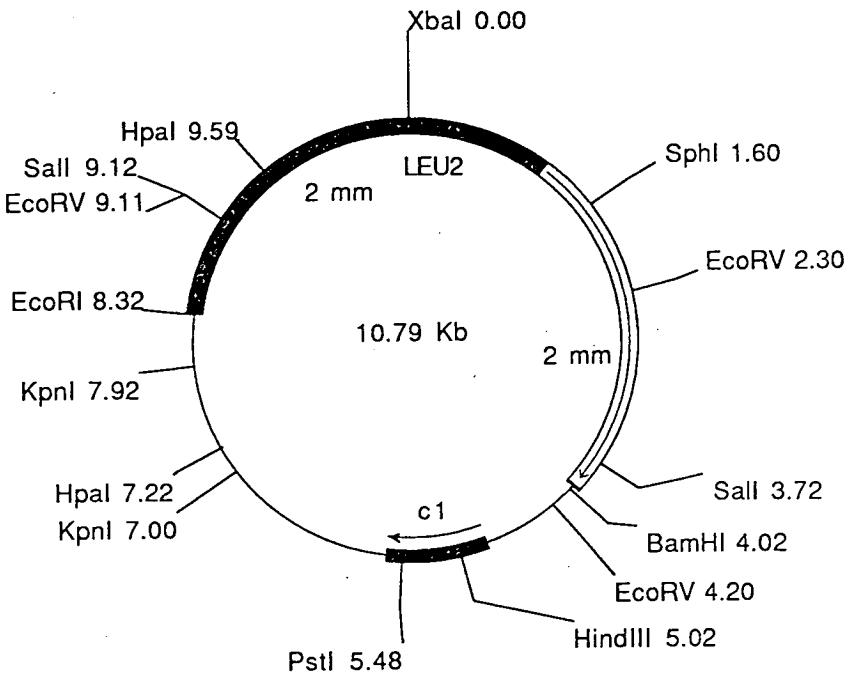


Figure 2 A pDB262

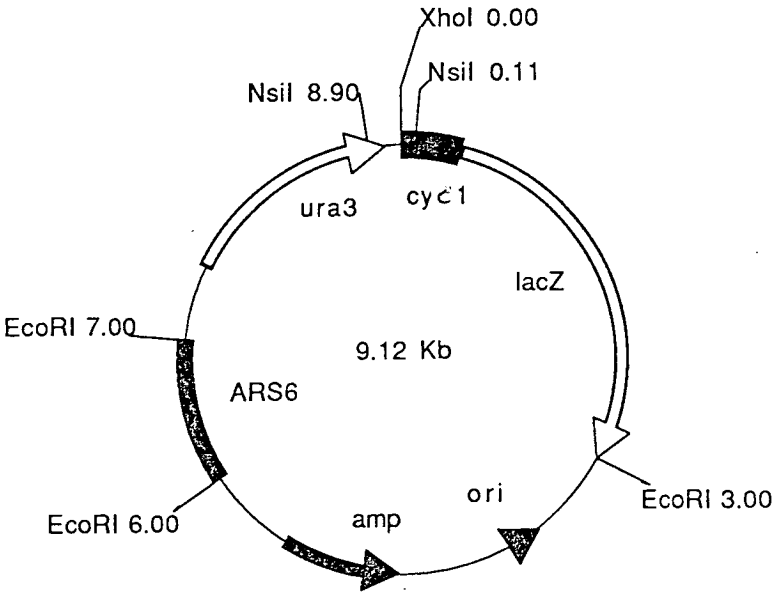


Figure 2 B pSPΔ178

pUC plasmids, but contain in addition the f1 origin of replication. Consequently, if the host cell is super-infected with the helper phage M13K07, replication will be initiated at the f1 origin, resulting in the excretion of phage coats containing single stranded DNA of the plasmid (Section 2g.ii). pBluescript SK/KS (Stratagene) plasmids have an alternative more extensive polylinker to the pTZ plasmids, and the ampicillin resistance gene has been replaced by the chloramphenicol resistance gene, for alternative selection.

pDB262

pDB262 (Fig. 2A) is capable of autonomous replication in both *E. coli* and *S. pombe* (Wright *et al.*, 1986). Cloning fragments into either the *Hin*dIII or *Bcl*I sites inactivates the λ*cI* suppressor gene product, which allows expression of the tetracycline resistance gene, under the control of the bacteriophage λ*Pr* promoter. This plasmid is especially useful for gene library construction, since selection for tetracycline resistance ensures that all *E. coli* transformants contain recombinant plasmids. The presence of the *leu2* gene permits selection for the plasmid in fission yeast.

pSPΔ178

pSPΔ178 (Fig. 2B) was developed to test *cis*-acting elements in fission yeast (Lowndes *et al.*, 1992a). pSPΔ178 is a derivative of pLGΔ178 (Guarente & Mason, 1983), in which the 2μ origin of replication is replaced by *ARS6*, a fission yeast chromosomal origin of replication (Maudrell *et al.*, 1988). pSPΔ178 contains a minimal promoter from a budding yeast cytochrome *c* gene, fused to the *lacZ* gene of *E. coli*. Selection is permitted in fission yeast *ura4* mutants by the presence of the budding yeast *URA3* gene. A unique cloning site (*Xho*I) allows the insertion of putative *cis*-acting elements directly 5' to the minimal promoter, and their activity can be assayed by monitoring *lacZ* gene expression.

2 c Molecular analysis of nucleic acids

i Restriction enzyme digestions

Restriction endonucleases were used, as recommended by the manufacturers, to cut DNA. For restriction enzyme buffers, the Gibco-BRL REact buffer system was used. Usually, 1-5 µg of DNA was incubated with 10 units of restriction enzyme and appropriate buffer, in a final volume of 10 µl, at 37°C for 1-15 hours. Digestions were stopped by the addition of $^1/_{25}$ volume of 0.5 M EDTA, and freezing at -20°C.

ii Ligations

Ligation of DNA was performed using T_4 DNA ligase (Boehringer Mannheim, 862509) and ligation buffer for cohesive termini, and concentrated T_4 ligase (New England Biloab 202CS) and ligation buffer for blunt termini. Routinely between 1-10 µg of DNA was ligated with 0.5 µl of ligase (1 or 100 units, respectively), in a volume of 10 µl. Ligation reactions were incubated for 16 hours at 14°C for cohesive termini, and at 25°C for blunt ended termini.

Cohesive termini were converted to blunt ended termini by treatment with the Klenow fragment of DNA polymerase I. DNA was incubated at 37°C in the presence of 1 unit of Klenow fragment (Boehringer Mannheim, 100840), ligase buffer (New England Biolab), 1mM DTT, and 2 mM dNTPs for 30 minutes, before the addition of 0.5 µl of concentrated T4 ligase, and incubation at 25°C for 16 hours.

iii Agarose gel electrophoresis

For the analysis of both cut and ligated DNA preparations of DNA fragments larger than 300 nucleotides, 0.6% (w/v) agarose gels in the horizontal Bio-Rad gel system were used.

Agarose (Type 2, medium EEO, Sigma A-6877) was dissolved in 1 x TBE or TAE buffer by brief boiling. DNA samples were mixed with a $^1/_5$ volume of loading buffer, and loaded into gel wells. Routinely 0.2 µg of molecular weight markers (1 kb ladder, Gibco-BRL 5615SA) was loaded to assess the size and amount of DNA fragments. Gels were run with an

applied voltage of 4-8 V cm^{-1} for 1-3 hours. DNA was stained with ethidium bromide at a final concentration of 0.5 µg ml^{-1}, visualized with a UV trans-illuminator, and photographs taken with a Mitsubishi Video Copy Processor Model P65B.

iv Acrylamide gel electrophoresis

For the analysis of both cut and ligated DNA preparations of DNA fragments less than 300 nucleotides in size, 6% acrylamide gels in the vertical Bio-Rad Gel system were used.

1. Acrylamide: bisacrylamide (Gibco-BRL, Ultrapure) was dissolved at a ratio of 125 g : 3 g (33: 1) in 206 ml of H_2O, and stored as a stock solution.
2. 5 ml of acrylamide stock was added to 50 ml of 1 x TBE buffer and mixed.
3. 200 µl of 10% (w/v) ammonium persulphate (Sigma A-9164) and 25 µl of TEMED (N,N,N',N'-Tetramethylethylenediamine, Bio-Rad 61-0800) were added, and the solution poured immediately.
4. DNA samples were mixed with a $^1/_5$ volume of loading buffer and loaded onto the gel: gels were run at 10 V cm^{-1} for 2-3 hours.
5. DNA was visualised and photographed as for agarose gels (Section 2c.iii).

v Purification of large DNA fragments

To purify fragments of DNA over 500 nucleotides in length from agarose gels the GeneClean kit was used (Volgelstein & Gillespie, 1979), according to the manufacturer's instructions.

vi Purification of small DNA fragments

This method permits the purification of small DNA fragments, less than 500 nucleotides in length (Sambrook *et al.*, 1989).

1. DNA was separated on a 6% acrylamide gel (Section 2c.iv), stained with ethidium bromide, and visualised under short-wave UV (to

minimise damage to DNA).

2. The desired fragment was excised with a scalpel.
3. The tip of a 500 µl Gilson tip was sealed with a Bunsen flame and a siliconized glass wool[1] plug inserted.
4. Acrylamide pieces were transferred to the Gilson tip, and mashed with a tooth pick.
5. Acrylamide mash was suspended in 200 µl of elution buffer[2], and the top of the Gilson tip sealed with NescoFilm: this was incubated overnight at 37°C.
6. The end of the Gilson tip was removed with a scalpel, and the solution allowed to elute into an Eppendorf tube: 200 µl more of elution buffer was added and this allowed to elute.
7. 2 volumes of ethanol and 1 µl of carrier glycogen was added to precipitate the DNA, the solution spun at high speed for 30 minutes, and the pellet was washed with 90% ethanol (Section 2b.iii).

1 Siliconized Glass Wool:

Glass wool (Sigma G-8389) was soaked in a 5% solution of trimethylchlorosilane (BDH 33164) and this allowed to evaporate (in a fume hood). The wool was then washed thoroughly in H_2O and baked dry at 180°C for 2 hours.

2 Elution Buffer:

	stock	100 ml
0.2 M NaOAc		2.72 g
1% SDS	20% (w/v)	5 ml
10 mM $MgCl_2$	1 M	1 ml.

vii Gel electrophoresis of RNA

RNA (Section 2e.vi) was separated using two methods: formaldehyde gels (Golberg, 1980) and glyoxal gels (Sambrook *et al.*, 1989). Gloves were worn during all procedures with RNA, to prevent RNase contamination.

Formaldehyde gels

1. 1.0 g agarose (Type 2, medium EEO, Sigma A-6877) was dissolved in 63 ml H_2O and 16 ml 5 x MNE[1] buffer by boiling: this was cooled to 60°C, 17 ml of 38% (w/v) solution of formaldehyde (BDH-28121) added, and this poured immediately into a horizontal gel mold.
2. Routinely, 10 µg of fission yeast RNA (Section 2e.vi) was run per lane: to each sample 15 µl of RNA buffer[2] and 1 µl of 0.5 mg ml^{-1} ethidium bromide was added, this incubated at 60°C for 5 minutes, and loaded onto the gel.
3. Gels were run in 1 x MNE buffer with an applied voltage of 10 V cm^{-1} for 3 hours.

1 5 x MNE buffer:

	500 ml
0.12 M MOPS (Sigma M-1254)	13.08 g
25 mM NaOAc	1.03 g
5 mM EDTA	0.19 g.

Adjusted to pH 7 with NaOH, and stored at 4°C in the dark.

2 RNA buffer:

formaldehyde	600 µl
formamide (BDH 28241)	200 µl
MNE Buffer	240 µl
H_2O	160 µl.

Glyoxal gels

1. A 1.0% (w/v) agarose gel (as above) was prepared in 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.5)[1].
2. 10 µg of fission yeast RNA (Section 2e.iv) was routinely run per lane: each sample was incubated in 10 µl glyoxal buffer[2], at 50°C for 15 minutes and loaded onto the gel.
3. The gels were run in 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.5), re-circularizing the buffer to maintain a pH less than 8.0.

1 10 mM Na_2HPO_4/NaH_2PO_4 (pH 6.5) was made by adding 3.9 ml of 1 M NaH_2PO_4 and 6.1 ml of 1 M Na_2HPO_4, in 90 ml of H_20.

2 Glyoxal buffer:

6.6 M glyoxal	2.5 ml
50% (v/v) DMSO	8.0 ml
15 mM Na_2HPO_4/NaH_2PO_4 (pH 6.5)	1. 6 ml.

De-ionized glyoxal (Sigma G3140, ready prepared at 6.6 M) was made by mixing 50 ml of glyoxal with 5 g of AG 501-X8 (D) mixed bead resin (Bio-Rad 142-6425), stirred at room temperature for 30 minutes and filtered to remove resin. This was stored in aliquots at -70°C.

viii Filter hybridization of RNA

Northern blotting

Northern blotting was performed using the capillary method onto GeneScreen membrane (New England Biolabs), following the manufacturer's instructions.

1. GeneScreen membrane was cut to slightly larger than gel size, and soaked in 0.025 M Na_2HPO_4/NaH_2PO_4 (pH 6.5) (made as above) for 20 minutes; the RNA gel was soaked in this buffer for the same time.
2. 10 pieces of filter paper (Whatman 3MM) were wetted with 0.025 M Na_2HPO_4/NaH_2PO_4 (pH 6.5), and placed on an elevated glass plate so that the ends formed a wick (Fig. 2C).

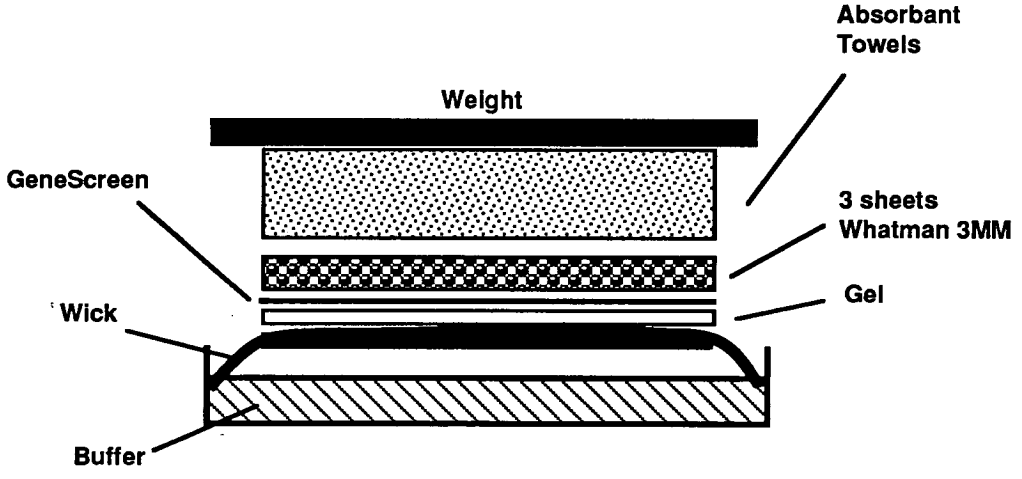


Figure 2 C Construction of a Northern blot.
Details of construction are described in the text.

3. The gel was placed on filter paper, avoiding air bubbles, and gel spacers placed along each side of gel.
4. GeneScreen membrane was placed on the gel ensuring there were no air bubbles.
5. 5 pieces of Whatman paper cut to the same size as the gel were placed on top of membrane.
6. 20 absorbent hand towels were placed on the filter paper, and a weight placed on top.
7. RNA was transferred for at least 12 hours.
8. Towels and Whatman paper were discarded.
9. Membrane was washed in 0.025 M Na_2HPO_4/NaH_2PO_4 (pH 6.5) buffer for 20 minutes with gentle agitation.
10. The membrane was dried on filter paper at room temperature, and then baked at 100°C for 3 hours: membranes were stored at this stage.

Hybridization

1. Membranes were pre-hybridized in hybridization buffer[1] for 12 hours at 42°C in a Techne hybridization oven.
2. Radio-labelled probe (Section 2d) was boiled for 5 minutes, and chilled on ice for 10 minutes: this was added to hybridization buffer and hybridized for 16 hours at 42°C.
3. Hybridization buffer was discarded, and the membrane washed in the Techne hybridization oven, as follows:
   twice with 100 ml of 2 x SSC[2] at room temperature for 5 minutes;
   twice with 100 ml of 2 x SSC plus 1.0% SDS at 65°C for 30 minutes;
   twice with 100 ml of 0.1 x SSC at room temperature for 30 minutes.
4. The membrane was never allowed to become dry at this stage, as the probe became covalently bound to the RNA, preventing further hybridizations.
5. The membrane was processed for autoradiography (Section 2c.ix).

1 Hybridization buffer:

10 ml de-ionized formamide, 4 ml P buffer, and 4 ml 50% (w/v) dextran sulphate were added together, mixed, and incubated at 42°C for 10 minutes. 1.16 g NaCl was added, mixed, and incubated at 42°C for a further 10 minutes until it had dissolved. 200 µl of denatured salmon sperm DNA was added, and thoroughly mixed.

P Buffer:

1.0% (w/v) bovine serum albumin (globulin free fraction, Sigma-A7638), 1.0% (w/v) polyvinyl-pyrrolidone (Sigma P-5288), 1.0% (w/v) ficoll (Sigma F-2637), 250 mM Tris. Cl (pH 7.5), 0.5% (w/v) tetra-sodium pyrophosphate (BDH 10261), and 5% (w/v) SDS.

50% (w/v) dextran sulphate (sodium salt, Pharmacia 17-0340-01) solution.

De-ionized formamide prepared by mixing 100 ml of formamide (BDH 28241) with 5 g of AG 501-X8 (D) mixed bead resin, stirred at room temperature for 30 minutes, and filtered to remove resin. This was stored in aliquots, at -20°C.

Salmon sperm DNA (Sigma D-1626):

this was dissolved at a concentration of 10 mg ml^{-1} in H_2O, and sonicated until the solution was liquid. Prior to use, DNA was denatured by boiling for 10 minutes, and chilling on ice for a further 10 minutes.

2 SSC was made as a 20 x stock, as follows, and stored at room temperature:

	5 l
3 M NaCl	876.6 g
0.3 M sodium citrate	441.15 g.

Stripping

Blots were stripped of radio-labelled probe, permitting further hybridizations with other probes.

1. Membranes were washed in 250 ml of strip solution[1] for 2 hours at 70°C in Techne hybridization ovens.
2. Membranes were air dried on Whatman paper (3MM) and stored.

1 Strip solution:

	stock	1 l
5 mM Tris. Cl (pH 8)	1 M	5 ml
0.2 mM EDTA	0.5 M	200 µl
0.05% (w/v) Na pyrophosphate		50 mg
0.02% (w/v) polyvinyl-pyrrolidone		20 mg
0.02% (w/v) bovine serum albumin		20 mg
0.02% (w/v) ficoll		20 mg.

ix Autoradiography

GeneScreen membranes sealed in SaranWrap and vacuum dried gels, were placed in contact with Fuji Blue X-ray film in cassettes. For the detection of ^{32}P, cassettes were placed at -70°C and intensifying screens used. For the detection of ^{35}S, cassettes were placed at room temperature without intensifying screens. Exposure times varied from 1 hour to 7 days. Films were developed using an X-ograph Compact X2.

2 d Radio-labelling of DNA fragments

Radiolabelled fragments of DNA were used as probes for a variety of methods, such as Northern blotting (Section 2c.viii), primer extension (Section 2i), and bandshift assays (Section 2j). The choice of radiolabelling method depended on the size of DNA fragment which was used as probe in each method. For DNA fragments larger than 500 base pairs random primer labelling was used; for DNA fragments smaller than 500 base pairs end labelling was suitable.

i Random-primed labelling

This method is based on that described by Feinberg and Vogelstein (1983). The method uses random hexa-nucleotides which bind to the DNA fragment, and initiate DNA polymerase reactions with the Klenow fragment of DNA polymerase I. One of the nucleotides incorporated is radiolabelled, and thus the newly synthesized DNA is radiolabelled.

1. DNA was cut with appropriate restriction endonuclease.
2. DNA fragments were separated in one lane of a 1.2% low melting point agarose gel (Gibco-BRL 5517UA) in 1 x TAE buffer.
2. DNA was visualised by ethidium staining, and the desired band excised and transferred to a pre-weighed Eppendorf tube: H_2O was added at a ratio of 3 µl mg^{-1} of gel.
3. The tube was boiled in water for 7 minutes to dissolve gel and denature the DNA: DNA was stored at -20°C at this stage: preparatory to subsequent labelling reactions, gels were re-boiled for
3 minutes and incubated for 10 minutes at 37°C, before use in the labelling reaction.
4. The labelling reaction consisted of:

H_2O	(to a total of 50 µl)	
OLB[1]	10 µl	
BSA[2]	2 µl	
DNA	10-20 ng	(up to 32.5 µl)
[α-^{32}P]dCTP	5 µl	(Amersham 10 µCi $µl^{-1}$)
Klenow	1 µl	(Boehringer Mannheim 100840, 2 U $µl^{-1}$)

incubated overnight at 25°C.

5. The reaction was stopped by addition of 200 µl stop buffer[3]: labelled DNA was stored at -20°C at this stage.

1 OLB:

OLB was made from the following components:

O: to 1 ml of 1.25 M Tris. Cl (pH 8.0) and 0.125 M $MgCl_2$, 18 µl of β-mercaptoethanol added, and 5 µl each of 100 mM dATP, dTTP, and dGTP (Pharmacia 27-2035-01).

L: 2 M hepes (Sigma H-3375), titrated to pH 6.6 with 4 M NaOH.

B: Hexa-deoxyribonucleotides at 90 OD units ml^{-1} (Pharmacia 27-2166-01).

Solution O: L: B mixed at a ratio of 100: 250: 150, to make OLB. OLB stored as 20 µl aliquots at -20°C.

2 Bovine serum albumin (BSA):

10 mg ml^{-1} (globulin free fraction, Sigma-A7638).

3 Stop buffer:

	stock	1 ml
20 mM NaCl	5M	4 µl
20 mM Tris. Cl	1 M	20 µl
2 mM EDTA	0.5 M	4 µl
0.25% SDS	20%	12.5 µl.

ii End-labelling

This method is based on that of Sambrook *et al.* (1989). End labelling uses bacteriophage T_4 polynucleotide kinase (PNK), which catalyses the transfer of the γ-phosphate of ATP to a 5' terminus of DNA or RNA. [γ-^{32}P]ATP is added in equimolar amounts to the number of ends of nucleic acid in the presence of PNK. This results in terminal γ-phosphates being replaced by [γ-^{32}P]-phosphates, and so the DNA fragment is radiolabelled.

1. The following reagents were added together:

DNA	10 pmoles	
PNK buffer[1]	1 µl	
H_2O	to 10 µl	
[γ-^{32}P]ATP	3 µl	(Amersham, 10 µCi μl^{-1})
T_4 PNK	1 µl	(Gibco-BRL, 10 U μl^{-1}).

2. The reaction mixture was usually incubated at 37°C for 30 minutes (although the time and temperature of incubation did vary with each DNA fragment), and stopped by incubating at 70°C for five minutes.

1 PNK Buffer:

	stock	100 ml
0.5 M Tris. Cl	1 M	500 µl
100 mM $MgCl_2$	1 M	100 µl
50 mM DTT	1 M	50 µl
1 mM spermidine	1 M	1 µl
1 mM EDTA	1 M	1 µl
H_2O		348 µl.

iii Purification of labelled DNA

Radiolabelled DNA was separated from un-incorporated radio-nucleotide by use of a G-50 matrix size exclusion column. G-50 matrix consists of small beads containing tiny holes. Small DNA fragments (<20 nucleotides) enter the holes in the beads and are retained, whereas larger DNA fragments cannot enter the holes and pass through the column. Thus, fragments of labelled DNA can be separated from nucleotides, by passing a labelling reaction through such a column: labelled DNA will pass through and be found in the eluate.

1. G-50 (Pharmacia 17-0042-01) beads were prepared by adding two volumes of TE and autoclaving.
2. The plunger of a 1 ml disposable syringe (Plastipak) was removed, and a small wad of siliconized glass wool (Section 2c.vi) pushed into the bottom.
3. G-50 was added until the syringe was full: the column was spun for 2 minutes at 2000 rpm, more G-50 was added and the column spun

again until the G-50 had shrunk to about 800 µl.

4. Labelled DNA was added (in a volume of about 100 µl, with 1 µl of glycogen carrier (Section 2b.iii), and spun again for 2 minutes at 2000 rpm.

5. If separation of labelled DNA fragment from unincorporated radionucleotide had occurred, a certain radioactive profile in the G-50 column was observed (see Fig. 2D).

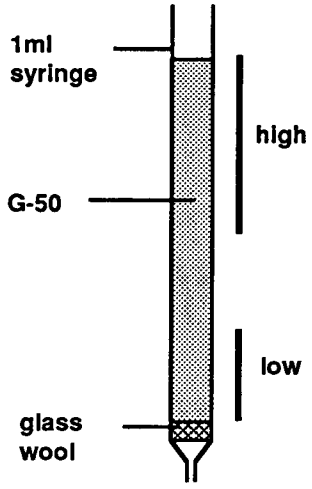


Figure 2 D **Radioactive profile observed in G-50 columns in which separation of radiolabelled DNA from nucleotide has occurred.**

High refers to high counts of radioactivity of unincorporated nucleotide, retained in the column. Low refers to low counts of radioactivity of labelled DNA excluded from the column.

6. DNA was sodium acetate precipitated and washed with 90% ethanol (Section 2b.iii).

2 e Fission yeast manipulations

i Strains

The wild type strain and mutant strains of the fission yeast *Schizosaccharomyces pombe* Linder, were all derived from the haploid heterothallic 972 (mating type h^-) and 975 (mating type h^+) isolates described by Leupold (1950).

Strain list

975 h+
972 h-
cdc2-33 h-
cdc10-C4 h-
cdc10-K28 h-
cdc10-129 h-
cdc10-C4 ura4-D18 h+
cdc10-K28 ura4-D18 h+
cdc2-33 ura4-D18 h+
ura4-D18 h+

cdc10-C4 mat2-102 ade6-M216 / cdc10-C4 leu1-32
cdc10-C4 mat2-102 ade6-M216 / cdc10-129 leu1-32
cdc10-129 leu1-32 / cdc10-129 lys1-131 mat2-102
cdc10-129 leu1-32 / mat2-102 ade6-M216
leu3-155 / mat2-102 ade6-M216
cdc10-C4 leu1-32 / mat2-102 ade6-M216

ii Media and growth conditions

Media

Strains of fission yeast were routinely grown on solid YE complex medium supplemented with adenine and uracil.

YE contains per litre:

glucose	30 g
yeast extract	5 g
adenine	75 mg
uracil	75 mg
Difco-Bacto agar	1.5 g

For complex liquid medium YE was used minus Difco-Bacto agar.

EMM (Moreno *et al.*, 1991) was used as minimal medium, and is a modification of EMM2 (Mitchison, 1970 and Nurse, 1975), and contains the following per litre:

glucose	20 g
KH phthalate	3 g
Na_2HPO_4	1.8 g
NH_4Cl	5 g
$NaSO_4$	100 mg
$CaCl_2$	15 mg
$MgCl_2$	1 g
vitamins	1 ml
minerals	100 µl.

Where vitamins are: inositol, 5 g; nicotinic acid, 5 g; calcium pantathenate, 0.5 g; and biotin, 5 mg all dissolved in 500 ml H_2O. Where minerals are: H_3BO_3 1 g; $MnSO_4$. 4 H_2O, 1.04 g; $ZnSO_4$. 7 H_20, 800 mg; $FeCl_3$. 6 H_2O, 400 mg; H_2MoO_4, 288 mg; $CuSO_4$. 5 H_2O, 80 mg; Citric acid, 2 g; and KI, 20 mg, all dissolved in 200 ml H_2O. Both were stored at 4°C.

Growth supplements such as adenine, uracil, lysine, and leucine were made as 50 x stock solutions of 375 mg ml^{-1}, and were added as required after autoclaving.

To generate fission yeast with thin cell walls to assist in spheroplasting for transformations, low glucose EMM was used. This is identical to EMM, except that glucose is at a concentration of 0.5% instead of 2%. 1.2 M sorbitol was included in solid media for the regeneration of osmotically sensitive spheroplasts.

For the induction of conjugation and consequent sporulation, nitrogen limiting medium ME, consisting of 30 g l^{-1} malt extract and 20 g l^{-1} agar, was used.

To check ploidy of cells, phloxin B was added, from a stock solution of 10 mg ml^{-1}, to a final concentration of 20 µg ml^{-1} in solid media. Diploid cells die faster than haploid cells and accumulate the dye more quickly, so can be differentiated by relative colour staining.

Storage

Short term storage of fission yeast (up to 4 weeks) was on solid medium at 4°C. Long term storage was in medium containing 20% (v/v) glycerol at -70°C.

iii Genetical analysis

Crossing strains

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

Random spore analysis

A loopful of mating mix was suspended in 1 ml of sterile distilled water containing 20 µl of a stock solution of the snail gut enzyme (Suc d'Helix pomatia, Industrie Biologique, France) and incubated overnight at 35°C. The stock solution is a $^1/_{10}$ dilution of the preparation supplied, which is stored at 4°C. The spore concentration was estimated using a haemocytometer and an appropriate dilution, to give about 200 spores per plate, spread onto solid YE media.

Analysis of phenotypes

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

iv Cell physiology

Growth in liquid cultures

For all experiments examining aspects of fission yeast cell physiology, actively growing cells were used. Such cells were undergoing exponential growth, and were at a concentration of 3 - 8 x 10^6 cells ml^{-1}. Exponentially growing cells were obtained by inoculating a single colony into a 10 ml YE pre-culture and incubating until the cells had entered the stationary phase of growth (usually 2 days). An aliquot of the pre-culture ($^1/_{100}$) was inoculated into 200 ml of EMM in a 500 ml conical flask, and incubated shaking for 14-18 hours at 25°C or 35°C. Cell number was monitored until cells reached mid-exponential stage.

Estimation of cell number

Cell number was estimated using a Coulter counter (Industrial D). A 100 µl sample of cell culture was removed and mixed in 10 ml of ISOTON solution (a $^1/_{1000}$ dilution), sonicated for 5 seconds at setting 2 on a Lucas Soniprobe Sonicator. Usually 2 counts of 500 µl volume were taken, and combined to give a cell count per ml.

Synchronous cultures

To obtain populations of fission yeast cells dividing synchronously, a Beckmann Elutriator rotor was used. These experiments were conducted with the kind and skilful help of Dr. Jim Creanor.

Temperature shift experiments

Cells were grown with shaking at the permissive temperature of 24°C, until the mid-exponential phase of growth (3 x 10^6 cells ml^{-1}) was reached, when the whole culture was transferred to the restrictive temperature of 36°C.

v Transformation

The method used for fission yeast transformation was essentially that of Beach & Nurse (1981), and yields up to 20,000 transformant colonies per μg of plasmid DNA.

1. 200 ml cultures, of mid-exponential stage of growth fission yeast cells (Section 2e.iv), were prepared in 0.5% glucose EMM.
2. Cells were transferred to 250 ml Beckman bottles and pelleted by centrifugation at 5000 rpm for 10 minutes.
3. The supernatants were discarded and cells resuspended in 10 ml of CPE[1].
4. Cells were centrifuged out and the supernatants discarded.
5. Pellets were resuspended in 5 ml of CPS[2].
6. Pellets were transferred to 50 ml screw cap tubes (Falcon 2070) and incubated at 37°C.
7. After 20 minutes, 5 μl of cells were removed to check spheroplast formation under the microscope. When spheroplasts had formed (cells appeared round as opposed to 'sausage' shaped) the next stage was started. If the cells had not spheroplasted the incubation was continued and the cells checked every ten minutes.
8. 15 ml of TS[3] was added, mixed, and 5 ml aliquoted into four 50 ml screw-cap tubes, and centrifuged at 2,000 rpm for 4 minutes.
9. Step 8 was repeated four more times.
10. Cells were finally resuspended at a density of 2.5×10^8 cells ml^{-1} in TCS[4].
11. 100 μl aliquots of cell suspension were transferred to 5 ml snap-cap polystyrene tubes (Falcon 2058) containing 1 and 5 μg of plasmid DNA, and incubated at room temperature for 15 minutes.
12. 1 ml of TCP[5] was added and cells incubated at room temperature for 15 minutes.
13. Cells were pelleted by centrifugation at 2,000 rpm for 5 minutes, and supernatants removed by aspiration.
14. Cells were resuspended in 500 ml of TCS with 0.5 mg ml^{-1} yeast extract and 5 mg ml^{-1} supplements.
15. Cells were incubated for 60 minutes at permissive temperature, and 250 μl plated onto each of two EMM plates with 1.2 M sorbitol.
16. Transformant colonies appeared after 4-6 days.

17. Non-integration of plasmids was checked by a stability test. Transformant cells were streaked to single colonies, grown on YE ('off selection') for 2 days, and then replica plated onto EMM ('on selection'). Cells containing non-integrated plasmids were evident having lost the ability to grow on EMM.

1 CPE:

20 mM citrate-phosphate buffer pH 5.6
40 mM EDTA
0.1% (v/v) β-mercaptoethanol (added immediately before use).

2 CPS:

50 mM citrate-phosphate buffer pH 5.6
1.2 M sorbitol
0.1% (v/v) β-mercaptoethanol (added immediately before use)
2 mg ml^{-1} Novozyme 234 (Novo Industries, Bagsvaerd, Denmark 69-289-01).

3 TS:

10 mM Tris. Cl pH 7.6
1.2 M sorbitol.

4 TCS:

10 mM Tris. Cl pH 7.6
10 mM $CaCl_2$
1.2 M sorbitol.

5 TCP:

10 mM Tris. Cl pH 7.6
10 mM $CaCl_2$
20% PEG-4000.

vi Preparation of nucleic acids from fission yeast

Genomic DNA

This method produced fission yeast genomic DNA of sufficient quality for PCR amplification, and is as described elsewhere Durkacz *et al.*, 1985).

1. 10 ml cultures of media were inoculated with a single yeast colony, and incubated until the the culture reached stationary phase (1-2 days).
2. Cells were harvested (5 minutes at 2500 rpm) in 15 ml centrifuge tubes (Falcon 2095).
3. Supernatants were discarded and cells resuspended in 1.5 ml CPS[1] containing 2.5 mg ml^{-1} zymolyase 20 T (Seikaguku Kogyo 120491): suspensions were transferred to Eppendorf tubes.
4. Cells were incubated at 37°C for 60 minutes.
5. Cells were harvested by centrifugation at high speed for 2 minutes.
6. Supernatants were discarded and cells resuspended in 300 µl of 5 x TE; 35 µl of 10% SDS (w/v) was added and the mixture incubated at 65°C for 5 minutes.
7. 100 µl of 5 M potassium acetate (pH 5.6) was added and the mixture incubated on ice for 30 minutes.
8. Cells were centrifuged at 4°C at high speed for 15 minutes: the supernatant was removed (400 µl) and to this 1 ml ice-cold 95% (v/v) ethanol added and mixed.
9. Centrifuged at high speed at 4°C for 10 minutes.
10. Supernatant was discarded and pellet resuspended in 400 µl of 5 x TE containing 100 µg ml^{-1} RNAase (bovine pancreatic, Sigma R-5000): incubated for 2-4 hours at 37°C.
11. Suspension was extracted once each with an equal volume of phenol, phenol/chloroform, and chloroform (Section 2b.ii).
12. The final aqueous phase was transferred to another Eppendorf tube, sodium acetate precipitated, and washed with 70% ethanol (Section 2b.iii).
13. Pellets were dried, dissolved in 100 µl of TE, and DNA quality and amount checked (Section 2b.iv).

1 CPS:

50 mM citrate-phosphate buffer pH 5.6
1.2 M sorbitol
0.1% (v/v) β-mercaptoethanol (added immediately before use)
2 mg ml^{-1} Novozyme 234 (Novo Industries, Bagsvaerd, Denmark 69-289-01).

Total RNA

This method, based on that of Kaufer *et al.* (1985), produced fission yeast total RNA which was used for Northern blotting (Sections 2c.vii and 2c.viii) and primer extension (Section 2i).

Gloves were worn during all RNA procedures to prevent RNAase contamination.

1. 200 ml cultures of mid-exponential stage of growth fission yeast cells were prepared (Section 2e.iv), and harvested by centrifugation at 5000 rpm for 10 minutes in four 50 ml screw cap tubes (Falcon 7020).
2. Each cell pellet was resuspended in 1 ml of sterile saline[1] and transferred to an Eppendorf tube; cells were pelleted by centrifugation at high speed, and the supernatant discarded (cells could be stored at -70°C at this stage).
3. Cells were resuspended in 75 µl of STE[2].
4. Chromic acid washed beads (425-600 micron, Sigma G-9268) were added to just beneath meniscus, and the cells briefly vortexed.
5. 600 µl of NTES[3] was added, and the mixture briefly vortexed again,
6. 500 µl of hot phenol (65°C) was added, and the mixture immediately vortexed.
7. The mixture was incubated at 65°C for 5 minutes with frequent vortexing.
8. The mixture was centrifuged for 1 minute at high speed, and the aqueous phase and protein interface were removed to a second 500 µl aliquot of hot phenol.
9. Incubated at 65°C for 2 minutes with frequent vortexing.
10. Centrifuged for 1 minute at high speed, and again the aqueous phase and interface were removed to a third 400 µl aliquot of hot

phenol.

11. Incubated at 65°C for 2 minutes with frequent vortexing.
12. Aqueous phase only was removed into 400 µl of phenol/chloroform at room temperature, vortexed, and spun at high speed for 1 minute.
13. Aqueous phase was re-extracted with 300 µl of chloroform, vortexed, and spun for one minute at high speed.
14. Aqueous phase was sodium acetate precipitated (Section 2b.iii).
15. Supernatant was discarded and the RNA pellet washed with 70% ethanol in DEPC H_2O; it was important that the pellet did not become completely dry, as this made re-dissolving difficult.
16. RNA pellet was resuspended in 55 µl of ice cold DEPC H_2O.
17. 5 µl of each sample was used to estimate amount and quality by spectrophotometry (Section 2b.iv).
18. RNA was stored at -20°C.

1 Sterile saline:

0.9% (w/v) NaCl, autoclaved.

2 STE:

0.32 M sucrose
20 mM Tris. Cl (pH 7.5)
10 mM EDTA (pH 8.0)
0.5 mg ml^{-1} heparin (Sigma H-7005) added solid just prior to use.

3 NTES:

100 mM NaCl
5 mM EDTA
50 mM Tris. Cl (pH 7.5)
1% (w/v) SDS
0.5 mg ml^{-1} heparin added as solid just prior to use.

vii Preparation of protein from fission yeast

This method was developed by Noel Lowndes and myself, and produced total non-denatured fission yeast protein extracts, which were used in bandshift assays (Section 2j).

1. 200 ml cultures of fission yeast cells in mid-exponential stage of growth were prepared (Section 2e.iv), and harvested by centrifugation at 5000 rpm for 10 minutes, in four 50 ml screw cap tubes (Falcon 7020).
2. Supernatants were discarded and each cell pellet resuspended in 100 µl of lysis buffer[1] in Eppendorf tubes: acid washed glass beads (425-600 micron, Sigma G-9268) were added to just beneath the meniscus.
3. Cells were broken by vigorous vortexing: 15 minutes of 30 seconds, with 30 second intervals on ice: cell breakage was monitored microscopically.
4. Cell walls were pelleted by centrifugation at high speed for 5 minutes at 4°C.
5. Supernatants were transferred to another Eppendorf tube, and the protein extract clarified by centrifugation at 17,000 rpm (Beckman JA-18) for 30 minutes at 4°C.
6. Supernatants were transferred to another Eppendorf tube, and 5 µl removed for protein assay: the remainder was snap frozen on solid CO_2 and stored at -70°C.

1 Lysis buffer:

	stock	1 ml
50 mM KCl	1 M	50 µl
50 mM Tris. Cl	1 M	50 µl
25% glycerol	50%	500 µl
2 mM DTT	1 M	2 µl
0.1% Triton X-100		1 µl
chymostatin (Sigma C-7268)		5 µl
antipain (Sigma A-6271)		5 µl
leupeptin (Sigma L-2884)		5 µl
aprotonin (Sigma A-6279)		5 µl
pepstatin (Sigma P-4265)		5 µl
H_2O		370 µl
0.2 mM PMSF	100 mM	2 µl (added at last moment).

DTT: dithiothreitol (Sigma D-0632) 1 M solution in H_2O, stored at -20°C.

PMSF: phenylmethanesulfonylfluoride (Sigma P-7626) 100 mM solution in isopropanol, stored at 4°C.

Protease inhibitors: each at 5 mg ml^{-1}: stored at -20°C.

Protein concentration was estimated using the Pierce BCA Protein Assay kit (23225) according to the manufacturer's instructions.

2 f *Escherichia coli* manipulations

i Strains

Two *Escherichia coli* strains were used in this work:

JM101 *supE thi D(lac-proAB) (F' traD36 proAB lacIqZDM15)*

JA226 *recBC leuB6 trpE5 hsdR- hsdM+ lacY600.*

The former was specifically used for the propagation of pUC-based plasmids and the production of single stranded DNA, as it has the f1 origin required for M13K07 helper phage infection (Section 2g.ii). The latter was used for routine plasmid propagation.

ii Media and growth conditions

Rich medium

All *E. coli* strains were routinely grown in rich medium LB consisting of, per litre:

Bacto-tryptone	10 g
NaCl	10 g
Yeast extract	5 g.

Glucose was added from a sterile stock solution to 1 g l^{-1} after autoclaving.
For solid plate medium 15 g l^{-1} Bactoagar (Difco 0140-01) was added before autoclaving.

Minimal medium

M9 minimal medium was used.

750 ml of H_2O containing 12 g of Bactoagar was autoclaved and cooled to 55°C in a water bath. Each of the following were autoclaved separately and then added together:

200 ml of 5 x M 9 salts[1]
2 ml of 1 M $MgSO_4$
100 µl of 1 M $CaCl_2$
20 ml of 20% glucose.

1 5 x M 9 salts:

$Na_2HPO_4.7H_2O$	64 g
KH_2PO_4	15 g
NaCl	2.5 g
NH_4Cl	5.0 g.

The solution was dissolved to final volume of 1 litre in H_2O, the pH adjusted to 7.4 with HCl, split into 200 ml aliquots, and autoclaved.

Thiamine was added as supplement at a final concentration of 1 mM thiamine-HCl (Sigma T-4625).

Selective antibiotics

Ampicillin

A 1000 x stock solution of 100 mg ml^{-1} of the sodium salt was dissolved in 50% ethanol.

Chloramphenicol

A 1000 x stock solution of 34 mg ml^{-1} chloramphenicol was dissolved in 100% ethanol.

Kanamycin

A 1000 x stock of 70 mg ml^{-1} kanamycin was dissolved in H_2O.

Tetracycline

A 500 x stock of 5 mg ml^{-1} of tetracycline was dissolved in 50% ethanol.

Antibiotic stock solutions were stored at -20°C, and were added to autoclaved media.

Colour selection

Where blue/white colour selection was available in cloning vectors (Section 2b.v), X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Sigma B-4252) to a final a concentration of 40 μg ml^{-1}, from a freshly made solution of 20 mg ml^{-1} in dimethyl formamide, and IPTG (isopropylthio-β-D-galactoside, Sigma I-6758) to a final concentration of 100 μM, from a stock of 100 mM in H_2O, were added to media.

Growth conditions

All bacteria were grown at 37°C, in an orbital shaking incubator.

Storage

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

iii Plasmid preparation

'Quick-preps'

This rapid method (Sambrook *et al.*, 1989) produced about 5 μg of relatively 'dirty' plasmid DNA, which was useful for screening large numbers of transformant colonies. The resulting DNA degrades within 30 minutes at 37°C, so restriction digests were incubated for 15 minutes at this temperature. This was not a problem as the DNA digested very quickly.

1. Single colonies were inoculated into 5 ml of LB with selective antibiotic, and grown overnight shaking at 37°C.
2. Cultures were transferred to disposable 20 ml test tubes (Sarstedt 55.466) and cells pelleted by centrifugation at 10,000 rpm for

10 minutes.

3. Supernatants were removed by aspiration, and each pellet re-suspended in 300 µl of STET[1]; suspensions were transferred to Eppendorf tubes and incubated on ice for 5 minutes.
4. A small hole was pierced in the lid of each Eppendorf tube to stop them 'popping' open at the next stage.
5. Eppendorf tubes were plunged into bubbling boiling water for 45 seconds, cooled on ice for 30 seconds, and immediately centrifuged at high speed for 10 minutes.
5. Gelatinous pellets were removed with a tooth-pick from each tube.
6. All remaining volumes were made up to 200 µl with STET, and 200 µl of isopropanol added, mixed, and frozen at -20°C for 20 minutes.
7. Solutions were centrifuged at high speed for 10 minutes, and supernatants discarded.
8. Precipitates were resuspended in 100 µl of STET, 100 µl of ethanol added, mixed carefully, and frozen at -20°C for 20 minutes.
9. Solutions were centrifuged at high speed for 10 minutes, supernatants discarded, and pellets resuspended in 50 µl of TE: these were frozen at -20°C immediately.

1 STET:

		stock	1 l
sucrose	8%		80 g
Triton X-100	5%		50 ml
EDTA	5 mM	0.5 M	10 ml
Tris. Cl	50 mM	1 M	50 ml.

'Midi-preps'

This method, based on that of Birboim & Doly (1979), produced 100 µg amounts of 'clean' plasmid DNA, which was used for all common molecular biology techniques.

1. Single colonies were inoculated into 100 ml of LB with appropriate antibiotic, and grown overnight shaking at 37°C.
2. Cultures were aliquoted into two 50 ml screw cap tubes (Falcon

7050) and cells pelleted by centrifugation at 10,000 rpm for 10 minutes.

3. Pellets were carefully resuspended in a final volume of 3 ml of TESL[1] by gentle pipetting, and incubated on ice water 20 minutes.
4. 6 ml of NS[2] was added, mixed carefully by inversion, and incubated on ice for 10 minutes.
5. Cells were pelleted by centrifugation at 15,000 rpms for 15 minutes, in a swing-out rotor, and the supernatant carefully removed; this was transferred to another 50 ml screw cap tube, 50 µl of RNAase[3] added and incubated for 20 minutes at 37°C.
6. Supernatants were phenol/chloroform extracted twice (Section 2b.ii).
7. Two volumes of ice cold ethanol were added to precipitate DNA, and DNA pelleted by centrifugation at 10,000 rpm for 10 minutes.
8. Supernatants were discarded, and pellets dissolved in 1.6 ml of H_20; 400 µl of 4 M NaCl added and mixed; 2 ml of 13% (w/v) PEG 6000 added, mixed, and the tubes incubated on ice water for 60 minutes.
9. DNA was pelleted by centrifugation at 10,000 rpm for 10 minutes, supernatants were discarded, washed pellets with 70% ethanol (Section 2b.iii), and pellets dissolved in 100 µl of TE.

1 TES:

	stock	100 ml
25 mM Tris. Cl (pH 8.0)	1 M	2.5 ml
10 mM EDTA	0.5 M	2 ml
15% sucrose		15 g
2 mg ml^{-1} lysosyme (Sigma L-6876).		

2 NS:

	stock	100 ml
0.2 M NaOH		800 mg
1% SDS	20% (w/v)	5 ml.

3 RNAse (Bovine pancreatic, Sigma R-5503)
Dissolved at a concentration of 10 mg ml^{-1} in Tris. Cl (pH 7.5) and 15 mM NaCl. Incubated at 100°C for 15 minutes on a heating block, and then allowed to cool slowly to room temperature.
Stored at -20°C.

iv Transformation

This method is based on that of Hanahan (1983), and produced transformation frequencies of up to 10^8 colonies per μg of plasmid DNA.

Competent cells

1. Pre-cultures were grown in LB overnight shaking at 37°C.
2. 200 ml of pre-culture was inoculated into 20 ml φ broth[1] and grown at 37°C shaking to an A_{550} of 0.25: this usually took about 2 hours.
3. 5 ml of cells were inoculated into 20 ml φ broth, and grown to an A_{550} of 0.48.
4. Cultures were cooled briefly on ice, and cells pelleted by centrifugation for 5 minutes at 9000 rpm at 4°C.
5. Pellets were resuspended in 33 ml of ice cold TfBI[2], and incubated on ice for 15 minutes.
6. Cells were pelleted again by centrifugation for 5 minutes at 9000 rpm at 4°C.
7. Pellets were resuspended in 4 ml of ice cold TfBII[3] and incubated on ice for 20 minutes.
8. 200 μl of cells aliquoted into ice-cold Eppendorf tubes, snap frozen on solid CO_2, and stored at -70°C.

1 φ broth was made of 10 ml of salts added to 490 ml tryptone/yeast extract.

10 g of tryptone and 2.5 g of yeast extract were dissolved in 490 ml of H_20, and autoclaved.

Salts:

	100 ml
1M $MgSO_4$. 7 H_2O	24.6 g
0.5 M NaCl	2.9 g
1.25 M KCl	9.3 g

and filter sterilized.

2 TfBI:

	500 ml
NaOAc	1.43 g
$CaCl_2$. 6 H_2O	1.1 g
glycerol	75 g

and pH to 5.9 with HOAc.

Added solid:

RbCl	6.0 g
$MnCl_2$	4.95 g

and autoclaved in 50 ml aliquots.

3 TfBII:

	100 ml
MOPS	209 mg
$RbCl_2$	120 mg
$CaCl_2$. 6 H_2O	1.64 g
glycerol	15 g

pH to 6.8 with KOH and autoclaved in 10 ml aliquots.

Transformation

1. Cells were thawed on ice for 10-15 minutes.
2. DNA was added in a minimum volume - less than 10 µl.
3. Cells were incubated on ice for 30 minutes.
4. Cells were heat-shocked cells in a 42°C water bath for 60 seconds.
5. 400 µl of LB was added and incubated at 37°C for 60 minutes.
6. 250 µl of cells were spread onto appropriate plates.

2 g DNA sequencing methods

Sequencing of DNA was performed by a combination of the phagemid system devised by Vieira and Messing (1982) and the chain termination sequencing system of Sanger *et al.* (1977).

i Deletions

A series of unidirectional nested deletions of all four *Hin*dIII *cdc22*$^+$ subclones (Section 3b; Fig. 3A) were made, using the method of Henikoff (1984). The Pharmacia Deletion kit was used, according to the manufacturer's instructions.

This method produces a group of unidirectional nested deletions using exonuclease III (Exo III). Exo III is an 3'-exonuclease which is only active on double stranded DNA: blunt and 5' overhanging ends are susceptible to digestion, while 3' overhanging ends of three or four bases in length are resistant to the enzyme. It is therefore possible to prepare linearized DNA which has only one susceptible end next to the DNA fragment of interest by cutting the DNA with two restriction enzymes, one of which produces a susceptible end and the other producing a resistant end. Incubation of this DNA with Exo III, under controlled conditions of salt and temperature, permits the progressive removal of nucleotides from one strand. By stopping the reaction at incremental times, a series of DNA pieces of varying length can be produced. The remaining single stranded DNA is removed using S1 nuclease, and the molecules are re-circularized using T4 DNA ligase. These nested deletions can then be sequenced using a common oligonucleotide primer, which hybridizes to a DNA sequence just internal to the resistant end.

N.B. Buffers typed in **bold** were included with the the Pharmacia deletion kit.

Restriction enzyme digestion

1. To create a linearized molecule with resistant and sensitive ends to Exo III, the following restriction digests were completed:

clone	resistant end	sensitive end
pCDC22. 10	*Sst*I	*Xba*I
pCDC22. 5	*Sst*I	*Bam*HI
pCDC22. 7	*Kpn*I	*Xba*I
pCDC22. 14	*Kpn*I	*Bam*HI

5 µg of DNA was cut in each digestion: the resistant end digestion was completed first, and monitored by gel electrophoresis to ensure completion; the sensitive end digestion was then performed. Both enzymes were then inactivated by freezing at -20°C.

Exo III digestion

1. Exo III digestion was performed at 30°C in 75 mM NaCl to give 300 nucleotide spaced deletions.

For Exo III digestion buffer of 75 mM NaCl added:

Cut DNA in REact III (100 mM NaCl)	20 µl
Exo III buffer	8 µl
0.3 M NaCl	4 µl
H_2O	12 µl

This mix was pre-warmed to 30°C and a 2µl sample removed and added to 3 µl of ice cold S1 Buffer[1].
1 µl of **Exo III** was added, mixed, and further 2 µl samples removed at 3 minute intervals, and each added to 3 µl of S1 Buffer.

2. After all timed samples have been removed, they were incubated simultaneously at room temperature for 30 minutes.
1 µl of **S1 stop solution** was added to each tube, mixed, and incubated at 65°C for 10 minutes.

Analysis of deletions

1. 3 µl of each sample was mixed with 3 µl of **loading buffer** and analysed by electrophoresis (see Fig. 2E).

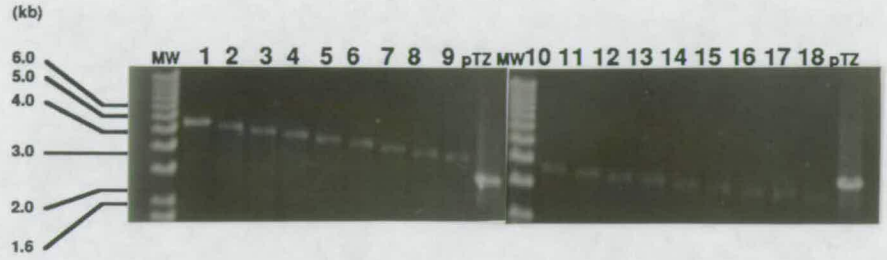


Figure 2 E Exonuclease III deletion series of pCDC22-14.

Exo III deletions of pCDC22-14 of 300 nucleotide incremental size (lanes 1-18) analysed on a 1.0% agarose gel. Molecular weight standards (MW) and linearized pTZ19 (pTZ) are indicated. For method see text.

Re-circularization and transformation

1. Ligation mix prepared by adding:

5 x ligase mix	85 µl
25% PEG	85 µl
H_2O	195 µl

2. 17 µl of ligation mix added to 3 µl remaining of each sample, gently mixed, and incubated at room temperature for 2 hours.

3. Each ligated sample was transformed into bacterial strain JM101 (Section 2f.iv).

4. Quick prep plasmid DNA was prepared (Section 2f.iii) from 12 transformant colonies from each time point, and the size of resultant inserts estimated. Initially, an *Eco*RI digestion was completed to ensure presence of the reverse sequencing primer site, adjacent to the polylinker. Those clones containing the primer site were then double digested with *Eco*RI and another restriction enzyme to release the DNA insert fragment which had been deleted by Exo III. Restriction enzymes cutting at sites progressively further into the clones were used, as they produced small DNA fragments, which could be accurately sized.

ii Production of single-stranded DNA

Single-stranded DNA (ssDNA) was produced using helper phage M13KO7. This method yielded about 5 µg of ssDNA template, suitable for dideoxy sequencing.

Cultures and infections

1. Single transformant colonies in *E. coli* strain JM101 were streaked onto M9 plates to enrich for the F9 episome.
2. Single colonies were inoculated into 5ml LB with appropriate antibiotic, and incubated overnight shaking at 37°C.
3. 400 µl of culture was inoculated into 10 ml of 2YT[1] in a 50 ml screw cap tube (Falcon 7020), and incubated shaking at 37°C.
4. Cultures were monitored until the A_{600} was between 0.5-1.0 (this usually took between 2 and 2.5 hours).
5. 2 ml of culture was transferred to another 50 ml screw cap tube, 5 µl of helper phage (Bio-Rad 1703578) added, and shaken vigorously at 37°C for 60 minutes.
6. 200 µl of culture was added to 10 ml of 2YT and 10 µl of kanomycin solution (Section 2f.ii) added, and this grown overnight at 37°C.

Preparation

7. Cultures were poured into 30 ml Corex tubes and centrifuged for 5 minutes at 9,000 rpm; supernatants were decanted into fresh 30 ml Corex tubes, and the spin repeated.
8. 9 ml of each supernatant was removed into fresh 30 ml Corex tubes and 1.2 ml of PS[1] added, mixed carefully, and incubated on ice for 60 minutes.
9. Helper phage was pelleted by centrifugation at 12,000 rpm for 10 minutes.
10. Supernatants were discarded and phage resuspended in 1.3 ml of TE; this suspension was aliquoted into two Eppendorf tubes each containing 650 µl.
11. 40 µl of 40% (w/v) PEG 6000 and 80 µl of 5 M NaCl were added to each tube, carefully mixed, and incubated at room temperature for 30 minutes.
12. Tubes were spun for 10 minutes at high speed and each pellet re-suspended in 300 µl of TE.
13. Each solution was phenol/chloroform extracted 3 times, DNA was sodium acetate precipitated, and the pellets washed with 70% ethanol (Section 2b.ii and iii).
14. Pellets were resuspended in 20 µl of TE.
15. 0.5 µl was run on an agarose gel in parallel with ssDNA from the Pharmacia T7 sequencing kit to assay amount.

1 2YT:

	1 l	
Bactopeptone	16 g	
yeast extract	10 g	
NaCl	5 g	and autoclaved.

2 PS:

	stock	100 ml
PEG 6000		27 g
NaCl	5 M	66 ml
H_2O		7 ml.

iii Sequencing

Dideoxy sequencing reactions using T7 DNA polymerase were performed with the Pharmacia T7 sequencing kit, according to the manufacturer's instructions.

This method depends on base-specific termination, by dideoxynucleotides, of enzyme catalysed primer extension reactions (Sanger *et al.*, 1977). Four reactions are performed, all containing primer, template, and four deoxyribonucleotides; each including a chain terminating dideoxynucleotide and T7 DNA polymerase. This results in stochastic mixture of extended fragments, each terminated by the particular dideoxyribonucleotide added to the reaction. When the products of the four reactions are separated by electrophoresis side by side, a 'ladder' of progressively larger fragments is formed. The base pair sequence of the DNA molecule can be deduced by reading the order of this ladder, which is visualised by radiolabelling.

N.B. Buffers printed in **bold** were supplied with the Pharmacia T7 sequencing kit.

Annealing of primer

1.

2 µg template DNA	10 µl
annealing buffer	2 µl
primer[1]	8.88 ng in 2 µl

were added together, and heated at 60°C for 10 minutes, and allowed to cool to room temperature for 15 minutes.

1 Primer:

universal primer supplied with the kit was dissolved at a concentration of 0.8 µM, or 4.44 µg ml^{-1}. Reverse sequencing primer (Amersham, NH512) was also used and dissolved to the same concentration. Other oligonucleotides were usually supplied at a concentration of 400 µg ml^{-1}, and were diluted $^1/_{250}$, to give the correct final concentration.

Sequencing reactions

1. To 'read short' (up to 500 nucleotides) or to 'read long' (50-1000 nucleotides) **mix-short** or **mix-long** reactions were used: four Eppendorf tubes were labelled 'A', 'C', 'G', and 'T': into each 2.5 µl of **'A' mix, 'C' mix, 'G' mix,** and **'T' mix,** was pipetted.
2. Labelling reaction: to each tube containing annealed template and primer, 6 µl of Enzyme pre-mix[1] was added, and incubated at room temperature for five minutes.
3. While this incubation was in progress, the four sequencing mixes just dispensed were incubated at 37°C for at least one minute.
4. After incubating the labelling reaction for 5 minutes, 4.5 µl of this reaction was transferred to each of the four pre-warmed sequencing mixes, mixed, and incubated at 37°C for 5 minutes.
5. 5 µl of **stop solution** was added to each sample and mixed.

1 Enzyme pre-mix
For one template mix together:

H_2O	1 µl
Labelling mix	3 µl
Diluted **T7 polymerase**	2 µl
[α-^{35}S]dATP (10µCi)	1 µl.

T7 polmerase was diluted using ice cold **enzyme dilution buffer,** to a final concentration of 1.5 units μl^{-1}.
[α-^{35}S]dATP: Amersham 37.0 M Bq 1.00 mCi

iv Gel electrophoresis

Electrophoresis was carried out using the BRL Model S2 sequencing gel electrophoresis system.

1. Glass plates were cleaned with H_2O and ethanol, treated with trimethylchlorosilane (BDH 33164), separated by spacers, and sealed with tape.
2. To 100 ml of sequencing mix[1], 1ml of freshly prepared 10% (w/v) ammonium persulphate (Sigma A-9164) and 25 µl of TEMED

(N,N,N',N'-tetramethylethylenediamine, Bio-Rad 61-0800) were added, the solution mixed, and immediately poured.

3. Loading wells were formed using 'shark's-tooth' combs, according to the manufacturer's instructions.
4. Gel were pre-run at a constant 43 Watts for 30 minutes in 1 x TBE buffer, the wells flushed out out with 1 x TBE buffer, and samples loaded after incubation at 75°C for 3 minutes.
5. Gels were run at 43 Watts for 6-12 hours.
6. Glass plates were separated so that the gel remained attached to one plate.
7. Gels were transferred to a sheet of Whatman filter paper. (3 MM), covered with SaranWrap, and dried on a vacuum gel dryer.
8. Gels were processed for autoradiography (Section 2c.ix).

1 Sequencing mix:

40% acrylamide (19:1 acrylamide: bisacrylamide)	75 ml
10 x TBE	50 ml
urea	250 g
H_2O	175 ml
total	500ml.

All reagents used were Gibco-BRL Ultra-Pure.

2 h Polymerase chain reaction

The polymerase chain reaction (PCR) was used for the amplification of DNA fragments, for a wide number number of uses.

Amplification was performed over 30 cycles using 0.1 μg of either chromosomal (Section 2e.vi) or plasmid DNA, with the GenAmp DNA reagent kit (Perkin Elmer Cetus N801-0055), in a Hybaid Thermal Reactor programmed as follows:

94°C for 2 minutes (denaturation)
55°C for 2 minutes (annealing)
72°C for 4 minutes (elongation)

and finishing with:

72°C for 5 minutes
25°C for 30 minutes.

Each reaction mixture, made up to the manufacturer's instructions, consisted of the following:

Template DNA	0.1 μg in 10 μl
primer 1	5 μl of 20 μM
primer 2	5 μl of 20 μM
Reaction Buffer[1]	10 μl
dNTP mix[2]	16 μl
H_2O	52.5 μl
Taq DNA polymerase	0.5 μl

To prevent evaporation each reaction mix was overlaid with 100 μl of light mineral oil (PCR Grade, Sigma M-3516).

After PCR reactions DNA product was removed from the reaction vessel, phenol/chloroform extracted twice, precipitated with sodium acetate, and monitored by electrophoresis (Sections 2b.ii-iv) .

The oligonucleotides used as primers in PCR reactions are listed in Appendix A.

For PCR, oligonucleotide primers were used at a concentration of 20 μM, which is equivalent to 1 n[illegible]in 50 μl.

18mer oligonucleotides came prepared at an A_{260} of about 20, where an OD unit of 1 = 20 μg DNA ml^{-1}.

Therefore an A_{260} of 20 = 400 μg DNA ml^{-1}, which is equivalent to 0.4 μg DNA μl^{-1}.

0.4 μg DNA μl^{-1} is equivalent to a concentration of 80 pM μl^{-1}, which is 0.08 n[illegible]μl^{-1}.

Therefore, used 2.5 μl per reaction.

1 Reaction buffer:

10 mM Tris. Cl (pH 8.3)
50 mM KCl
1.5 mM $MgCl_2$
0.01% gelatin.

2 dNTP mix:

Working solution of dNTP's was made up from 125 μl each of 10 mM dATP, dCTP, dGTP, and dTTP (Pharmacia 27-2035-01): made up to 1 ml with H_2O.

2 i Primer extension

A method to determine the site of transcriptional initiation was developed by myself, based on the methods of Jones *et al.* (1988) and Sambrook *et al.* (1989).

The method works by annealing a labelled antisense oligonucleotide to the mRNA of the gene of interest, and extending this 3' to 5', using reverse transcriptase, to the 5' end of the mRNA. The size of this extended product is measured on a denaturing gel, and the site of transcriptional initiation estimated relative to the oligonucleotide's position.

Labelling and purification of oligonucleotide

1. Antisense oligonucleotides were end labelled using T_4 polynucleotide kinase (PNK) and [γ–^{32}P]ATP (Section 2d.ii):

Oligonucleotide (10 pmole μl^{-1})	1.0 µl
PNK Buffer	2.0 µl
[γ–^{32}P]ATP 5000Ci mmole^{-1}	3.0 µl
H_2O	13.4 µl.

2. To this 10 units (1 µl) of T_4 PNK (Gibco-BRL 8004SA) was added, mixed, and incubated at 25°C for 20 minutes.
3. Reactions were stopped by heating the mixture at 70°C for 10 minutes.
4. 20 µl of loading buffer was added to each reaction and this incubated at 80°C for three minutes and loaded into a 19% urea-acrylamide gel[1]. Gels were run at 200 volts until the xylene cyanol had run about 5 cm: an 18mer oligonucleotide runs just behind the bromophenol blue at this acrylamide concentration.
5. Gels were removed, wrapped in Saran-Wrap and exposed to X-ray film for 1 minute. This located the labelled oligonucleotide.
6. Labelled oligonucleotides were cut out of the gel, placed in Eppendorf tubes, mashed with a tooth pick, 1 ml of elution buffer[2] added, and inverted overnight at room temperature.
7. Gel fragments were centrifuged at high speed for 1 minute, and

supernatants transferred to other Eppendorf tubes. Another 500 µl of elution buffer was added to the gel fragments, mixed, and spun again; the supernatants were removed and placed in other Eppendorf tubes. 0.5 µl of carrier glycogen (20 mg ml^{-1}, Boehringer Mannheim 12463420) and two volumes of ethanol was added to each tube, these frozen for 20 minutes at -20°C, and spun at high speed in the cold for 30 minutes.

8. Supernatants were discarded and the pellets re-dissolved in 200 µl of DEPC H_2O.
9. Pellets were sodium acetate precipitated and rinsed with -20°C 90% ethanol (v/v), dried, and re-dissolved in 100 µl of DEPC H_2O (Section 2b.iii).

Primer extension

1. 50µl of labelled oligonucleotide was mixed with 20 µg of total *S. pombe* RNA (Section 2e.vi), sodium acetate precipitated, and the pellet rinsed with 100 µl 70% ethanol with DEPC H_2O , and dried.
2. Pellets were dissolved in 10 µl of hybridization buffer[3] containing 35 units (1 µl) of RNAguard (Pharmacia 27-0815-01): this was covered with 30 µl of light mineral oil (Sigma M-3516), and incubated at 70°C for 5 minutes and 50°C for 1 hour.
3. 10 units (1µl) of reverse transcriptase (AMV, Promega) was added to 25 µl of reverse transcriptase buffer[4], this added to the hybridization mixture through the mineral oil, and incubated at 42°C for 1 hour.
4. The reaction was stopped by heating to 70°C for 10 minutes.
5. 5 µl of RNAase[5] was added and incubated at 42°C for 15 minutes.
6. The bottom phase was transferred to another Eppendorf tube and 200 µl of TE added: this was phenol/chloroform extracted twice, to give a final volume of 200 µl (Section 2b.ii).
7. Solution was sodium acetate precipitated, the pellet washed with 70% ethanol, and dried at room temperature (Section 2b.iii).
8. Pellets were dissolved in 3 µl of TE and 3 µl of loading buffer[5] added.
9. DNA was denatured by heating to 75°C for 3 minutes, loaded onto a 6% urea acrylamide gel (Section 2g.iv), and run in parallel with a sequencing reaction using the same oligonucleotide (Section 2g.iii).

10. The gel was dried on a vacuum drier, and processed for autoradiography (Section 2c.ix).

1 19% acrylamide Gel:

Acrylamide: bisacrylamide (19:1)	37.5 ml
Urea	31.5 g
TBE	7.5 ml
H_2O	7.5 ml.

Polymerized by the addition of 1 ml of freshly prepared 10% (w/v) ammonium persulphate (Sigma A-9164), 50 µl of TEMED (N,N,N',N'-tetramethylethylenediamine, Bio-Rad 61-0800), and poured immediately.

2 Elution buffer:

	stock	100 ml
0.5 M NH_4OAc		3.85 g
10 mM MgOAc		214 mg
1 mM EDTA	0.5 M	200 µl
0.1% (w/v) SDS		100 mg.

3 Hybridization buffer:

	stock	
0.25 M KCl		186 mg
10 mM Tris. Cl (pH 8.0)	1 M	100 µl
1m M EDTA	0.5 M	20 µl
DEPC H_2O		9. 88 ml.

4 Reverse transcriptase buffer:

	stock	1 ml
20 mM Tris. Cl (pH 8.5)	1 M	20 µl
10 $MgCl_2$	1 M	10 µl
0.25 mM dNTPs each	10 mM	25 µl
5 mM DTT	1 M	5 µl
DEPC H_2O		865 µl.

5 Loading buffer:

Loading buffer from the Pharmacia T7 sequencing kit was used (Section 2g.iii).

2j Bandshift assay

The bandshift method permits the assay of *in vitro* binding properties of proteins to DNA fragments (Fried & Crothers, 1981; Garner & Revzin, 1981).

The DNA fragment is radioactively labelled and mixed with protein extract. This mixture is subjected to electrophoresis under non-denaturing conditions. Labelled 'free' DNA runs in the gel proportional to its size and forms a band. Any protein that binds to the DNA sufficiently strongly will be carried into the gel. This DNA-protein complex will be larger than the 'free' DNA, and consequently runs less far: the band will be shifted to form a new band, and hence the name of the technique.

Specificity of binding of the protein to the DNA can be tested by adding unlabelled competitor DNAs to the reaction mix, prior to electrophoresis. If the competitor DNA binds protein in any of the shifted bands, they disappear.

End-labelling of DNA fragment

1. DNA fragments were end labelled using polynucleotide kinase and [γ-^{32}P]ATP (Section 2d.ii); and purified using a G-50 column (Section 2d.iii), to a specific activity of 1000 cps per ng of DNA.

Bandshift

1. 4% acrylamide gels (Section 2c.iv) in 0.6% TBE, using vertical moulds, were pre-run at 180 V for 30 minutes.
2. To Eppendorf tubes on ice was added:

binding buffer[1]	5 µl
poly dI. dC[2]	1 µl
fission yeast protein	5-15 µg (Section 2e.vii)
H_2O	to 10 µl.

3. Samples were gently mixed, and incubated on ice for 5 minutes.
4. 1 µl of end-labelled DNA fragment was added to each tube, mixed gently and incubated at room temperature for 5 minutes.

5. Wells of the acrylamide gel were washed with running buffer, and 5 µl of each sample loaded.
6. Gels were run for 2 hours at 180 V.
7. Gel plates were split, the gel transferred to Whatman filter paper (3MM), covered in SaranWrap, dried on a vacuum gel-drier, and processed for autoradiography (Section 2c.ix).

1 Binding buffer:

	stock	250 µl
50 mM KCl	1 M	25 µl
50 mM Tris. HCl (pH 7.5)	1 M	25 µl
50% (v/v) glycerol		125 µl
2 mM DTT	1 M	2 µl
2 mM PMSF	100 mM	2 µl
25 mM $MgCl_2$	1 M	12.5 µl
Protease Inhibitors	(see 2e.vii)	2.5 µl of each
H_2O		75 µl.

2 Poly dI. dC (Pharmacia 27-7880):
dissolved at a concentration of 1 µg μl^{-1}, and sonicated until ~400 nucleotides in length.

Chapter 3: Sequence analysis of *cdc22+*

3a Introduction

Why sequence a gene? The nucleotide sequence of a gene can provide a considerable amount of information, such as genetic structure, predicted amino acid sequence, and gene function. Furthermore, sequence data permits more molecular analysis as it creates an exhaustive restriction map, allowing such manipulations as deletion and over-expression of the gene.

The genetic structure of the gene can be inferred from the nucleotide sequence. An open reading frame (ORF) can be predicted, initiating with a start codon and terminating with a stop codon. Consensus nucleotide sequences are known in some organisms for the branch and splice sites of intervening sequences (introns), and so their presence interrupting the ORF can be detected.

The ORF provides information about the precise size and amino acid composition of the predicted gene product. The function of some genes can be suggested by comparing the peptide sequence with other sequences of known biochemical function in a data base. Three dimensional structures can often be postulated from the peptide sequence: such structures can sometimes give information on potential functions for the gene product. For example, regions of hydrophobic amino acids within polypeptides are often associated with transmembrane domains; and conserved motifs in DNA binding proteins, such as zinc fingers, can be suggested.

Sequencing data on flanking regions of the gene can provide information on control of expression of the gene. 5' to the gene, potential *cis*-acting transcriptional control regions such as TATA boxes and upstream activating sequences (UAS) can be identified. 3' to the gene, DNA sequences thought to be important in mediating mRNA stability and processing, such as polyadenylation, may be present.

This chapter describes the determination of the nucleotide sequence of the *cdc22+* gene, and a comparative sequence analysis of the predicted polypeptide product.

3b Experimental strategy

The original plasmid containing *cdc22*$^+$, pCDC22-1, consists of two adjacent *Hin*dIII genomic fragments cloned into the *Hin*dIII site of pDB262, which have been shown to be co-linear in the genome (Gordon & Fantes, 1986).

The two *Hin*dIII fragments were subcloned into the polylinker *Hin*dIII site of pTZ18R (Section2b.v), with each fragment cloned in both orientations, and named pCDC22-7,pCDC22-10, pCDC22-5, and pCDC22-14 (Fig. 3A). These subclones were made for sequencing, as the pTZ vector contains both the f1 origin of replication which permits the generation of single-stranded DNA used in the chain termination sequencing method of Sanger *et al.* (1977), and the reverse sequencing primer adjacent to the polylinker for priming sequencing reactions.

An extensive map of restriction enzymes that cut pCDC22-1 was constructed for subsequent subcloning, and is shown in Figure 3A.

To prepare a series of subclones suitable for sequencing, nested deletions were made of the four *Hin*dIII subclones using Exonuclease III, by the method of Henikoff (1984). The method used for the construction of the nested deletions is described in Section 2g.i.

Additionally, three sub-clones of pCDC22-1 were made for sequencing. The first subclone was made to permit sequencing across the central *Hin*dIII junction. pCDC22-1 was digested with *Pst*I and *Xba*I, and the resultant DNA ligated into similarly cut pBluescript II SK. *Sal*I/*Xba*I and *Bam*HI/*Xba*I subclones, for sequencing from the *Sal*I and *Bam*HI restriction sites, respectivley, were made by digestion with the appropriate restriction enzymes, and ligated into similarly digested pBluescript II SK.

Where no sub-clones or nested deletions were available, synthetic oligonucleotides were used as primers to initiate sequencing reactions.

The position and orientation of deletions, subclones and oligonucleotides used in the sequencing strategy are summarised in Figure 3B.

Once suitable sub-clones had been generated, they were used for the generation of single-stranded DNA (Section 2g.ii). Single-stranded DNA was used in sequencing reactions, and the sequence determined by urea-

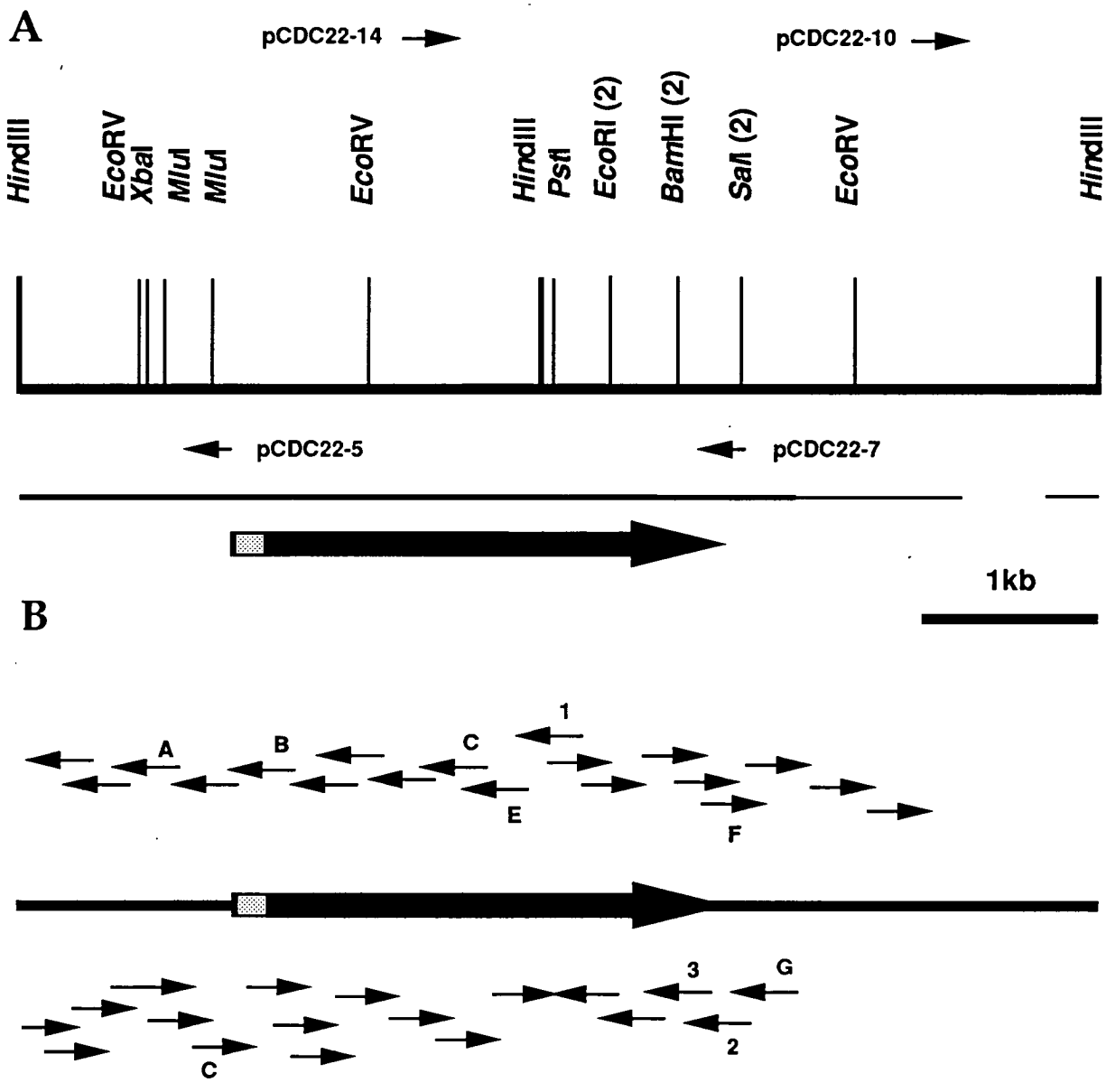


Figure 3 **Restriction map and sequencing strategy of pCDC22-1.**

A Outline map of restriction enzymes that cut pCDC22-1. Enzymes that do not cut pCDC22-1 include *Kpn*I, *Sma*I, and *Xho*I. Position and orientation of pCDC22-1 *Hin*dIII subclones are shown (small arrows). The region sequenced on both strands (line) and one strand (thin line), predicted *cdc22*$^+$ open reading frame (thick arrow), and intron (dotted) are indicated.

B Sequencing strategy for pCDC22-1. Arrows indicate approximate length of each subclone sequenced. Numbers indicate subclones referred to in the text: 1, *Pst*I/*Hin*dIII; 2, *Sal*I/*Xba*I; 3, *Bam*HI/*Xba*I. Letters indicate oligonucleotides - see Appendix A. Remainder indicate Exo III subclones.

acrylamide gel electrophoresis, followed by the detection of radio-labelled DNA by autoradiography (Sections 2g.iii and iv.). Routinely, between 300 and 500 nucleotides were obtained from each sequencing reaction. Regions of sequence overlap between adjacent clones were ascertained; and the sequences assembled to form the whole continuous sequence, using the UWGCG Gelassemble program.

3 c Sequence

DNA was sequenced on both strands for 4136 base pairs, from the left-hand *Hin*dIII site to beyond the right-hand *Sal*I site (Fig. 3A). The nucleotide sequence, sequenced on both strands, is shown in Figure 3C. Additionally, DNA was sequenced on one strand for a further 912 base pairs from beyond the right-hand *Sal*I site to beyond the right-hand *Eco*RV site, and for 312 nucleotides from the right-hand *Hin*dIII site (Fig. 3A).

Initial confirmation that the DNA fragment sequenced was correct, was provided by the presence and distribution of restriction enzyme recognition sites that agreed with those sites that had previously been mapped. The only surprise was the repetition of pairs of close sites cut by the same restriction enzyme, such as *Bam*HI, *Sal*I, and *Eco*RI (Fig. 3C). These extra sites were not detected by mapping since, being so close to one another, they released only small DNA fragments (about 100 nucleotides) after digestion, that were not detected by agarose gel electrophoresis.

i Open reading frame

An open reading frame (ORF) for *cdc22*$^+$ of 2750 nucleotides is contained within the sequenced region, if the presence of a single intron is taken into account (Fig. 3D).

The putative start codon (ATG) most likely to initiate translation is indicated in Figure 3C and 3D. 5' to the ATG is a region of 133 nucleotides containing no ATG's in any reading frame, or consensus sequences for fission yeast introns (Section 3c.ii), and stop codons in all three frames.

Translation has been shown to start at the first ATG codon of the ORF of genes, in the majority of cases (Kozak, 1983). An optimum

Figure 3 C Nucleotide sequence of pCDC22-1.

Nucleotide sequence of pCDC22-1 sequenced on both strands. Sites cut by restriction enzymes referred to in the text are underlined, and predicted start and stop codons are in bold typeface.

*Hin*dIII

-1307 AAGCTTTCTCACAGCACGTAATTGCTTATTATCGTGGAACTTTAGCTGGGTCTCCAATTT -1248

-1247 TGCACTAGAATTATTGTAGAAAATTGAAGCGACATTATCTCTGTTGATAACCTTGCTGGT -1188

-1187 ATACAGGCGCTTGGTATTTTTATACACGTTGATAGTCGAAGTAGCAGATAGCCGTAGTTG -1128

-1127 ATGGTTTAAACATTTTGTATTTTGCGGAAATTTGCACACTGTTTATATCTCATTCTCTTT -1068

-1067 ACGTCAAGCATACCAATGTTTGCAATTTCATGTTTTGAAGGTGACCGTTGTTATTCTATC -1008

-1007 TATGCATTTGTATTACCATTTGATTTTCACTCTGTCTACAGGCAAGATTATCAGATGAAG -948

-947 ATATAAGCATTGTTGACTTTACCTAAAAAAAAAGGATAATTGCGAGGGAGCAAATAGTGAA -888

-887 TTAAAGGGAAAAGAACAAAGTTTTTGTAGCTACTAAAAGCTGTTTGTCTATCTCTATGTG -828

-827 TACCTTTTATAGTGCTTATAAGTAAAATGCAGAGTCGAAATCTTCATGCTTTAATGTAAA -768

-767 GCATTAGTAAGGTTACATATTACAACAGAAATGGGAATCAAAATCTTAAATCTAAAAGTT -708

-707 GAGAGGTCTTAACAAAAAAGCTAACTCAACTTAGCTTTACAATTTATAAATCAAGGAAAC -648

-647 AATACTTTATCAATTGTATGTTATGATCTTCTCCAACAGGTAATTGACAACATCAAAAAG -588

*Eco*RV *Xba*I

-587 TAATATAAAGAACAAGGGGAAATTGATATCTAGATATATTAGAGAAATTAGTAGTTCAAT -528

-527 CTCATAGAGCAGGTTGGTAGTCGGGTTGGACCGCGTGTTTAATTTATGTAAACAGTCGCG -468

*Mlu*I

-467 TCGCGTTGCAATTGAGACGCGTAAATAAATATTTAATTTATTACATTCAGTCGTAAACAG -408

-407 AGTATTTATAAACACTTTTTTTATGTTTAATAAAAGATAAATGTAACAGTTGAATGTATT -348

-347 GTATCAGGTCAGACCACTTCAACATGTTTAATCGCGTTTTTTTAAAAAAATATTTTTTTA -288

-287 ATTTTTAAAAGTCGGACTTATTTTAGCGGAACTTTGATGTTCAGAAGTGAAAAAGATAAA -228

-227 TCTATTTAGCAAGTCTTAATTAACGTCTTTTAGATATAGTAGAGCTACAAAAATGATCCG -168

-167 GTTTCCACTCTTAGCTTTATTTACATTGATCAACATGACTTAAAGTTCGGATGACGCGAC -108

*Mlu*I

-107 GGGCCACATCTCAACCGGTTTAATTAATTAGCGTGACGCGTCTGAACGCTTTTTCATTTA -48

-47 CTATAAATATTCCCGGTATTTACCACCTTACAAACTAGAACAAACACG**ATG**TTTGTATAC 12

13 AAAAGAGGTATGAAACAGGGAAATAAAATACAGTTAGAAGATTAGTAGAATTTTGTTACA 72

73 CGTTGCCGTTTCGAAAAATGTCGTGATTTTACTGGGGAGTAAATGTTTAAAGAAAGTGAG 132

133 ATTTTTCTATTTGTTTAAGCGTGTCATTCAGTATTCACTGTTTGTTGTGTGCAGAGATGT 192

193 TTAAGCAAATGTTCTTTTAGCCGTTTGCTCTACTTCCAAGTGTTCGGTTTGATTGCTGAC 252

253 AGATTCGTTGCTGGTGGCGTTGTATTAAAATTGACTGACTGTTGACATTGAATCGAAAAG 312

313 CAATAATGTACTAACTGTATAGACGGACGTCAAGAGAAAGTGGCCTTTGACAAAATCACT 372

373 GCTCGTGTTTCGAGGCTTTGTTATGGCCTTGATTCCGATCATGTTGATCCCGTCGAAATC 432

433 ACTCAAAAAGTCATTTCTGGTGTGTATCCCGGTGTTACCACTATTGAATTGGATAATTTA 492

493 GCTGCTGAAACTGCCGCGACTATGACTACAAAGCATCCTGATTATGCTATTTTGGCTGCT 552

553 AGAATTGCTGTTTCCAACTTGCATAAGCAAACCGAGAAGGTGTTTTCAACTGTCGTTCAA 612

613 CAGCTTCATGACTATGTAAATCCCAAAACAGACAAGCCTGCCCCCATGATCTCTGACAAA 672

*Eco*RV

673 ATCTACGATATCGTAATGAAACACAAGGACGAATTAGATAGTGCTATCATATACGATCGT 732

733 GATTTCACTTACAATTTCTTTGGCTTCAAGACTTTAGAGCGTTCTTATTTATTGCGTATC 792

793 GACGGCAAAGTTGCTGAGCGCCCTCAGCATATGATTATGCGTGTCGCCGTTGGTATCCAC 852

853 GGTGAGGACATTGAAGCTGCTATTGAAACATACAATTTGATGTCTCAACGCTACTTTACC 912

913 CATGCTTCACCAACTCTTTTCAATGCCGGTACTCCTCGTCCCCAACTCTCTTCTTGTTTC 972

973 CTCGTTACGATGAAAGACGACTCTATTGAAGGAATCTACGACACACTCAAGATGTGTGCT 1032

1033 ATGATCTCCAAGACCGCTGGCGGTATTGGTATTTATATTCACAACATTAGAGCAACCGGT 1092

1093 TCGTATATTGCTGGAACTAATGGTACTTCCAACGGAATTGTCCCCATGATTCGTGTTTAC 1152

1153 AACAACACAGCTCGTTATGTTGACCAAGGAGGTAATAAGCGTCCTGGTGCCTTTGCAGCC 1212

1213 TATTTGGAGCCTTGGCATGCCGATGTTATGGACTTTTTAGAACTTCGAAAGACTCATGGC 1272

1273 AACGAGGATTTCCGCGCACGGCAAATGTTCTATGCCTTGTGGATTCCTGACTTGTTTATG 1332

1333 CAACGTGTTGAACGTAACGAGCAATGGACCTTCTTCTGCCCCAATGAGGCTCCAGGTTTG 1392

1393 GCAGATGTTTGGGGTGACGAGTTTGTTGCCCTCTACGAAAAGTATGAGAAGGAGAACCGT 1452

1453 GGTCGTCGTTCATTACCTGCTCAAAAAGTTTGGTACGCCATTCTTCAATCACAAGTTGAA 1512

1513 ACTGGTAATCCTTTCATGTTGTATAAGGATTCATGCAACCGTAAGTCCAATCAGAAGAAT 1572

1573 GTTGGTACTATTCGCAGCTCAAACCTGTGTACTGAGATTGTTGAATACTCTTCTCCTGAC 1632

1633 GAAGTTGCTGTTTGTAACCTTGCTTCAGTTGCTTTGCCCACATTTATTAAAGACGGAAAG 1692

*Hin*dIII

1693 TACAATTTCCAAAAGCTTCACGATGTCGTGAAGGTCGTTACTCGTAACCTCAACAAGATT 1752

1753 ATCGACGTTAACTATTATCCTGTTCCTGAAGCTCGCCGTAGCAATATGCGCCATCGTCCC 1812

1813 GTTGGTTTGGGTGTTCAAGGTCTTGCCGATGCTTTCTTCGCTCTTCGTTTACCATTCGAA 1872

*Pst*I

1873 TCTGCAGGTGCCAAGAAGCTAAACATCCAAATCTTCGAAACAATTTACCACGCAGCCCTT 1932

1933 GAGGCCTCTTGCGAGATTGCCCAGGTAGAGGGTACATATGAGAGTTATGAAGGATCTCCC 1992

1993 TGCATCCAGGGTATTTTACAGTATGACATGTGGAACGTTAATCCCACCGATCTTTGGGAT 2052

*Eco*RI

2053 TGGGCCGAGTTCAAGGAAAAGATTGCTAAACACGGAATTCGTAATTCTTTGTTAGTAGCA 2112

2113 CCAATGCCTACTGCTTCAACATCTCAAATCCTTGGTTTCAATGAGTGTTTTGAGCCATAT 2172

*Eco*RI

2173 ACTTCTAACATGTACCAACGCCGTGTTCTTTCTGGTGAATTCCAAATTGTCAATCCATGG 2232

2233 CTGTTGAAGGATCTCGTTGAGCGTGATTTGTGGAATGAGGACATGAAGAACAAACTTGTT 2292

*Bam*HI

2293 ATGCTTGATGGATCCATCCAAGCGATTCCTGAAATTCCTCAAGACCTTAAGGATCTTTAC 2352

2353 AAAACCGTATGGGAGATTAGTCAAAAGACTGTTATTGACTATGCTGCCGACCGTGGCCCA 2412

*Bam*HI

2413 TTCATTGATCAATCCCAAAGTTTGAACATTCACTTGAAGGATCCTTCCTACGGAAAAATT 2472

2473 ACTAGTATGCACTTTTATGGCTGGAAGAAGGGTCTTAAGACTGGAATGTACTATCTTCGT 2532

*Sal*I

2533 ACTATGGCTGCTTCTGCTGCTATCAAATTTACGGTCGACCCTGTGGCCTTGCGCGCTCGT 2592

2593 AATGAAGAATCTAATGAAGAGAATAAGAAGCCAGTTATAAAGAATGGAAAGGCTGAAATT 2652

2653 TCTGCAGAGCCCACTAAAGAAGAGATTGACATTTATAATGAAAAAGTGCTCGCATGCTCC 2712

2713 ATCAAGAACCCTGAGGCTTGCGAAATGTGCTCAGCC**TAA**TTTACTGTTGCAAAAATGGAT 2772

2773 TTGTTAAATCCCATCCATGTTTAATATCCTACATTTTATTGTTTCTTTTAATGTTTTTAT 2832

*Sal*I

2833 TCAATATTCTTTTCATTTGTCGACATACATAATTGTGTCTTAAATTCAACCTTGGTTTTC 2892

2893 CATTCTAGTGAGCAGGCTCAGAATGGTTCTAATACATAAATTAGATTACGAATT 2946

sequence for the site of initiation of translation by higher eukaryotic ribosomes has been determined (Kozak, 1986), with a consensus of 5'-ACCATGG-3'. The base sequence that includes the proposed ATG of *cdc22*+ is 5'-ACGATGT-3', and shows some similarity (2 nucleotides out of 4). The ORF terminates with a stop codon (TAA) 2750 nucleotides downstream of the proposed start codon.

The length of *cdc22*+ transcript found in fission yeast is 3.3 kb (Fig. 4E, Gordon & Fantes, 1986), sufficient to contain the predicted translated region of 2433 nucleotides.

Corroboration of the predicted *cdc22*+ ORF is provided by the high similarity of the predicted peptide sequence with the large subunit of ribonucleotide reductase (RNR) in different organisms (Section 3d). The large subunit of RNR is remarkably constant in size and sequence in a number of organisms, and the predicted *cdc22*+ polypeptide is consistent with these.

ii Intron

The *cdc22*+ ORF is interrupted by a single putative intron of 315 nucleotides in length (Fig. 3D). This intron starts at nucleotide 19 of the coding region, and finishes at nucleotide 333. Within it are sequences showing high similarity with intron splicing regions in fission yeast (Russell, 1989). The putative 5' splice site at nucleotides 19-23 is GTATG (consensus GTANG); the branch site at nucleotides 320-326 is TACTAAC (consensus NNCTPuAN); and the 3' splice site at nucleotides 331-326 is TAG (consensus NAG). The distance between the branch site and the 3' end of the intron is 14 base pairs, within the consensus range of 11-23.

Two independent pieces of evidence support the position and size of the putative intron in *cdc22*+.

Evidence supporting the position of the intron is given by the similarity between the predicted peptide sequence of *cdc22*+ with the large subunit of ribonucleotide reductase (Section 3d). The amino acid sequence across the predicted intron splice sites is highly conserved (KRDGR, Fig. 3D), and the intron spliced out of the predicted *cdc22*+ ORF would result in exactly this peptide sequence.

Figure 3 D Nucleotide sequence and predicted peptide sequence of *cdc22+*.

Nucleotides are numbered relative to the predicted start codon. Predicted start and stop codons are in bold typeface. Predicted intron is italisized, with consensus splice and branch sequences underlined.

```
-44  AAATATTCCCGGTATTTACCACCTTACAAACTAGAACAAACACGATGTTTGTATACAAAA  15
                                                  M  F  V  Y  K  R

 16  GAGGTATGAAACAGGGAAATAAAATACAGTTAGAAGATTAGTAGAATTTTGTTACACGTT  75
       D/
 76  GCCGTTTCGAAAAATGTCGTGATTTTACTGGGGAGTAAATGTTTAAAGAAAGTGAGATTT  135

136  TTCTATTTGTTTAAGCGTGTCATTCAGTATTCACTGTTTGTTGTGTGCAGAGATGTTTAA  195

196  GCAAATGTTCTTTTAGCCGTTTGCTCTACTTCCAAGTGTTCGGTTTGATTGCTGACAGAT  255

256  TCGTTGCTGGTGGCGTTGTATTAAAATTGACTGACTGTTGACATTGAATCGAAAAGCAAT  315

316  AATGTACTAACTGTATAGACGGACGTCAAGAGAAAGTGGCCTTTGACAAAATCACTGCTC  375
                     /D  G  R  Q  E  K  V  A  F  D  K  I  T  A  R

376  GTGTTTCGAGGCTTTGTTATGGCCTTGATTCCGATCATGTTGATCCCGTCGAAATCACTC  435
       V  S  R  L  C  Y  G  L  D  S  D  H  V  D  P  V  E  I  T  Q

436  AAAAAGTCATTTCTGGTGTGTATCCCGGTGTTACCACTATTGAATTGGATAATTTAGCTG  495
       K  V  I  S  G  V  Y  P  G  V  T  T  I  E  L  D  N  L  A  A

496  CTGAAACTGCCGCGACTATGACTACAAAGCATCCTGATTATGCTATTTTGGCTGCTAGAA  555
       E  T  A  A  T  M  T  T  K  H  P  D  Y  A  I  L  A  A  R  I

556  TTGCTGTTTCCAACTTGCATAAGCAAACCGAGAAGGTGTTTTCAACTGTCGTTCAACAGC  615
       A  V  S  N  L  H  K  Q  T  E  K  V  F  S  T  V  V  Q  Q  L

616  TTCATGACTATGTAAATCCCAAAACAGACAAGCCTGCCCCCATGATCTCTGACAAAATCT  675
       H  D  Y  V  N  P  K  T  D  K  P  A  P  M  I  S  D  K  I  Y

676  ACGATATCGTAATGAAACACAAGGACGAATTAGATAGTGCTATCATATACGATCGTGATT  735
       D  I  V  M  K  H  K  D  E  L  D  S  A  I  I  Y  D  R  D  F

736  TCACTTACAATTTCTTTGGCTTCAAGACTTTAGAGCGTTCTTATTTATTGCGTATCGACG  795
       T  Y  N  F  F  G  F  K  T  L  E  R  S  Y  L  L  R  I  D  G

796  GCAAAGTTGCTGAGCGCCCTCAGCATATGATTATGCGTGTCGCCGTTGGTATCCACGGTG  855
       K  V  A  E  R  P  Q  H  M  I  M  R  V  A  V  G  I  H  G  E

856  AGGACATTGAAGCTGCTATTGAAACATACAATTTGATGTCTCAACGCTACTTTACCCATG  915
       D  I  E  A  A  I  E  T  Y  N  L  M  S  Q  R  Y  F  T  H  A

916  CTTCACCAACTCTTTTCAATGCCGGTACTCCTCGTCCCCAACTCTCTTCTTGTTTCCTCG  975
       S  P  T  L  F  N  A  G  T  P  R  P  Q  L  S  S  C  F  L  V

976  TTACGATGAAAGACGACTCTATTGAAGGAATCTACGACACACTCAAGATGTGTGCTATGA  1035
       T  M  K  D  D  S  I  E  G  I  Y  D  T  L  K  M  C  A  M  I

1036 TCTCCAAGACCGCTGGCGGTATTGGTATTTATATTCACAACATTAGAGCAACCGGTTCGT  1095
       S  K  T  A  G  G  I  G  I  Y  I  H  N  I  R  A  T  G  S  Y

1096 ATATTGCTGGAACTAATGGTACTTCCAACGGAATTGTCCCCATGATTCGTGTTTACAACA  1155
       I  A  G  T  N  G  T  S  N  G  I  V  P  M  I  R  V  Y  N  N

1156 ACACAGCTCGTTATGTTGACCAAGGAGGTAATAAGCGTCCTGGTGCCTTTGCAGCCTATT  1215
       T  A  R  Y  V  D  Q  G  G  N  K  R  P  G  A  F  A  A  Y  L

1216 TGGAGCCTTGGCATGCCGATGTTATGGACTTTTTAGAACTTCGAAAGACTCATGGCAACG  1275
       E  P  W  H  A  D  V  M  D  F  L  E  L  R  K  T  H  G  N  E

1276 AGGATTTCCGCGCACGGCAAATGTTCTATGCCTTGTGGATTCCTGACTTGTTTATGCAAC  1335
       D  F  R  A  R  Q  M  F  Y  A  L  W  I  P  D  L  F  M  Q  R

1336 GTGTTGAACGTAACGAGCAATGGACCTTCTTCTGCCCCAATGAGGCTCCAGGTTTGGCAG  1395
       V  E  R  N  E  Q  W  T  F  F  C  P  N  E  A  P  G  L  A  D

1396 ATGTTTGGGGTGACGAGTTTGTTGCCCTCTACGAAAAGTATGAGAAGGAGAACCGTGGTC  1455
       V  W  G  D  E  F  V  A  L  Y  E  K  Y  E  K  E  N  R  G  R
```

```
1456  GTCGTTCATTACCTGCTCAAAAAGTTTGGTACGCCATTCTTCAATCACAAGTTGAAACTG  1515
        R  S  L  P  A  Q  K  V  W  Y  A  I  L  Q  S  Q  V  E  T  G

1516  GTAATCCTTTCATGTTGTATAAGGATTCATGCAACCGTAAGTCCAATCAGAAGAATGTTG  1575
        N  P  F  M  L  Y  K  D  S  C  N  R  K  S  N  Q  K  N  V  G

1576  GTACTATTCGCAGCTCAAACCTGTGTACTGAGATTGTTGAATACTCTTCTCCTGACGAAG  1635
        T  I  R  S  S  N  L  C  T  E  I  V  E  Y  S  S  P  D  E  V

1636  TTGCTGTTTGTAACCTTGCTTCAGTTGCTTTGCCCACATTTATTAAAGACGGAAAGTACA  1695
        A  V  C  N  L  A  S  V  A  L  P  T  F  I  K  D  G  K  Y  N

1696  ATTTCCAAAAGCTTCACGATGTCGTGAAGGTCGTTACTCGTAACCTCAACAAGATTATCG  1755
        F  Q  K  L  H  D  V  V  K  V  V  T  R  N  L  N  K  I  I  D

1756  ACGTTAACTATTATCCTGTTCCTGAAGCTCGCCGTAGCAATATGCGCCATCGTCCCGTTG  1815
        V  N  Y  Y  P  V  P  E  A  R  R  S  N  M  R  H  R  P  V  G

1816  GTTTGGGTGTTCAAGGTCTTGCCGATGCTTTCTTCGCTCTTCGTTTACCATTCGAATCTG  1875
        L  G  V  Q  G  L  A  D  A  F  F  A  L  R  L  P  F  E  S  A

1876  CAGGTGCCAAGAAGCTAAACATCCAAATCTTCGAAACAATTTACCACGCAGCCCTTGAGG  1935
        G  A  K  K  L  N  I  Q  I  F  E  T  I  Y  H  A  A  L  E  A

1936  CCTCTTGCGAGATTGCCCAGGTAGAGGGTACATATGAGAGTTATGAAGGATCTCCCTGCA  1995
        S  C  E  I  A  Q  V  E  G  T  Y  E  S  Y  E  G  S  P  C  I

1996  TCCAGGGTATTTTACAGTATGACATGTGGAACGTTAATCCCACCGATCTTTGGGATTGGG  2055
        Q  G  I  L  Q  Y  D  M  W  N  V  N  P  T  D  L  W  D  W  A

2056  CCGAGTTCAAGGAAAAGATTGCTAAACACGGAATTCGTAATTCTTTGTTAGTAGCACCAA  2115
        E  F  K  E  K  I  A  K  H  G  I  R  N  S  L  L  V  A  P  M

2116  TGCCTACTGCTTCAACATCTCAAATCCTTGGTTTCAATGAGTGTTTTGAGCCATATACTT  2175
        P  T  A  S  T  S  Q  I  L  G  F  N  E  C  F  E  P  Y  T  S

2176  CTAACATGTACCAACGCCGTGTTCTTTCTGGTGAATTCCAAATTGTCAATCCATGGCTGT  2235
        N  M  Y  Q  R  R  V  L  S  G  E  F  Q  I  V  N  P  W  L  L

2236  TGAAGGATCTCGTTGAGCGTGATTTGTGGAATGAGGACATGAAGAACAAACTTGTTATGC  2295
        K  D  L  V  E  R  D  L  W  N  E  D  M  K  N  K  L  V  M  L

2296  TTGATGGATCCATCCAAGCGATTCCTGAAATTCCTCAAGACCTTAAGGATCTTTACAAAA  2355
        D  G  S  I  Q  A  I  P  E  I  P  Q  D  L  K  D  L  Y  K  T

2356  CCGTATGGGAGATTAGTCAAAAGACTGTTATTGACTATGCTGCCGACCGTGGCCCATTCA  2415
        V  W  E  I  S  Q  K  T  V  I  D  Y  A  A  D  R  G  P  F  I

2416  TTGATCAATCCCAAAGTTTGAACATTCACTTGAAGGATCCTTCCTACGGAAAAATTACTA  2475
        D  Q  S  Q  S  L  N  I  H  L  K  D  P  S  Y  G  K  I  T  S

2476  GTATGCACTTTTATGGCTGGAAGAAGGGTCTTAAGACTGGAATGTACTATCTTCGTACTA  2535
        M  H  F  Y  G  W  K  K  G  L  K  T  G  M  Y  Y  L  R  T  M

2536  TGGCTGCTTCTGCTGCTATCAAATTTACGGTCGACCCTGTGGCCTTGCGCGCTCGTAATG  2595
        A  A  S  A  A  I  K  F  T  V  D  P  V  A  L  R  A  R  N  E

2596  AAGAATCTAATGAAGAGAATAAGAAGCCAGTTATAAAGAATGGAAAGGCTGAAATTTCTG  2655
        E  S  N  E  E  N  K  K  P  V  I  K  N  G  K  A  E  I  S  A

2656  CAGAGCCCACTAAAGAAGAGATTGACATTTATAATGAAAAAGTGCTCGCATGCTCCATCA  2715
        E  P  T  K  E  E  I  D  I  Y  N  E  K  V  L  A  C  S  I  K

2716  AGAACCCTGAGGCTTGCGAAATGTGCTCAGCCTAATTTACTGTTGCAAAAATGGATTTGT  2775
        N  P  E  A  C  E  M  C  S  A  *

2776  TAAATCCCATCCATGTTTAA  2795
```

Evidence supporting the size of the intron is provided by experiments mapping the start of transcription of *cdc22*+ by primer extension (Section 4b). Two oligonucleotides, one 5' and one 3' to the putative intron, were used to initiate separate reverse transcriptase reactions (Fig. 4A). Both reactions predict nearly identical start sites for transcription, provided the presence of the intron, 315 nucleotides in length, is allowed for.

iii 5' sequence

A detailed analysis of the nucleotide sequence 5' to the *cdc22*+ ORF will be given in the next chapter.

iv 3' sequence

Most higher eukaryotic mRNAs have a polyadenylated 3' terminus that starts approximately 20 base pairs downstream of a transcriptional terminator sequence related to 5'-AAUAAA-3'. Transcription may proceed beyond the terminator sequence, with endonucleolytic cleavage and poly-A addition generating the mature 3' end (Birnstiel *et al.*, 1985). Although nearly all higher eukaryotic genes contain the 5'-AATAAA-3' terminator sequence, many genes in budding and fission yeast lack the sequence altogether (Russell, 1989). In budding yeast an alternative terminator sequence 5'-TAG...TATGT...TTT-3' has been proposed that can be positioned up to 160 base pairs downstream of the stop codon (Zaret & Sherman, 1982). The nucleotide sequence 3' to *cdc22*+ does not contain either of these sequences, suggesting that it, and perhaps fission yeast genes in general, have different terminator sequences, which are at present unknown.

3 d Sequence comparisons

Translation of the predicted ORF, allowing for the presence of the intron, predicts a polypeptide of 92.2 kDa containing 811 amino acids.

The homology search algorithm FASTA (Lipman & Pearson, 1985) was used to search the NBRF and SwissProt protein sequence data bases for proteins showing similarity to the predicted *cdc22*$^+$ product. The search revealed that *cdc22*$^+$ encodes a predicted polypeptide with very high similarity to the large subunit of ribonucleotide reductase (RNR) from a number of organisms: alignment between the predicted gene products of *cdc22*$^+$, *RNR3* from budding yeast (Yagle & McEntee, 1990, Elledge & Davis, 1990), and M1 from mouse (Caras *et al.*, 1985) is shown in Figure 3E. The degree of similarity is very high throughout the coding region: the predicted *cdc22*$^+$ product shows 69% amino acid identity with the M1 and *RNR3* gene products.

RNR is an enzyme essential for DNA precursor metabolism, providing the only means of generating deoxynucleotides from the corresponding ribonucleotides (Thelander & Reichard, 1979). Its structure is highly conserved through evolution, and consists of a heterodimer of two large and two small subunits.

The predicted *cdc22*$^+$ polypeptide contains all the amino acid motifs thought essential for biological function of the large subunit of RNR, which include both catalytic and regulatory binding sites for deoxynucleotides and ribonucleotides (Eriksson & Sjoberg, 1989). These motifs include cysteines at positions 218, 429, and 444, involved in the active site; and the C-terminal cysteines at positions 806 and 809 thought to interact with thioredoxin and glutaredoxin (Fig. 3E).

The proposed function of *cdc22*$^+$ is consistent with the arrest point of *cdc22* mutants during the cell cycle. *cdc22*$^+$ is required at the G_1-S phase boundary (Nasmyth & Nurse, 1981), and it is at this stage when its transcript is maximally abundant (Gordon & Fantes, 1986). RNR is likely to be required at the onset of, and during S-phase, when DNA precursors are in high demand.

Figure 3 E **Comparison between the predicted *cdc22+* product and large subunits of ribonucleotide reductase from budding yeast and mouse.**

Alignment generated using the UWGCG Lineup program, between the *cdc22+* product (*S.pombe*), the *RNR3* gene product of budding yeast (*S. cerevisiae*), and the M1 subunit of mouse (*Mus*). Numbering relates to the *cdc22+* sequence. RNR represents a consensus sequence of the three: identity ($^3/_3$) is indicated by upper case letters; $^2/_3$ consensus by lower case. Conserved residues referred to in the text are boxed.

```
        1                                                        50
   RNR  M.ViKRDGRq E.V.FDKITs Ri.rLcYGLd .d.vDpv.iT qkvIsGvYsG
Spombe  MFVYKRDGRQ EKVAFDKITA RVSRLCYGLD SDHVDPVEIT QKVISGVYPG
Scerev  MYVIKRDGRK EPVQFDKITS RITRLSYGLD PNRIDAVKVT QRIISGVYSG
  Mus   MHVIKRDGRQ ERVMFDKITS RIQKLCYGLN MDFVDPAQIT MKVIQGLYSG

        51                                                      100
   RNR  VTTvELDnLA AETaAtmTTk HPDYAiLAAR IAvSNLHKqT .KvFS.V.ed
Spombe  VTTIELDNLA AETAATMTTK HPDYAILAAR IAVSNLHKQT EKVFSTVVQQ
Scerev  VTTVELDNLA AETCAYMTTV HPDYATLAAR IAISNLHKQT TKQFSKVIED
  Mus   VTTVELDTLA AETAATLTTK HPDYAILAAR IAVSNLHKET KKVFSDVMED

        101                                                     150
   RNR  LhdyiNP.tg khaPMisd.i ydIVM..kd. LnSAIiYDRD F.YnyFGFKT
Spombe  LHDYVNPKTD KPAPMISDKI YDIVMKHKDE LDSAIIYDRD FTYNFFGFKT
Scerev  LHDWINPATG KHAPMISDEI YNIVMETRY. LNSAIVYDRD FQYTYFGFKT
  Mus   LYNYINPHNG RHSPMVASST LDIVMANKDR LNSAIIYDRD FSYNYFGFKT

        151                                                     200
   RNR  LERSYLLrin GkVAERPQHm .MRVavGIHg eDIeaaieTY NLmS.ryFTH
Spombe  LERSYLLRID GKVAERPQHM IMRVAVGIHG EDIEAAIETY NLMSQRYFTH
Scerev  LERSYLLRLN GEVAERPQHL VMRVALGIHG SDIESVLKTY NLMSLRYFTH
  Mus   LERSYLLKIN GKVAERPQHM LMRVSVGIHK EDIDAAIETY NLLSEKWFTH

        201                                                     250
   RNR  AsPTLFNAGT prPQlSSCFL ..MKDDSIEG IYDTLK.CAm ISKtAGvl..
Spombe  ASPTLFNAGT PRPQLSSCFL VTMKDDSIEG IYDTLKMCAM ISKTAGVLVF
Scerev  ASPTLFNAGT PLPQMSSCFL IAMKDDSIEG IYDTLKECAM ISKTAGVL.S
  Mus   APPTLFNAGT NRPQLSSCFL LSMKDDSIEG IYDTLKQCAL ISKSAGGIGV

        251                                                     300
   RNR  .i.nIRaTGS YIAGTNGtSN GlvPMiRVyN NTARYVdqgg nkrpgafa.y
Spombe  NIHNIRATGS YIAGTNGTSN GIVPMIRVYN NTARYVDQGG NKRPGAFAAY
Scerev  HINNIRSTGS YIAGTNGTSN GLIPMIRVFN NTARYVTRVV TRDLVLSPFS
  Mus   AVSCIRATGS YIAGTNGNSN GLVPMLRVYN NTARYVDQGG NKRPGAFAIY

        301                                                     350
   RNR  lepwh.d... fldlrKthGk Ee.RARdlF. ALWIPDLFMk RvE.n..Wtl
Spombe  LEPWHADVMD FLELRKTHGN EDFRARQMFY ALWIPDLFMQ RVERNEQWTF
Scerev  WSHGMQISST KFDIRKTHGK EEIRARDLFP ALWIPDLFMK RQEDGP.WTL
  Mus   LEPWHLDIFE FLDLKKNTGK EEQRARDLFF ALWIPDLFMK RVETNQDWSL

        351                                                     400
   RNR  fcPneaPGLd dVWGdEFe.L Ye.YEkegRg r....AQklW YAIlqsQtET
Spombe  FCPNEAPGLA DVWGDEFVAL YEKYEKENRG RRSLPAQKVW YAILQSQVET
Scerev  FSPSAAPGLD DVWGDEFEEL YTRYEREGRG KTIK.AQKLW YAILQAQTET
  Mus   MCPNECPGLD EVWGEEFEKL YESYEKQGRV RKVVKAQQLW YAIIESQTET

        401                                                     450
   RNR  GtPfMlYKDs CNRKsNQqNl GTIksSNLCt EIVEYssSpDE VAVCNLAS.A
Spombe  GNPFMLYKDS CNRKSNQKNV GTIRSSNLCT EIVEYSSPDE VAVCNLASVA
Scerev  GTPFMVYKDA CNRKTNQQNL GTIKSSNLCC EIVEYSSPDE VAVCNLASIA
  Mus   GTPYMLYKDS CNRKSNQQNL GTIKCSNLCT EIVEYTSKDE VAVCNLASLA
```

```
          451                                                  500
   RNR    Lp.fv..... .k...YnFek Lhev.KVitr NLNkiID.NY YPvPEAr.SN
Spombe    LPTFI..... .KDGKYNFQK LHDVVKVVTR NLNKIIDVNY YPVPEARRSN
Scerev    LPAFVEVSED GKTASYNFER LHEIAKVITH NLNRVIDRNY YPVPEARNSN
   Mus    LNMYV..... TPEHTYDFEK LAEVTKVIVR NLNKIIDINY YPIPEAHLSN

          501                                                  550
   RNR    mrHRPiglGV QGLADaf..l RlPFES.eAq .LNkQIFETI YhaaLEASCE
Spombe    MRHRPVGLGV QGLADAFFAL RLPFESAGAK KLNIQIFETI YHAALEASCE
Scerev    MKHRPIALGV QGLADTYMML RLPFESEEAQ TLNKQIFETI YHATLEASCE
   Mus    KRHRPIGIGV QGLADAFILM RYPFESPEAQ LLNKQIFETI YYGALEASCE

          551                                                  600
   RNR    lAq.eG.Yet yEGSP.skgI LQyDMWNv.P tdlWDW..lk ekIaKhGiRN
Spombe    IAQVEGTYES YEGSPCIQGI LQYDMWNVNP TDLWDWAEFK EKIAKHGIRN
Scerev    LAQKEGKYST FEGSPASK.I LQFDMWNAKP FGMWDWETLR KDIVKHGLRN
   Mus    LAKEYGPYET YEGSPVSKGI LQYDMWNVAP TDLWDWKPLK EKIAKYGIRN

          601                                                  650
   RNR    SLl.APMPTA STsQILG.NE cfEPyTSNmY .RRVLSGEFQ iVNP.LLkDL
Spombe    SLLVAPMPTA STSQILGFNE CFEPYTSNMY QRRVLSGEFQ IVNPWLLKDL
Scerev    SLTMAPMPTA STSQILGYNE CFEPVTSNMY SRRVLSGEFQ VVNPYLLRDL
   Mus    SLLIAPMPTA STAQILGNNE SIEPYTSNIY TRRVLSGEFQ IVNPHLLKDL

          651                                                  700
   RNR    verglWne.M Kn.li..nGS IQ.iPeiPqd LK.LYKTVWE ISQKTvi.mA
Spombe    VERDLWNEDM KNKLVMLDGS IQAIPEIPQD LKDLYKTVWE ISQKTVIDYA
Scerev    VDLGIWDDSM KQYLITQNGS IQGLPNVPQE LKELYKTVWE ISQKTIINMA
   Mus    TERGLWNEEM KNQIIACNGS IQSIPEIPDD LKQLYKTVWE ISQKTVLKMA

          701                                                  750
   RNR    AdRg.fIDQS qSLNihl..P syGKiTSMHF YGWKkGLKtG MYYLRT.aAs
Spombe    ADRGPFIDQS QSLNIHLKDP SYGKITSMHF YGWKKGLKTG MYYLRTMAAS
Scerev    ADRAIYIDQS HSLNLFLQAP SMGKITSMHF YGWKKGLKYG MYYLRTQAAS
   Mus    AERGAFIDQS QSLNIHIAEP NYGKLTSMHF YGWKQGLKTG MYYLRTRPAA

          751                                                  800
   RNR    aaIqFT.d.. .L..... ..... ..... KEE.....N.
Spombe    AAIKFTVDPV ALRARNEESN EENKKPVIKN GKAEISAEPT KEE.IDIYNE
Scerev    AAIQFTID
   Mus    NPIQFTLNKE KLK..... ..... ....DKEKAL KEEEEKERNT

          801              819
   RNR    ....CS..N. E.C.MC..*
Spombe    KVLACSIKNP EACEMCSA*
   Mus    AAMVCSLENR EECLMCGS*
```

3 e Conclusions

This chapter describes the determination of the functional region of *cdc22*$^+$ by DNA and protein sequence analysis.

The sequence of a fragment of DNA of 4136 nucleotides is presented which contains an ORF of 2750 nucleotides, interrupted by one intron of 315 nucleotides in length.

The *cdc22*$^+$ predicted polypeptide, of 811 amino acids, shows very high similarity with the large subunit of ribonucleotide reductase from a number of species, and contains all the peptide motifs thought to be required for its activity. Thus it is likely that *cdc22*$^+$ encodes a large subunit of ribonucleotide reductase in fission yeast.

Chapter 4: *cis*- and *trans*-acting components controlling *cdc22*+ expression

4 a Introduction

The molecular mechanisms that control gene expression in eukaryotes have been well studied, and a general model proposed (Review: Guarente & Bermingham-McDonough, 1992; Section 1f). Gene expression is mediated by two interacting components: *cis*-acting sequences 5' of the gene, and *trans*-acting transcription complexes which bind to them. Together these components stimulate general transcription factors, to induce gene expression at the appropriate time.

cdc22+ transcript varies in abundance during the cell cycle with a maximum at the G_1-S phase boundary (Gordon & Fantes, 1986). This chapter describes experiments which try to understand the molecular mechanisms that control *cdc22*+ transcription during the cell cycle.

Initially, the *cdc22*+ promoter region is studied to characterize *cis*-acting elements that control *cdc22*+ expression. Experiments are described that map positions of transcriptional initiation of *cdc22*+. Next, the identification of putative *cis*-acting elements, related to budding yeast MCBs, in the *cdc22*+ promoter is shown. MCBs are then demonstrated to be physiologically relevant in fission yeast and, significantly, shown to be able to confer cell cycle gene expression.

Subsequently, experiments are described that characterize a *trans*-acting factor which specifically binds MCBs in fission yeast. We have have called this factor DSP1 for DNA synthesis control in *S. pombe*, as it is related to DSC1, a MCB binding factor previously identified in budding yeast (Lowndes *et al.*, 1991). DSP1 is shown to bind MCBs constitutively during the cell cycle.

4b Mapping transcriptional start

i Introduction

Transcriptional start was mapped using two methods. The first method, primer extension, uses a complementary oligonucleotide hybridized to *cdc22*+ mRNA to initiate a reverse transcriptase reaction (Sambrook *et al.*, 1989). The size of the DNA produced from such a reaction can be estimated, and the start of transcription calculated relative to the position of the oligonucleotide. The second method involves hybridizing *cdc22*+ mRNA with probes of DNA fragments 5' and 3' to the predicted transcription start sites. Only those probes within the *cdc22*+ transcribed region would be predicted to hybridize to *cdc22*+ mRNA.

ii Results

Primer extension

Two complementary oligonucleotides were annealed to the *cdc22*+ mRNA to initiate separate reverse transcriptase reactions. The first oligonucleotide 5' to the predicted intron (L, 5'-GGTGGTAAATACCGG GAA-3') and the second downstream of the intron (A, 5'-GATCAACA TGATCGGAAT-3'), at nucleotides -38 to -21 and 403 to 420, respectively (see Figs. 3C, 4A, and Appendix A). The method used is described in Section 2i.

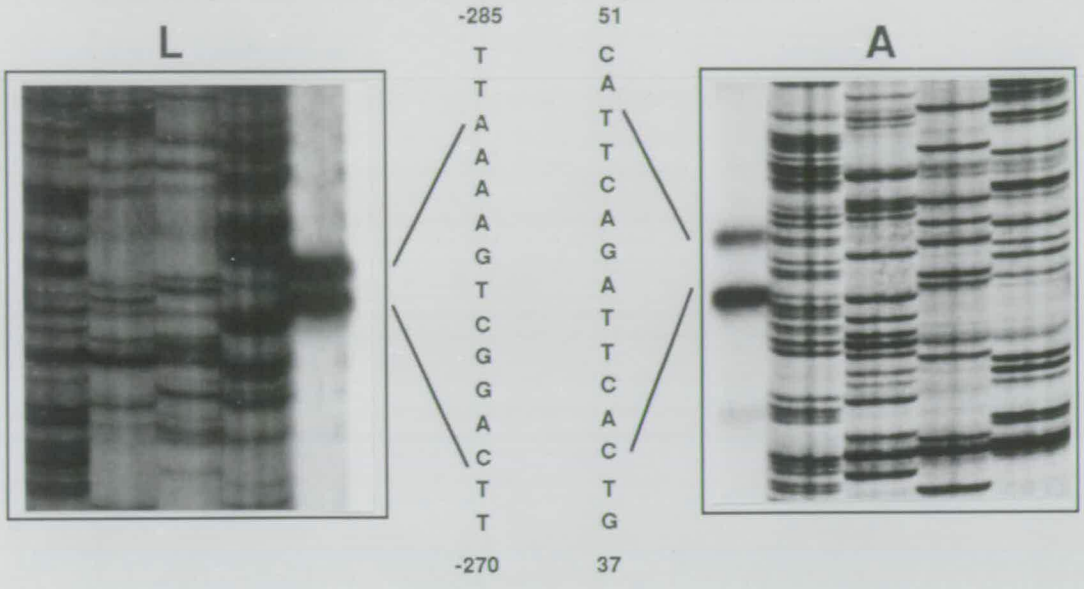
The result of the primer extensions are presented in Figure 4A. Both primer extension reactions predicted two transcription start sites at very similar positions for *cdc22*+. The reaction initiated with the oligonucleotide 5' to the putative intron (L) predicted two start sites at positions -283 and -269 relative to the start codon of *cdc22*+ (Fig 3C). The reaction initiated with an oligonucleotide 3' to the putative intron (A) predicted two starts sites at positions +35 and +49, but when the intron of 315 nucleotides was allowed for, this gave start sites of -280 and -266.

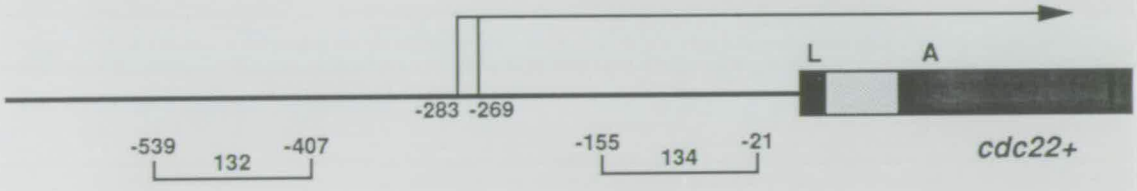
Figure 4 **Mapping transcriptional start of *cdc22*$^+$.**

A Separate primer extension reactions primed with oligonucleotides 5' and 3' to the *cdc22*$^+$ putative intron (L and A, respectively - Appendix A). The method used is described in Section 2i. The two principal extended products from each reaction, are labelled according to the position of their initiation relative to the start codon, estimated from the adjacent sequencing ladders, which were primed using the same oligonucleotide.

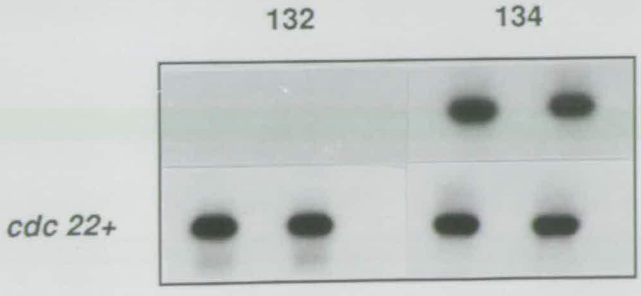
B Northern blot of wild-type (*972h*$^-$) fission yeast RNA probed with DNA fragments 5' (132) and 3' (134) to the predicted transcriptional start sites. The 132 and 134 nucleotide fragments were made using the polymerase chain reaction with oligonucleotides H, I, and L, M, respectively (Appendix A), and end-labelled using T4 polynucleotide kinase and [γ–^{32}P]ATP (Section 2d.ii). Equal amounts of *cdc22*$^+$ mRNA in all lanes was shown by subsequent hybridization with a *cdc22*$^+$ probe, consisting of equimolar amounts of the *Hin*dIII insert fragments of pCDC22-1 (Fig. 3A).

A


L
A
-285
T
T
A
A
A
A
G
T
C
G
G
A
C
T
T
-270
51
C
A
T
T
C
A
G
A
T
T
C
A
C
T
G
37


L
A
-283
-269
-539
132
-407
-155
134
-21
cdc22+

B


132
134
cdc 22+

Northern blots

Total RNA was prepared from exponentially-growing wild-type (*972h⁻*) fission yeast (Section 2e.vi). Four 10 μg samples were separated by formaldehyde agarose gel electrophoresis, and transferred to GeneScreen membrane (Sections 2c.vii and viii). The Northern blot was split in two: one half hybridized with a DNA fragment 5' (132 nucleotides) to the predicted transcription start sites; and the other half hybridized to a DNA fragment 3' (134 nucleotides) to the predicted transcription start sites (Fig. 4B). After this hybridization, both blots were stripped and re-hybridized with a *cdc22⁺* probe.

The result of the Northern blots are presented in Figure 4B.

The Northern blots probed with DNA fragments 5' and 3' to the predicted transcription start sites confirmed the position of transcriptional starts. Only the probe 3' to the predicted start sites (134) hybridized to *cdc22⁺* mRNA. Subsequent hybridization of all lanes with a *cdc22⁺* probe confirmed the presence of equal amounts of *cdc22⁺* mRNA.

The predicted start sites for transcription of *cdc22⁺* agree well with other fission genes that have had start sites mapped (Russell, 1989). Transcription has been found to initiate usually within 250 nucleotides 5' to the ORF, and there are often two start sites within a 10 nucleotide region. TATA sequence motifs have been suggested for genes that are transcribed at moderate or high levels, located about 35 - 45 nucleotides upstream of the start sites (Russell, 1989). The consensus for fission yeast TATA motifs is very weak and no such sequences are apparent 5' to the *cdc22⁺* transcriptional start.

4c MCBs

i Introduction

cdc22+ transcript varies in abundance during the cell cycle with a maximum level at the G_1-S phase boundary (Gordon & Fantes, 1986). A group of genes with similar expression patterns to *cdc22*+ have been identified in budding yeast (Johnston, 1990). This group of genes has been found to share a common 5' sequence, named the *Mlu*I cell-cycle box (MCB, Verma *et al.*, 1992). MCBs are defined as the restriction endonuclease *Mlu*I recognition site ACGCGT, or a $^5/_6$ match with the 4 nucleotides CGCG intact; some MCBs have adenine at position 8 (McIntosh *et al.*, 1991; Gordon & Campbell, 1991). A trimer of MCBs has been shown to confer periodic expression in G_1 on a heterologous gene in budding yeast (Lowndes *et al.*, 1991), implicating MCBs in the cell cycle control of genes.

The Find program of the UWGCG facility in the Daresbury VAX was used to search for MCBs in the *cdc22*+ promoter region. The distribution and types of MCBs found are shown in Figure 4C and 4D. Two clusters of MCBs are apparent: one cluster 5' to transcriptional start with one *Mlu*I site and three $^5/_6$ matches; and one cluster 3' to transcriptional start with one *Mlu*I site and one $^5/_6$ match. One lone $^5/_6$ match is present between these two clusters. Only the *Mlu*I site 5' to transcriptonal start has an adenine at position 8.

Having identified putative MCBs in the promoter region of *cdc22*+, it was necessary to test whether they were physiologically relevant in fission yeast.

ii Results

A 36 base pair synthetic oligonucleotide containing three *Mlu*I recognition sites (Appendix A) was cloned into the *Xho*I site of the test plasmid pSPΔ178 (Section 2b.v) to create pSPΔ178.3M, and transformed into a wild-type fission yeast strain carrying *ura4-D18*, for plasmid selection (Section 2e.v). This test plasmid contains a unique cloning site

C

```
      CTTTATCAATTGTATGTTATGATCTTCTCCAACAGGTAATTGACAACATCAAAAAGTAAT
-644  ----+---------+---------+---------+---------+---------+-----  -585

      ATAAAGAACAAGGGGAAATTGATATCTAGATATATTAGAGAAATTAGTAGTTCAATCTCA
-584  ----+---------+---------+---------+---------+---------+-----  -525

      TAGAGCAGGTTGGTAGTCGGGTTGGACCGCGTGTTTAATTTATGTAAACAGTCGCGTCGC
-524  ----+---------+---------+---------+---------+---------+-----  -465

      GTTGCAATTGAGACGCGTAAATAAATATTTAATTTATTACATTCAGTCGTAAACAGAGTA
-464  ----+---------+---------+---------+---------+---------+-----  -405

      TTTATAAACACTTTTTTTATGTTTAATAAAAGATAAATGTAACAGTTGAATGTATTGTAT
-404  ----+---------+---------+---------+---------+---------+-----  -345

      CAGGTCAGACCACTTCAACATGTTTAATCGCGTTTTTTTAAAAAAATATTTTTTTAATTT
-344  ----+---------+---------+---------+---------+---------+-----  -285
        *         *
      TTAAAAGTCGGACTTATTTTAGCGGAACTTTGATGTTCAGAAGTGAAAAAGATAAATCTA
-284  ----+---------+---------+---------+---------+---------+-----  -225

      TTTAGCAAGTCTTAATTAACGTCTTTTAGATATAGTAGAGCTACAAAAATGATCCGGTTT
-224  ----+---------+---------+---------+---------+---------+-----  -165

      CCACTCTTAGCTTTATTTACATTGATCAACATGACTTAAAGTTCGGATGACGCGACGGGC
-164  ----+---------+---------+---------+---------+---------+-----  -105

      CACATCTCAACCGGTTTAATTAATTAGCGTGACGCGTCTGAACGCTTTTTCATTTACTAT
-104  ----+---------+---------+---------+---------+---------+-----  -45

      AAATATTCCCGGTATTTACCACCTTACAAACTAGAACAAACACGATGTTTGTATACAAAA
 -44  ----+---------+---------+---------+---------+---------+-----  15
                                                  M  F  V  Y  K
```

D

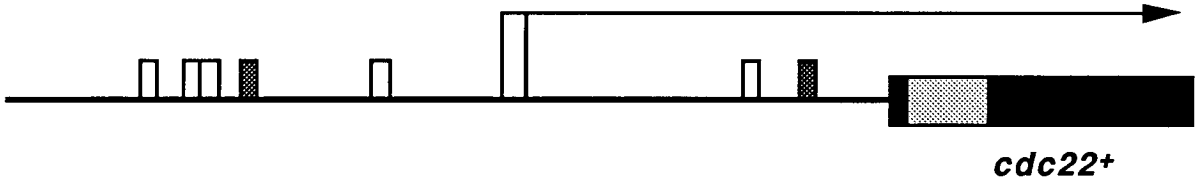


Figure 4 Distribution of MCBs in the promoter region of *cdc22*$^+$.

C The nucleotide sequence 5' to the *cdc22*$^+$ ORF numbered relative to the predicted start codon (in bold typeface). *Mlu*I recognition sites are underlined, *Mlu*I $^5/_6$ matches are wavy underlined, and transcriptional start sites are indicated by asterisks.

D A schematic diagram of the *cdc22*$^+$ promoter region. *Mlu*I sites (filled boxes), *Mlu*I $^5/_6$ matches (open boxes), the *cdc22*$^+$ open reading frame (black) and intron (dots), and transcriptional start sites (arrow) are indicated.

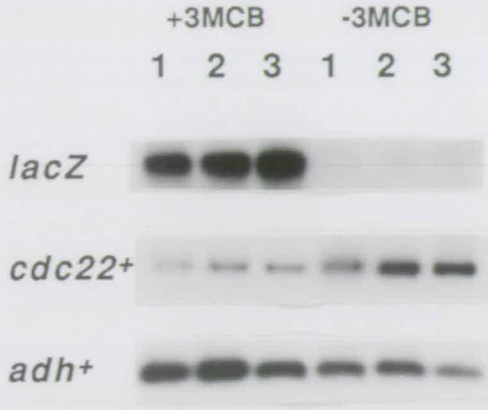


Figure 4 E MCBs are physiologically relevant in fission yeast.

Northern blot of RNA prepared from three clones each of wild-type fission yeast transformed with pSPΔ178.3M (+3MCB), and pSPΔ178 (-3MCB). The blot was hybridized consecutively with *lacZ* (a 2.2 kb *Eco*RI/*Xba*I fragment from pSPΔ178, Fig. 2B), *cdc22*$^+$ (Section 4b) and *adh1*$^+$ (a 1.1 Kb *Eco*RI fragment from the fission yeast *adh1*$^+$ gene) probes.

(*Xho*I) immediately 5' to a budding yeast minimal cytochrome *c* promoter and the *lacZ* gene of *E. coli* (Lowndes *et al.*, 1992). Potential *cis*-acting elements were inserted into the *Xho*I site and their effects on *lacZ* transcription assayed. A control plasmid, pSPΔ178 without the *Mlu*I trimer, was also transformed into fission yeast. Three colonies from each transformation were grown to mid-exponential stage, and the cells harvested. Total RNA was prepared from the cells (Section 2e.vi) and this subjected to glyoxal gel electrophoresis, and transferred to GeneScreen membrane (Sections 2c.vii and viii). The Northern blot was hybridized consecutively with *lacZ*, *cdc22*$^+$ and *adh1*$^+$ probes.

The results of this experiment are shown in Figure 4E.

A *lacZ* transcript was produced that is specific to those cells that contained pSPΔ178.3M. This transcript was absent in control cells which contained pSPΔ178. Thus, MCBs are physiologically relevant in fission yeast.

Interestingly, *cdc22*$^+$ transcript was reduced in those cells that contained pSPΔ178.3M, in comparison with those cells that contained pSPΔ178. Perhaps multiple MCBs in the test plasmid titrated out the transcription factor common to both genes, and consequently reduced *cdc22*$^+$ transcription? Growth rate, however, was not affected in pSPΔ178.3M carrying cells (P. Kersey, pers. comm.), suggesting that reduced *cdc22*$^+$ transcription was not affecting cell cycle progress.

4 d MCBs confer cell cycle gene expression in fission yeast

i Introduction

Having shown that MCBs are physiologically relevant in fission yeast, the next question to address was whether they can confer cell cycle gene expression. Such ability has already been demonstrated for an MCB trimer in budding yeast (Lowndes *et al.*, 1991).

ii Results

Wild-type fission yeast cells containing pSPΔ178.3M were grown to mid-exponential stage of growth at 32°C, and a population of small cells selected using Beckman elutriator rotor (Section 2e.iv). The resulting culture was incubated at 32°C for 6 hours to follow two divisions, and samples removed every 20 minutes. Total RNA was prepared from the cells (Section 2e.vi), 10 μg of each sample subjected to glyoxal gel electrophoresis, and transferred to GeneScreen membrane (Sections 2c.vii and viii). The Northern blot was hybridized consecutively with *lacZ*, *cdc22*$^+$ and *adh1*$^+$ probes (Fig. 4E).

The results of this experiment are shown in Figure 4F.

The *lacZ* transcript specifically produced by the MCB trimer was expressed in a cell cycle specific manner with a maximum when cells were beginning to divide, which in rapidly growing fission yeast cells corresponds to the G_1-S phase boundary. In the first cycle sampled after synchronization, expression of *lacZ* was slightly perturbed, as it did not precisely coincide with *cdc22*$^+$ expression. But in the second and the start of the third cycles *lacZ* expression paralleled *cdc22*$^+$ expression. Thus, an MCB trimer can confer expression at the G_1-S phase boundary on a reporter gene in fission yeast, as it does in budding yeast.

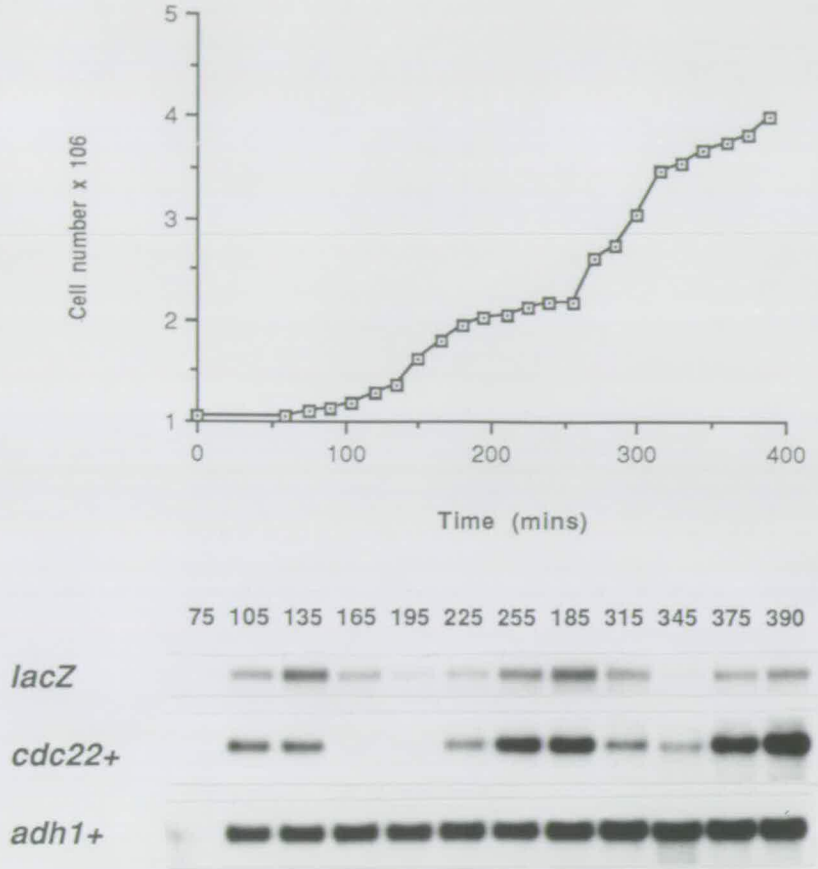


Figure 4 F MCBs confer cell cycle gene expression in fission yeast.

Northern blot of RNA prepared from samples of sychronously dividing wild-type fission yeast cells, transformed with pSPΔ178.3M. Small cells were selected by elutriation and grown at 32°C for $6^1/_2$ hours, to follow two divisions. Samples were removed from the resulting culture every 20 minutes, and total RNA prepared. A plot of cell number against time is shown, and the time in minutes at which samples were taken is indicated above each lane. The RNA was subjected to Northern blot analysis, and the blot hybridized consecutively with *lacZ*, *cdc22*$^+$, and *adh1*$^+$ probes (Section 4d). Synchronization was performed with the help of Dr. Jim Creanor, and in this experiment Northern blotting was conducted by Antony Johnson.

4 e Identification of a DSC1-like complex in fission yeast: DSP1

i Introduction

Budding yeast DSC1 (for DNA synthesis control) was identified using a synthetic oligonucleotide containing three MCBs as substrate with budding yeast protein extracts, in bandshift assays (Lowndes *et al.*, 1991).

The bandshift method characterizes *in vitro* binding properties of proteins to DNA (Fried & Crothers, 1981; Garner & Revzin, 1981). Radio-labelled DNA fragments are mixed with protein extracts, and the reaction subjected to electrophoresis. Free DNA runs into the gel a distance proportional to its size to form a band; whereas complexes of proteins bound to DNA run less far, with the consequence that a new shifted band is formed. Specificity of binding is tested by the addition of unlabelled competitor DNA in excess to the reaction mixture before electrophoresis. If the competitor DNA binds the protein complex the shifted band disappears.

To identify a protein complex in fission yeast that binds to MCBs, the synthetic oligonucleotide containing three MCBs was initially used as substrate with fission yeast protein extracts in bandshift assays (Appendix A). Subsequently, a fragment from the promoter region of *cdc22*$^+$, 5' to transcriptional start, containing four MCBs (132) was used as substrate (Figs. 4A and 4H). Bandshifts were also attempted with a fragment of the promoter region of *cdc22*$^+$, 3' to transcriptional start, containing two MCBs (134).

ii Results

The method used for bandshift assays is described in Section 2j.

Bandshifts were conducted with budding and fission yeast protein extracts using three radiolabelled DNA fragments as substrates: a synthetic oligonucleotide containing three MCBs, of 73 nucleotides; a DNA fragment 5' to transcriptional start of *cdc22*$^+$ containing four MCBs, of 132 nucleotides; and a DNA fragment 3' to transcriptional start of *cdc22*$^+$ containing two MCBs, of 134 nucleotides (Figs. 4A and 4H)

All three DNA fragments were made using the polymerase chain

reaction (Section 2h). The synthetic *Mlu*I trimer cloned into pSPΔ178 (pSPΔ178.3M, Section 4c) was amplified using two flanking oligonucleotides O and P (Appendix A). The 134 and 132 nucleotide fragments were amplified from pCDC22-1 (Section 3b) with oligonucleotides H, I, and L, M, respectively (Appendix A).

All DNA fragments were end-labelled to a specific activity of approximately 1000 counts per second per ng of DNA, and purified using a G-50 molecular size exclusion column (Section 2d.ii and iii). 0.5 ng of DNA was used per reaction.

Protein extracts were made from exponentially-growing wild-type budding (CB001 S100) and fission (*972h*$^-$) yeast cells (Section 2e.vii). For bandshifts using the synthetic *Mlu*I trimer as substrate, 20 μg of budding or 5 μg of fission yeast protein extract was used per reaction, to normalize exposure times for radiography. For bandshifts using the upstream *cdc22*$^+$ fragments of DNA as substrate, 15 μg of fission yeast protein was used per reaction.

In competition experiments 10 x and 100 x molar excesses of unlabelled DNA fragments were added to reaction mixtures prior to electrophoresis.

All DNA fragments for competition experiments were produced using the polymerase chain reaction. The synthetic *Mlu*I trimer, 132 and 134 nucleotide fragments were made as described above. A DNA fragment from the promoter region of *cdc22*$^+$ containing one MCB, of 166 nucleotides, was amplified using the oligonucleotides K and J from pCDC22-1 (Appendix A).

Two other DNA fragments were used in control competition experiments:

1) a synthetic oligonucleotide identical to the *Mlu*I trimer, except that the central GC of all three MCBs was replaced by AT (3Mut);

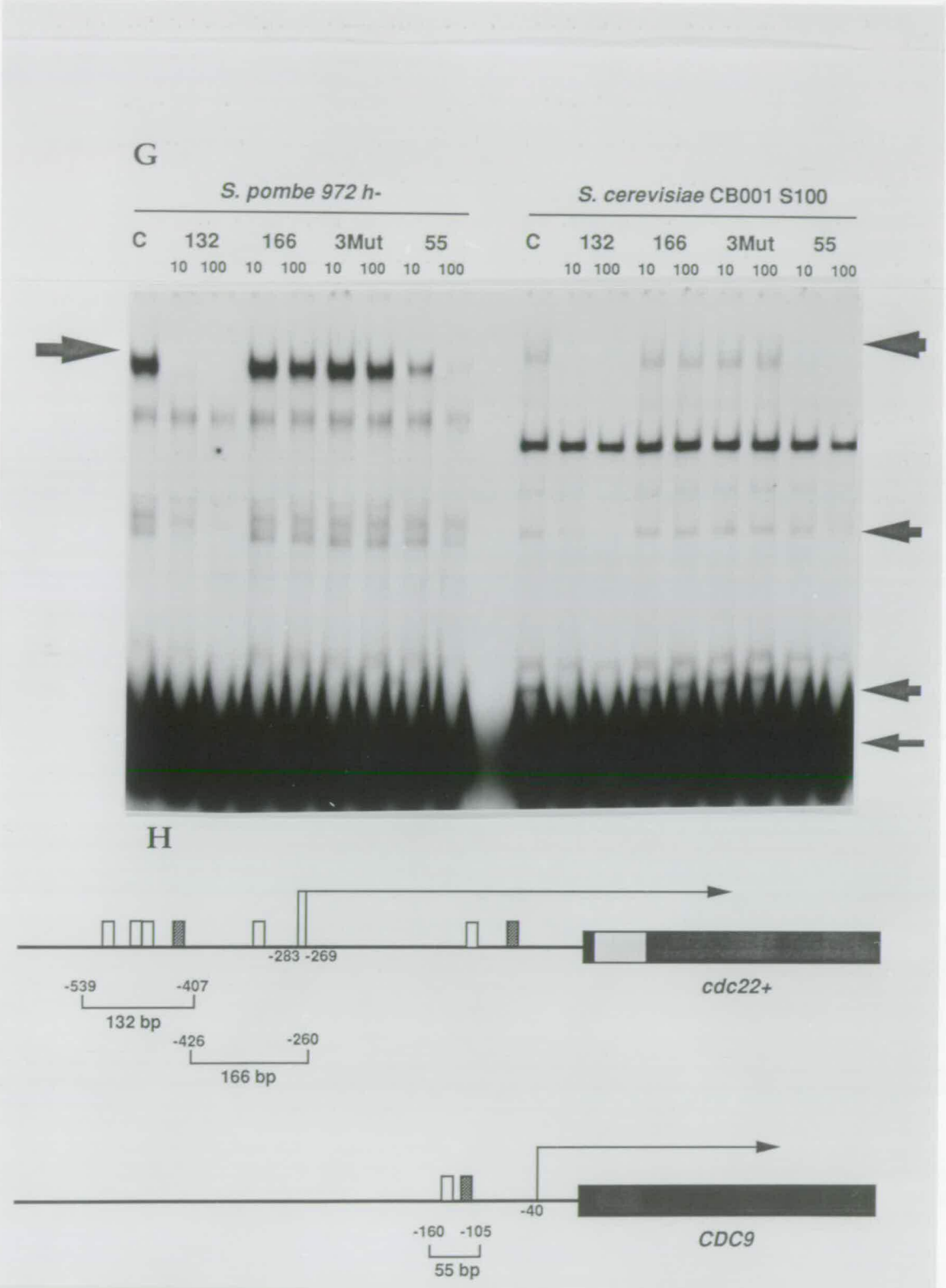
2) a DNA fragment from the budding yeast *CDC9* promoter (55) containing two MCBs, of 55 nucleotides. *CDC9* encodes DNA ligase in budding yeast and is periodically expressed at the G_1-S phase boundary (White *et al.*, 1986).

Both of these DNAs were previously cloned into pLGΔ178 (Lowndes *et al.*, 1991) and amplified using the same flanking oligonucleotides as were used for the amplification of the synthetic *Mlu*I trimer.

Figure 4 **Identification of a DSC1-like complex in fission yeast: DSP1.**

G Bandshift analysis showing binding of specific proteins from fission and budding yeast to multiples of the ACGCGT sequence element. Wild-type yeast protein extracts were mixed with a labelled synthetic oligonucleotide containing three MCBs, generated from psΔ178.3M by the the polymerase chain reaction. Control lanes (C) show reactions where no competitor DNA was added. Competitor DNA (in 10 and 100 molar excesses) added to reactions was as follows: fragments from the *cdc22*$^{+}$ promoter containing four and one MCBs (132 and 166, respectively); three mutated MCBs (3Mut); and a fragment from the *CDC9* promoter containing two MCBs (55). Large and medium arrows indicate specific DSC1 and DSP1 shifted complexes, smaller arrow indicates unbound DNA.

H Promoter regions of *cdc22*$^{+}$ from fission yeast and of *CDC9* from budding yeast, showing distribution of MCBs, and DNA fragments used in competition experiments. Filled boxes represent *Mlu*I recognition sites, and open boxes *Mlu*I $^{5}/_{6}$ matches. Transcriptional start sites are indicated by arrowed lines. Numbers refer to nucleotide positions relative to the start codons.


G
S. pombe 972 h-
S. cerevisiae CB001 S100
C
132
166
3Mut
55
10 100 10 100 10 100 10 100
C
132
166
3Mut
55
10 100 10 100 10 100 10 100
H
-283 -269
-539
-407
132 bp
-426
-260
166 bp
cdc22+
-40
-160 -105
55 bp
CDC9

Synthetic *Mlu*I trimer

The results of bandshift assays using the synthetic *Mlu*I trimer as substrate, with fission and budding yeast protein extracts, are shown Figure 4G.

In the control lanes (C), where no competitor DNA has been added, a number of shifted complexes are observed for protein extracts from both yeasts. In budding yeast extracts it is the lowest mobility complex that is DSC1 (Lowndes *et al.*, 1991). A complex of similarly low mobility is also present in fission yeast extracts. We suggest calling this complex DSP1, for DNA synthesis control in *S. pombe*.

In competition experiments it is the lowest mobility complex from both budding and fission yeast extracts (indicated in Fig. 4G by a large arrow) that disappears in the presence of molar excess of *cdc22*+ (132) and *CDC9* (55) promoter regions. Both of these promoters contain multiple MCBs, and thus the two complexes recognize MCBs in their normal DNA context. Furthermore, the binding specificities of the two complexes are interspecific: DSC1 and DSP1 can both bind budding and fission yeast promoter fragments containing MCBs, suggesting structural conservation. Additionally, shifted bands of fastest mobility, from both fission and budding yeast proteins extracts are competed by MCBs (medium arrows in Fig. 4G). Potentially, these are partial DSP1/DSC1 complexes.

No effect is observed on the bandshift profile in the presence of molar excess of a 5' region of *cdc22*+ containing only a single MCB (166), and the mutated synthetic *Mlu*I trimer (3Mut), suggesting that these fragments do not bind any of the protein complexes in the bands. Thus DSC1 and DSP1 recognize only multiples of the CGCG core, and not other sequences in the oligonucleotide used as substrate.

***cdc22*+ promoter fragment containing four MCBs**

The results of bandshift assays using a *cdc22*+ promoter fragment containing four MCBs (132, Fig. 4J) as substrate with wild-type fission yeast protein extracts, are shown in Figure 4I. This bandshift was deliberately over-exposed to X-ray film to show lower abundance shifted bands.

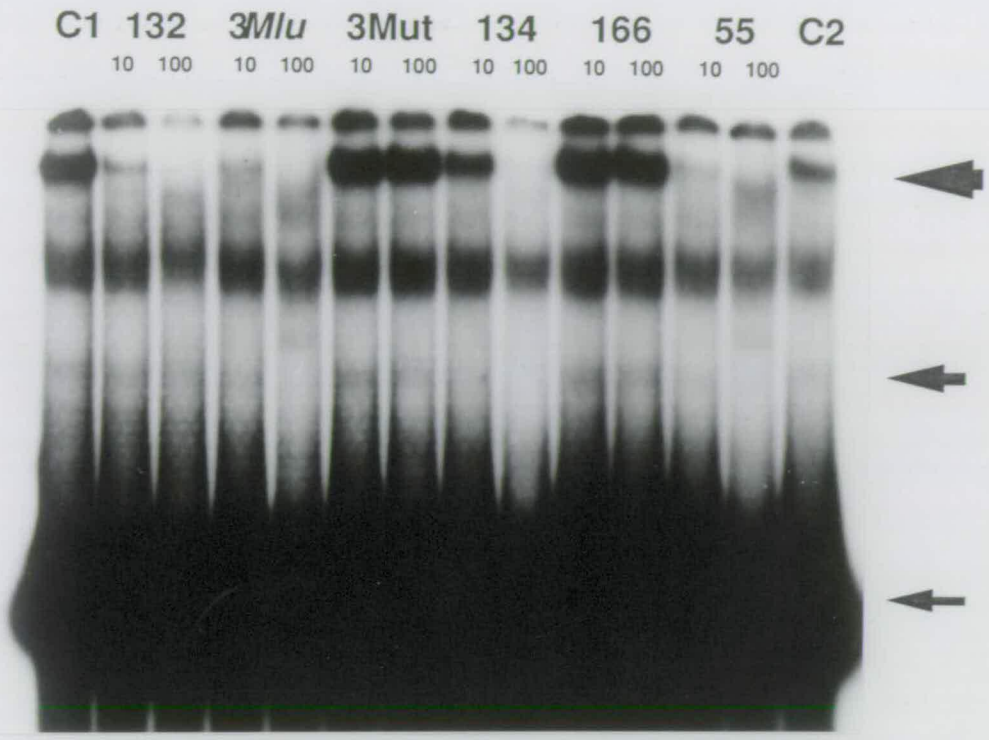
A similar bandshift profile is observed in control lanes (C1 and C2 with 15 and 8 μg of protein, respectively) where no competitor DNA has been added, to that observed with the *Mlu*I trimer as substrate, although

Figure 4 **A *cdc22*$^+$ promoter fragment containing four MCBs binds DSP1 in bandshift assays.**

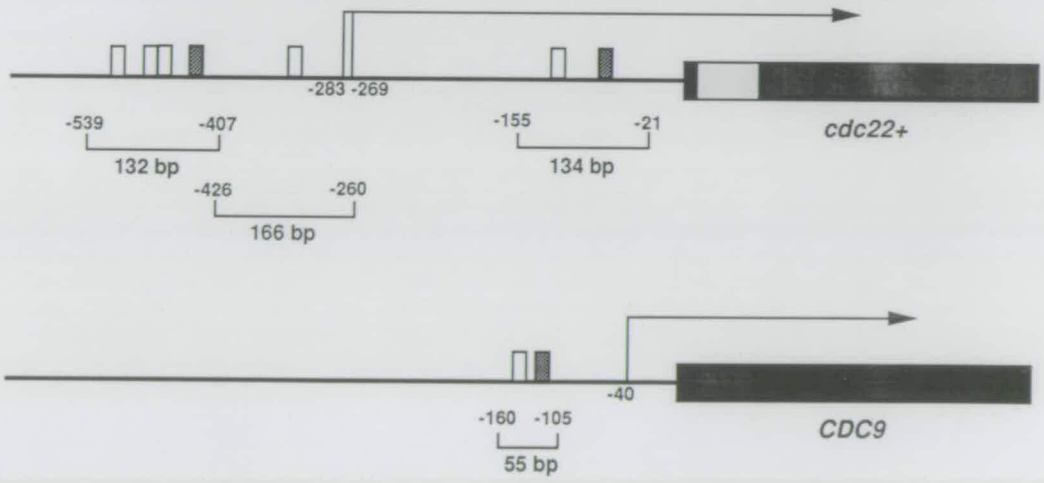
I Bandshift analysis showing specific binding of proteins from fission yeast to a promoter fragment of *cdc22*$^+$ containing four MCBs. Wild-type fission yeast (*972h*$^-$) protein extracts were mixed with a 132 nucleotide labelled fragment generated from pCDC22-1 by the polymerase chain reaction. Control lanes (C1 and C2: 15 and 8 μg of protein, respectively) show reactions where no competitor DNA was added. Competitor DNA added to reactions (in 10 and 100 molar excesses) was as follows: fragments from the *cdc22*$^+$ promoter containing four, one, and two MCBs (132, 166, and 134 respectively); synthetic *Mlu*I trimer (3*Mlu*); mutated *Mlu*I trimer (3Mut); and a fragment from the *CDC9* promoter containing two MCBs (55). Large and medium arrows indicates specific DSP1 shifted complexes, smaller arrow indicates unbound DNA.

J Promoter regions of *cdc22*$^+$ from fission yeast and of *CDC9* from budding yeast, showing distribution of MCBs and DNA fragments used in bandshifts. Filled boxes represent *Mlu*I recognition sites and open boxes *Mlu*I $^5/_6$ matches. Transcriptional start sites are indicated by arrowed lines. Numbers refer to nucleotide positions relative to the start codons.

I


C1
132
3Mlu
3Mut
134
166
55
C2
10 100 10 100 10 100 10 100 10 100 10 100

J


-283 -269
-539
-407
132 bp
-155
-21
134 bp
cdc22+
-426
-260
166 bp
-40
-160 -105
55 bp
CDC9

fewer faster mobility bands are apparent.

As in bandshifts using the synthetic *Mlu*I trimer, it is the lowest mobility band (indicated in Fig. 4I by a large arrow) that disappears only in reactions containing molar excess of unlabelled DNA containing multiples of MCBs. This band disappears in the presence of the *cdc22*+ promoter fragment containing four MCBs (132), the synthetic trimer (3*Mlu*), and the *CDC9* promoter fragment (55). Interestingly, the lowest mobility band (medium arrow) also disappears in the presence of an unlabelled *cdc22*+ promoter fragment containing a pair of MCBs from a region 3' to transcriptional start (134), suggesting that this DNA also binds to the complex. This complex is perhaps a degradation product of DSP1.

As expected, the presence of DNA fragments, such as the mutated *Mlu*I trimer (3Mut) and the promoter region of *cdc22*+ containing only one MCB (166), have no effect on the band shift profile.

Thus, the *cdc22*+ promoter fragment containing four MCBs from a region 5' to transcriptional start (132), recognizes DSP1 when used as substrate in the bandshift assay.

cdc22+ promoter fragment containing two MCBs

The results of bandshift assays using a *cdc22*+ promoter fragment containing two MCBs (134, Fig. 4J) as substrate with wild-type fission yeast protein extracts, are shown in Figure 4K.

Two control lanes (C1 and C2, with 8 and 15 μg of fission yeast protein, respectively) show bandshift reactions using the *cdc22*+ promoter fragment containing four MCBs (132) as substrate. Both show the specific low mobility band, demonstrating that DSP1 is present in these protein extracts.

In the absence of competitor DNAs (lane C), a different bandshift profile is observed using the *cdc22*+ promoter fragment containing two MCBs (134) as substrate. The low mobility band is not observed. None of the observed bands disappear in the presence of competitor DNAs, suggesting that these complexes do not specifically bind MCBs in the substrate.

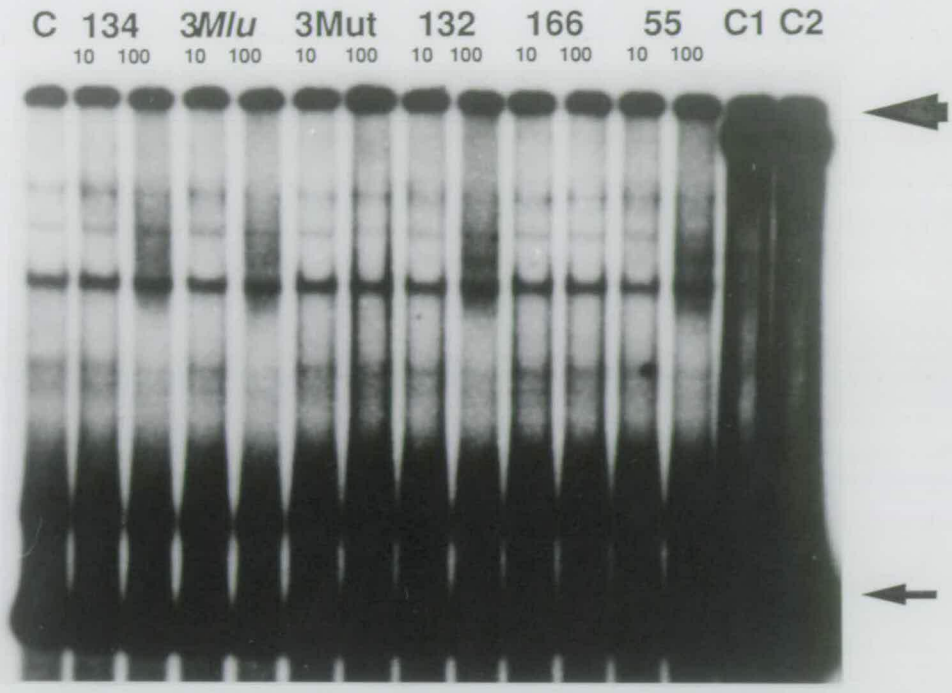
Thus a *cdc22*+ promoter fragment containing only two MCBs, from a region 3' to transcriptional start (134), does not recognize DSP1, when used as substrate in the bandshift assay.

Figure 4 **A *cdc22*$^+$ promoter fragment containing two MCBs does not bind the DSP1 in bandshift assays.**

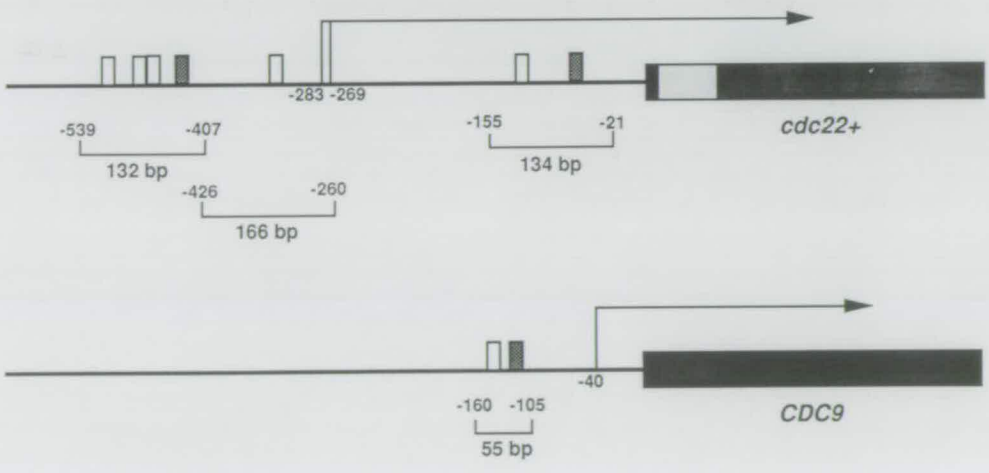
K Bandshift analysis showing absence of specific binding of proteins from fission yeast to a promoter fragment of *cdc22*$^+$ from within the transcribed region, containing two MCBs. Wild-type fission yeast (*972h*$^-$) protein extracts were mixed with a 134 nucleotide labelled fragment, generated from pCDC22-1 by the polymerase chain reaction. Control lane (C) show reactions where no competitor DNA was added. The competitor DNA added to reactions was as follows, where 10 and 100 molar excesses were used: fragments from the *cdc22*$^+$ promoter containing four, one, and two MCBs (132, 166, and 134 respectively); synthetic *Mlu*I trimer (3*Mlu*); mutated *Mlu*I trimer (3Mut); and a fragment from the *CDC9* promoter containg two MCBs (55). The two last lanes show controls using the *cdc22*$^+$ promoter fragment containing four MCBs as substrate with 15 and 8 µg of protein (C1 and C2, respectively). Large arrow indicates specific DSP1 shifted complex, smaller arrow indicates unbound DNA .

L Promoter regions of *cdc22*$^+$ from fission yeast and of *CDC9* from budding yeast, showing the distribution of MCBSs, and DNA fragments used in the bandshifts. Filled boxes represent *Mlu*I recognition sites and open boxes *Mlu*I $^5/_6$ matches. Transcriptional start sites are indicated by arrowed lines. Numbers refer to nucleotide positions relative to the start codons.

K


C
134
10 100
3Mlu
10 100
3Mut
10 100
132
10 100
166
10 100
55
10 100
C1 C2

L


-283 -269
-539
-407
132 bp
-426
-260
166 bp
-155
-21
134 bp
cdc22+
-40
-160 -105
55 bp
CDC9

Bandshift assays are a stringent *in vitro* assay for proteins' ability to bind DNA. DNA and protein have to bind very tightly for bandshift to work, as the complex must remain intact under conditions of electrophoresis. Many weaker DNA-protein interactions will not be detected using this technique. The *cdc22*$^+$ promoter fragment containing two MCBs does bind to DSP1, as it can compete in bandshifts using other substrates. Presumably it does not bind DSP1 tightly enough for itself to be a substrate. This observation is consistent with the lack of success in attempting to use promoter fragments from budding yeast genes containing two or less MCBs, as bandshift substrates (N. Lowndes, pers. comm.).

4 f Cell cycle behaviour of DSP1

i Introduction

Budding yeast DSC1 has been shown to bind MCBs in a cell cycle specific manner coincident with transcription of genes that are expressed at the G_1-S phase boundary (Lowndes *et al.*, 1991). Recently, however, DSC1 has been found to bind invariantly in sychronized cells after α factor block and release (Dirick *et al.*, 1992).

This experiment analyses the cell cycle binding activity of DSP1.

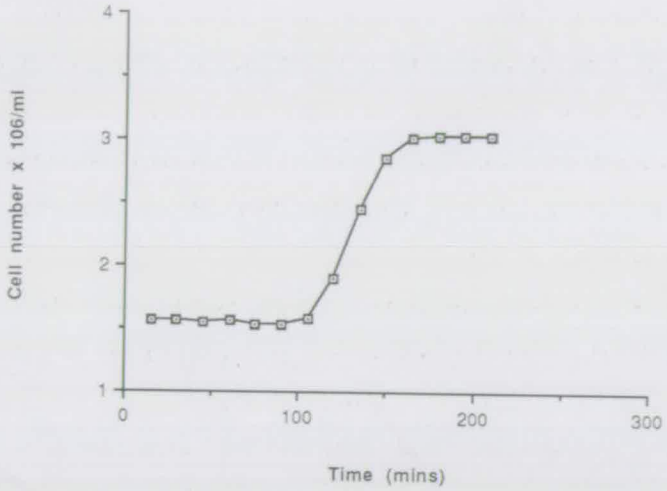
ii Results

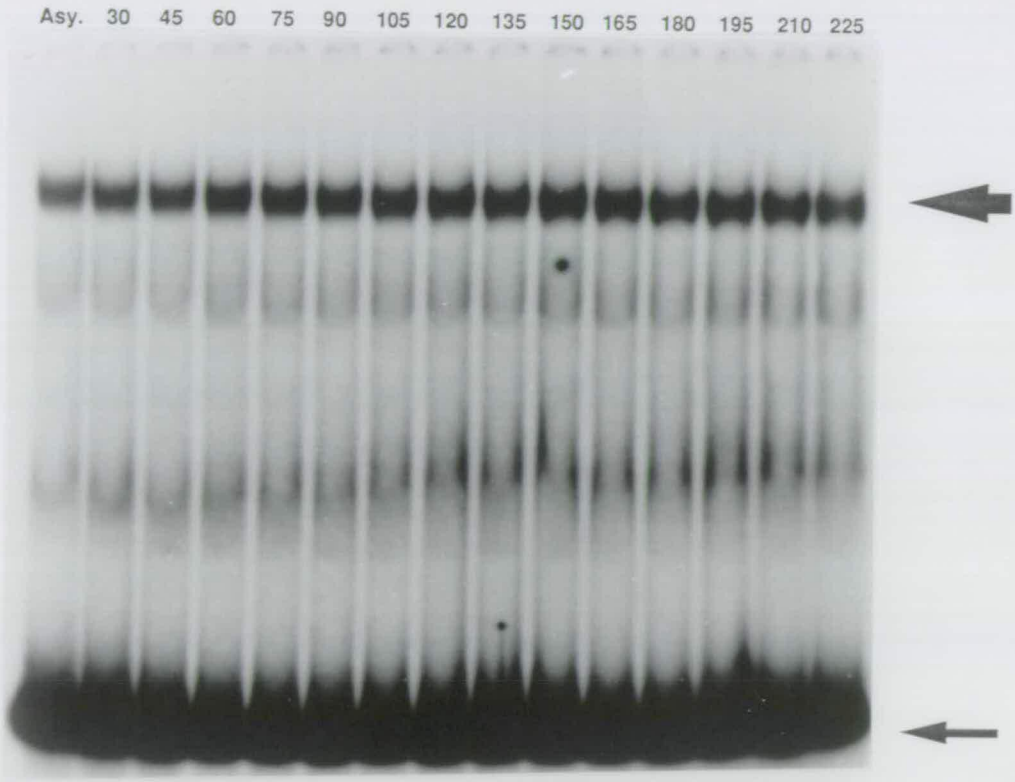
Wild-type fission yeast cells (*972h*$^-$) were grown to mid-exponential stage of growth at 32°C, and a population of small cells selected, using a Beckman Elutriator rotor (Section 2e.iv). The resulting culture was incubated at 32°C to follow one division, and duplicate samples removed every 20 minutes for 3 $^1/_2$ hours: protein extracts were prepared from one sample (Section 2e.vii), and total RNA from the other (Section 2e.vi).

Bandshift assays were performed with 15 μg of protein from each sample, using the 132 nucleotide *cdc22*$^+$ promoter fragment as substrate (Sections 2j and 4e). In separate bandshift reactions, 10 molar excess of unlabelled *cdc22*$^+$ promoter fragment containing two MCBs (134) was added to each reaction mixture, prior to electrophoresis.

Figure 4 M DSP1 binds MCBs constitutively during the cell cycle.

Bandshift analysis of DSP1 binding activity from samples of synchronously dividing wild-type (*972h⁻*) fission yeast cells. A population of small cells were selected by elutriation, and grown at 32°C. Duplicate samples were removed from the culture every 20 minutes for 3 hours, to follow one division cycle. A plot of cell number against time is shown, and the time in minutes at which samples were taken is indicated above each lane. From one set of cell samples protein extract was prepared, and from the other total RNA. 15 μg of each protein was used per bandshift reaction, with the *cdc22⁺* promoter fragment containing four MCBs (132) as substrate. The RNA was subjected to Northern blot analysis, and the blot hybridized consecutively with *cdc22⁺* and *adh1⁺* probes (Fig. 4E). A control lane of protein and RNA from asynchronous cell (Asy.) is shown.


Cell number x 106/ml
4
3
2
1
0
100
200
300
Time (mins)


Asy. 30 45 60 75 90 105 120 135 150 165 180 195 210 225


cdc22+
adh1+

10 μg of each RNA sample was separated by electrophoresis in a formaldehyde gel, and transferred to GeneScreen membrane (Sections 2c.vii and viii). This Nothern blot was probed with *cdc22*$^+$ and *adh1*$^+$ probes (Fig. 4E).

The results of this experiment are shown in Figure 4M.

DSP1 binds to the *cdc22*$^+$ promoter fragment at a constant level in protein extracts from synchronously dividing fission yeast cells. Similar results have been obtained using the synthetic *Mlu*I trimer as substrate (N. Lowndes, pers. comm.). The nucleotide fragment containing two MCBs from 3' to transcriptional start (134) competed DSP1 equally in all samples (data not shown), suggesting that DSP1 also binds this DNA fragment invariantly during the cell cycle. Thus, DSP1 binds MCBs from the promoter region of *cdc22*$^+$ constitutively during the cell cycle. As a control, *cdc22*$^+$ transcript levels are periodic, demonstrating that the cells are dividing synchronously.

4 g Conclusions

In this chapter both *cis*-acting elements and a *trans*-acting factor that are involved in the control of *cdc22*+ expression have been described.

Sequences resembling budding yeast *Mlu*I cell cycle boxes (MCBs), have been found 5' to the *cdc22*+ ORF. A synthetic oligonucleotide containing three MCBs has been shown to confer periodic expression of a heterologous gene in fission yeast, suggesting that MCBs are *cis*-acting elements that have a role the control of *cdc22*+ periodic expression.

An activity that specifically binds MCBs in the promoter region of *cdc22*+ has been identified in fission yeast, by bandshift assays. This complex has very similar bandshift properties to DSC1, a budding yeast MCB binding activity, and so we have named it DSP1, for DNA synthesis control in *S. pombe*. Both DSP1 and DSC1 have low mobilities under electrophoresis and so are large proteins. DSP1 and DSC1 are likely to contain a number of components, as faster mobility complexes, probably representing partial complexes, are also observed.

DSC1 and DSP1 recognize only multiples of MCBs in bandshift assays. Both complexes only recognize at least three MCBs in substrate DNA, but can be competed by DNA fragments containing two MCBs. DNA containing single MCBs cannot even act as competitor DNA. Structural conservation of DSC1 and DSP1 is suggested by their ability to recognize promoter fragments containing MCBs from the promoters of genes from both yeast species. DSP1 binds MCBs constitutively during the cell cycle. Both DSC1 and DSP1 are probably *trans*-acting transcription complexes which are involved in the control of MCB genes at the G1-S phase boundary.

In the next chapter experiments will be described which identify a component of DSP1.

Chapter 5: *cdc10*$^+$

5 a Introduction

In Chapter 4 experiments were described showing the discovery of a binding activity from fission yeast which specifically recognizes MCBs. This activity has been called DSP1, as it is closely related to DSC1, an MCB binding activity previously characterized in budding yeast (Lowndes *et al.*, 1991). It is likely that DSP1 is a *trans*-acting transcription complex which controls *cdc22*$^+$ expression by binding *cis*-acting MCBs in its promoter region.

A transcription complex which is involved in the control of expression of a gene required for cell cycle progress may itself be required for cell cycle progress. One could predict, therefore, that cells defective in such a complex would show the cdc mutant phenotype (Section 1c.i). Two fission yeast *cdc* genes are required before *cdc22*$^+$ in the cell cycle: *cdc2*$^+$ and *cdc10*$^+$ (Nurse *et al.*, 1976). Both of these genes are needed for transition through the 'START' event (Nurse & Bissett, 1981), a point during G_1 at which cells decide to enter either the vegetative or sexual life cycles (Section 1b.ii). *cdc2*$^+$ functions have been well characterized at the onset of mitosis, although less is known about its role in G_1 (Nurse, 1990). *cdc10*$^+$ is more enigmatic, although its gene product contains regions that are homologous to parts of the budding yeast *SWI4* and *SWI6* gene products, which are believed to be transcriptional activators of cell cycle regulated genes (Breeden & Nasmyth, 1987b; Andrews & Herskowitz, 1989b).

In the first part of this chapter experiments are described which demonstrate that the *cdc10*$^+$ gene product, p87^{cdc10} (Simanis & Nurse, 1989), is part of DSP1. Then a mutant form of p87^{cdc10}, p87$^{cdc10\text{-}C4}$, is characterized: p87$^{cdc10\text{-}C4}$ appears to be constitutively hyperactive, resulting in *cdc22*$^+$ over-expression throughout the cell cycle. This mutant phenotype is genetically recessive to wild-type, but dominant to *cdc10-129*.

5b *In vivo* temperature shift of *cdc10* mutants

i Introduction

The three *cdc10* mutants used in this work are temperature-sensitive conditional-lethals. Such mutants, at the permissive temperature of 24°C behave apparently normally, whereas at the restrictive temperature of 36°C display the cdc phenotype, where they are no longer able to divide, but continue to grow, and so elongate. This experiment analyses the effect of an *in vivo* temperature shift on DSP1 binding activity, from both *cdc10* mutant and wild-type cells.

ii Results

972h⁻ (wild-type) and *cdc10-129* fission yeast cells were grown to mid-exponential stage of growth at the permissive temperature of 24°C, and subjected to a temperature shift to the restrictive temperature of 36°C (Section 2e.iv). Samples of cells were removed from the culture during the shift at time intervals of 0, 5, 30, 60, 120 and 210 minutes, and protein extracts prepared from each sample (Section 2e.vii). Bandshift assays were conducted with these extracts using either the synthetic *Mlu*I trimer or the *cdc22*+ promoter fragment containing four MCBs as substrates (Sections 2j and 4e).

The result of an *in vivo* temperature shift on DSP1 binding activity, from wild-type and *cdc10-129* cells, is shown in Figures 5A and 5B.

DSP1 binding activity was rapidly lost in *cdc10-129* cells when shifted to the restrictive temperature, using either the synthetic *Mlu*I trimer or the *cdc22*+ promoter fragment as substrates. This loss in activity was not observed in similarly treated wild-type cells. Slight variation in the time taken to lose activity was apparent using the two substrates. With the *Mlu*I trimer, loss was evident after only 5 minutes at 36°C, and there was no detectable activity after 30 minutes. Using the *cdc22*+ promoter fragment, loss of activity was not apparent till after 30 minutes at the restrictive temperature. This may reflect differences in the binding properties of DSP1 to the two substrates.

The temperature-sensitive effect observed on DSP1 binding activity

Figure 5 **Bandshift analysis of DSP1 binding activity in *cdc10-129* : *in vivo* temperature shift.**

Wild-type (*972h-*) and *cdc10-129* cells were grown to mid-exponential stage of growth at 24°C, shifted to 36°C, and samples taken at 0, 5, 30, 60, 120, and 210 minutes. Protein extracts were prepared and assayed for DSP1 activity. In separate experiments the *Mlu*I trimer (**A**) and *cdc22+* promoter fragment (**B**) were used as substrates. Arrows indicate the specific DSP1 shifted band. In experiment 5A probe preparation and electrophoresis were performed by Noel Lowndes.

suggests that $p87^{cdc10}$ is either part of the DSP1 itself, or, is at least required for activation or regulation of the complex. In both cases, loss of activity was well within the period for one cell cycle for fission yeast at 36°C, so the loss cannot be a secondary consequence of a cell cycle block.

5 c *In vitro* temperature shift of *cdc10* mutants

i Introduction

This experiment was designed to confirm and extend the result of the *in vivo* temperature shifts. Extracts were taken from wild-type and *cdc10* mutant strains grown at the permissive temperature. These extracts were then subjected to an brief *in vitro* temperature shift, and DSP1 binding activity tested.

ii Results

The fission yeast strains *cdc10-129*, *cdc10-C4*, *cdc10-K28* and*972h*$^-$ (wild-type) were grown at the permissive temperature of 24°C to mid-exponential stage of growth, and protein extracts prepared (Section 2e.vii). These extracts were subsequently subjected to an *in vitro* temperature shift, and samples removed at time intervals of 0, 5, 10, 15, and 30 minutes. Each sample was processed for bandshift assay, using either the *Mlu*I trimer or the *cdc22*$^+$ promoter fragment as substrates (Sections 2j and 4e). The temperature shift was varied depending on the substrate used: 28°C for the synthetic *Mlu*I trimer, and 31°C for the *cdc22*$^+$ promoter fragment.

The results of *in vitro* temperature shifts on protein extracts from wild-type and *cdc10* mutant strains is shown in Figures 5C and 5D.

Two alleles, *cdc10-129* and *cdc10-K28*, produced reduced DSP1 binding activity, which was rapidly lost *in vitro*. Thus the temperature sensitive loss of DSP1 activity in *cdc10* mutants is due a temperature sensitive gene product, and strongly suggests that $p87^{cdc10}$ is a component of DCP1, and not an ancillary regulatory protein.

DSP1 binding activity was not detectable in *cdc10-C4* even at the

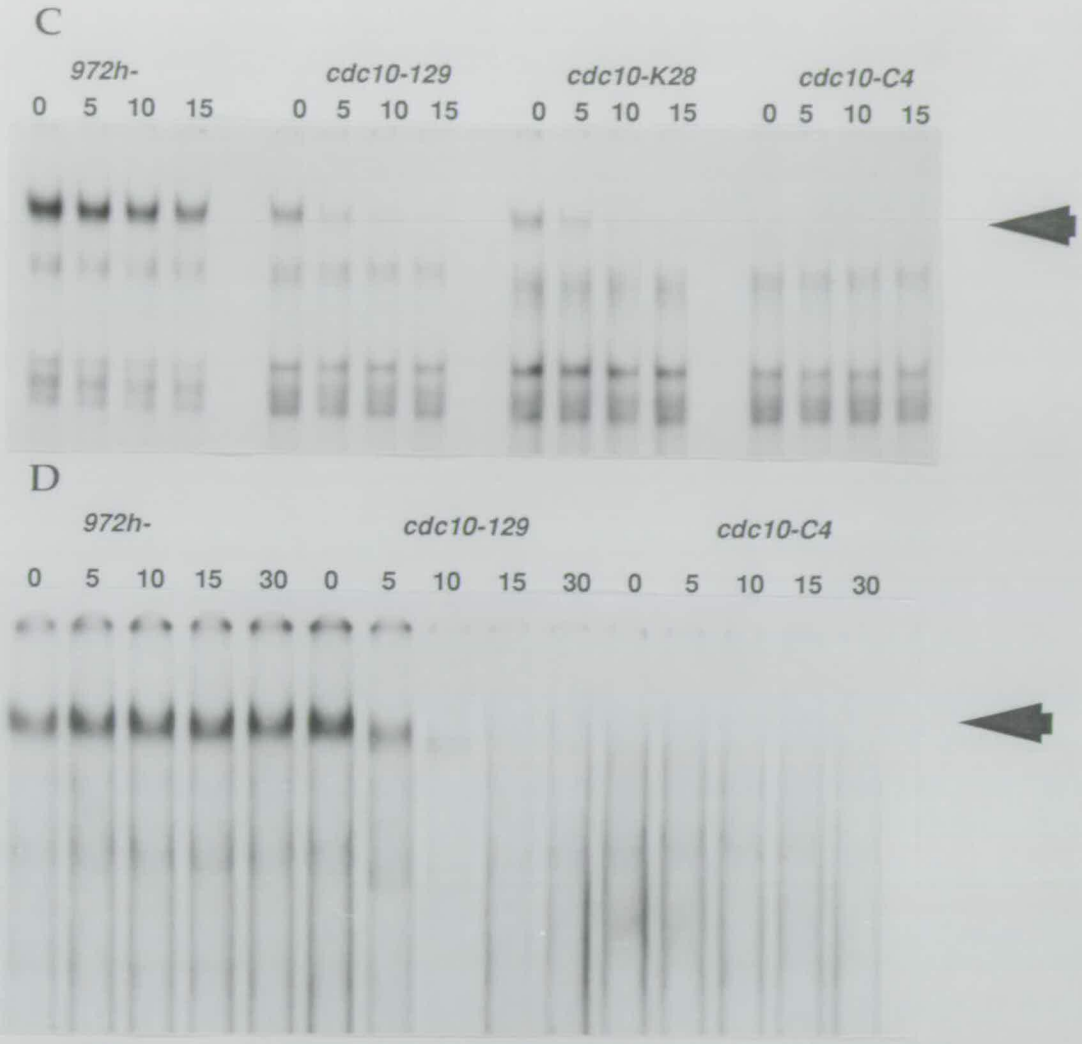


Figure 5 **Bandshift analysis of DSP1 binding activity in *cdc10* mutants: *in vitro* temperature shift.**

Wild-type (*972h-*), *cdc10-129*, *cdc10-K28*, and *cdc10-C4* cells were grown to mid-exponential stage of growth at 24°C, samples taken, and protein extracts prepared. In separate experiments the *Mlu*I trimer (C) and *cdc22*$^{+}$ promoter fragment (D) were used as substrates. The protein extracts were incubated at 28°C and 31°C (for the *Mlu*I trimer and *cdc22*$^{+}$ promoter fragment, respectively), for 0. 5, 19 and 15 minutes, and assayed for DSP1 activity. Arrows indicate the specific DSP1 shifted band. In experiment 5C probe preparation and electrophoresis were performed by Noel Lowndes.

permissive temperature using both substrates, which strongly implies that the protein complex is extremely unstable in this mutant form.

No temperature effect is observed on DSP1 activity with extracts from wild-type cells using either substrate, in agreement with the *in vivo* temperature shift.

5 d Supershift of DSP1

i Introduction

The two previous experiments demonstrate that p87^{cdc10} is likely to be a component of DSP1. To confirm this result, anti-p87^{cdc10} polyclonal serum was used in supershift experiments.

If antibody is added to a bandshift reaction prior to electrophoresis, and it binds a protein that is part of one of the shifted bands, the band may be further shifted - 'supershifted' - to form a new band. This new band has even lower mobility band than the original, as the complex contains bound antibody, which may even crosslink complexes to form oligomers. Thus, components of shifted bands can be identified if suitable antiserum is available.

ii Results

Wild-type fission yeast cells (*972h*$^-$) were grown to mid-exponential stage of growth, and protein extracts prepared (Section 2e.vii). These extracts were used in bandshifts, with the *cdc22*$^+$ promoter fragment as substrate (Sections 2j and 4e). Prior to electrophoresis, 1 µl of anti-p87^{cdc10} polyclonal antibody was added to reaction mixtures at a series of concentrations from $^1/_{12.5}$ to $^1/_{200}$, with controls of 1 µl of $^1/_{12.5}$ of pre-immune serum, and no antibody.

The result of the supershift experiment using p87^{cdc10} antibody on DSP1 activity is shown in Figure 5E.

When anti-polyclonal serum was added to the binding reaction it caused a supershift of the DSP1 shifted complex, an effect not seen with pre-immune serum. Thus, *p87^{cdc10}* is indeed part of DSP1. When high

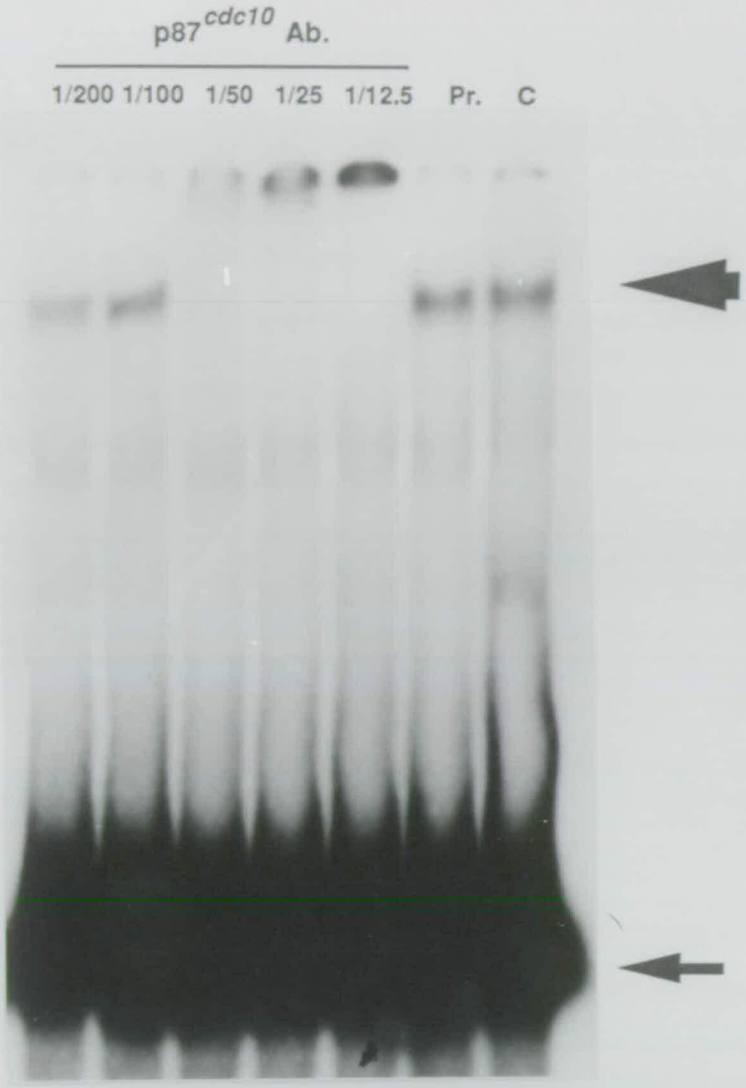


Figure 5 E DSP1 contains the *cdc10*$^{+}$ gene product, p87^{cdc10}.

Bandshifts were conducted using fission yeast wild-type (*972h*$^{-}$) protein extracts and the *cdc22*$^{+}$ promoter fragment as substrate. Prior to electrophoresis, to separate binding reactions, were added the following: C, binding buffer; Pr., $^{1}/_{12.5}$ dilution of preimmune serum; p87^{cdc10} Ab., a dilution series of anti-p87^{cdc10} polyclonal antibody, as indicated. Both the preimmune and immune sera were from the same rabbit, and were a kind gift from Uli Deuschle and David Beach.

concentrations of anti-p87^{cdc10} serum were used, the supershifted complex did not even enter the gel, suggesting that oligomers of DSP1 were formed by crosslinking. When the antibody was diluted the electrophoretic mobility of the supershifted complex increased, presumably because fewer of the available epitopes were bound, and so monomers of DSP1 bound to antibody were present, which would have lower mobility than free DSP1.

5 e Effects of *cdc10* mutants on *cdc22*$^+$ and reporter gene expression

i Introduction

The previous experiments have demonstrated that p87^{cdc10} is part of DSP1, and that the binding properties of this activity, at least in bandshift assays, are affected in *cdc10* mutants.

As DSP1 specifically binds MCBs, and it is MCBs 5' to the *cdc22*$^+$ ORF which are implicated in the control of *cdc22*$^+$ expression, one could predict that DSP1 is involved in the control of *cdc22*$^+$ expression. The next experiment tests this prediction by observing the effect of *cdc10* mutants on *cdc22*$^+$ expression. Furthermore, the effect of *cdc10* mutants on a reporter gene under the control of the *Mlu*I trimer is also tested (Section 4c).

ii Results

Four fission yeast strains, each carrying *ura4-D18* for plasmid selection, were transformed with pSPΔ178.3M (Sections 4c and 2e.v): *cdc10-C4*, *cdc10-129*, *cdc2-33*, and wild-type (*cdc*$^+$).

Three clones of *cdc10-129* and *cdc10-C4*, two clones of *cdc2-33*, and one clone of wild-type, all containing pSPΔ178.3M, were grown to mid-exponential stage of growth at the permissive temperature of 24°C. Samples of cells were removed from each culture at 0 and 3 $^1/_2$ hours after a temperature shift to 36°C. Total RNA was extracted from all samples (Section 2e.vi), and this subjected to glyoxal gel electrophoresis, and transferred to GeneScreen membrane (Section 2c.vii and viii). This Northern blot was hybridized consecutively with *cdc22*$^+$, *lacZ* and *adh1*$^+$ probes (Fig. 4E).

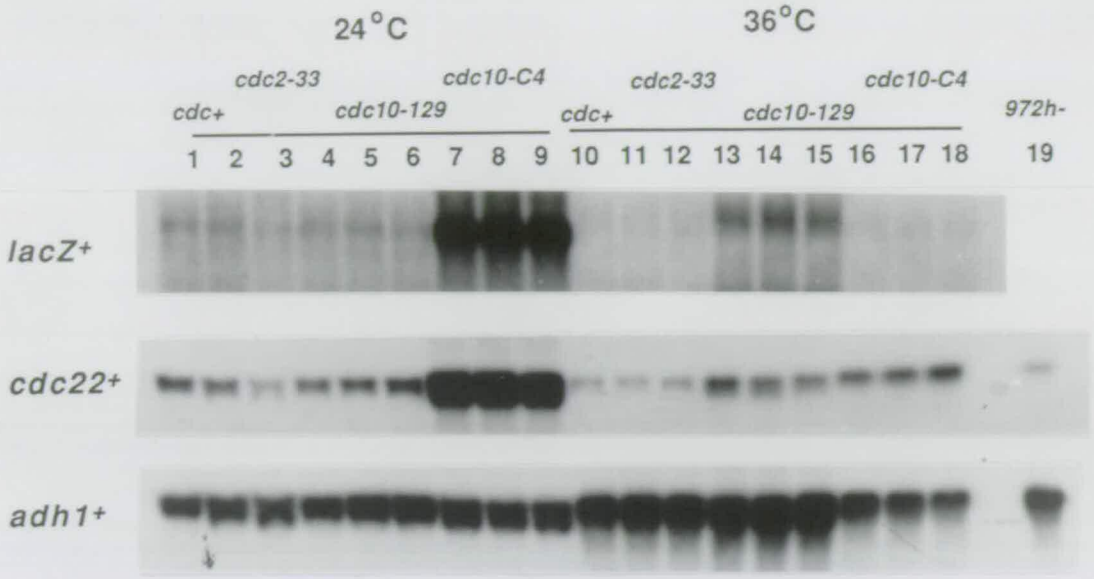


Figure 5 F Effect of *cdc10* mutants on *cdc22*$^+$ and reporter gene expression.

Strains of fission yeast wild-type (*972h*$^-$), *cdc2-33*, *cdc10-129*, and *cdc10-C4*, *ura4*$^-$ for plasmid selection, were transformed with pSPΔ178.3M, and checked for unstable plasmids. One clone of *cdc*$^+$, two clones of *cdc2.33*, and three clones of *cdc10-129* and *cdc10-C4*, were grown to mid-exponential stage of growth at 24°C, and subjected to a temperature shift to 36°C. Samples of cells were removed at 0 and $3^1/_2$ hours, total RNA extracted, which was separated on a glyoxal gel, and transferred to GeneScreen membrane. The Northern blot was hybridized consecutively with *cdc22*$^+$, *lacZ*$^+$, and *adh1*$^+$ probes (Section 4d).

The results of this experiment are shown in Figure 5F.

No changes were observed in *cdc22*$^+$ and reporter gene transcript levels in *cdc10-129* at the permissive and restrictive temperatures (lanes 4-6 and 13-15, respectively). *lacZ* does appear to be expressed at slightly higher levels at the restrictive temperature, but this can accounted for in slight overloading in these lanes.

Although no reduction in *cdc22*$^+$ transcript levels was observed in *cdc10-129* after $3^1/_2$ hours, cells did cdc arrest. This suggests that absolute *cdc22*$^+$ transcript levels are not limiting for cell cycle progress. *cdc10*$^+$ may have other functions required for cell cycle progress which, at the restrictive temperature in *cdc10* mutants, result in cell cycle arrest. If *cdc10-129* cells are synchronized for division by elutriation, and then subjected to a temperature arrest, *cdc22*$^+$ transcript levels are reduced (M-J. Fernandez-Sarabia, pers. comm.)

If p87^{cdc10} is part of DSP1 which controls *cdc22*$^+$ expression, why is there no reduction in *cdc22*$^+$ transcript levels when DSP1 binding activity is lost (Fig 5A and 5B)? The lack of correlation between the two observations may be a reflection on the two techniques used. DSP1 is rapidly thermolabile in *cdc10-129* at the the restrictive temperature *in vitro* (in bandshift assays). In cells, however, DSP1 could take longer to lose binding activity, and this loss may have a delayed effect on *cdc22*$^+$ expression. The rapid thermolability observed in bandshifts may, therefore, be an *in vitro* artifact.

cdc10-C4 shows a surprising and interesting result. At the permissive temperature both *cdc22*$^+$ and *lacZ* transcripts are highly over-expressed, relative to wild-type and other *cdc* strains carrying the test plasmid (lanes 7-9). This over-expression is reduced to wild-type levels at the restrictive temperature (lanes 16-18). β-galactosidase levels parallel *lacZ* transcript levels in these mutant cells (P. Kersey, pers. comm.) One explanation for this observation is that the *cdc10-C4* form of p87^{cdc10} is hyperactive as part of DSP1 at this temperature, with the consequence that both *cdc22*$^+$ and *lacZ* are over-expressed. The two genes have different promoters both containing MCBs, so perhaps any genes under MCB control are over-expressed in this mutant.

This explanation is difficult to reconcile with the absence of DSP1 activity in bandshift assays in this mutant (Figs. 5C and 5D). One would

expect there to be a positive correlation between binding activity of DSP1 and expression levels of genes under MCB control: DSP1 might be expected to bind tighter to MCBs, to induce higher expression of genes under its control. This paradox will be further discussed in the next experiment.

The observed over-expression of *cdc22*$^+$ and *lacZ* in *cdc10-C4* does fulfil the prediction that was to be tested by this experiment. MCB gene transcripts are specifically over-expressed in *cdc10-C4*, consistent with the hypothesis that p87^{cdc10} is part of a transcription complex that controls MCB gene expression.

5 f Effect of *cdc10-C4* on *cdc22*$^+$ transcript levels

i Introduction

In the previous experiment it was shown that *cdc22*$^+$ transcript was over-expressed in *cdc10-C4* cells at the permissive temperature of 24°C. Mutant cells were transformed with the test plasmid pSPΔ178.3M carrying the *lacZ* gene, whose transcript was also over-expressed.

It was proposed that p87$^{cdc10\text{-}C4}$, as part of DSP1, forms a hyperactive transcription complex resulting in over-expression of MCB controlled genes. An alternative explanation is that the combination of the *cdc10-C4* mutant allele with artificially high numbers of MCBs in the test plasmid created a deregulated system, resulting in over-expression of *cdc22*$^+$ and *lacZ*.

This experiment was designed to exclude the second of the two possible explanations, by examining *cdc22*$^+$ expression in *cdc10-C4* cells in the absence of pSPΔ178.3M.

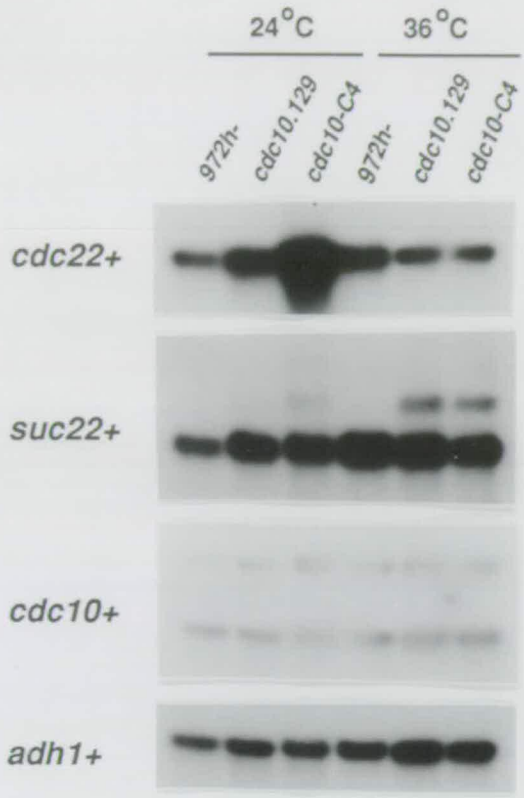


Figure 5 G Over-expression of *cdc22*+ transcript in *cdc10-C4*.

Wild-type (*972h*-), *cdc10-129*, and *cdc10-C4* cells were grown to mid-exponential stage of growth at 24°C, and subjected to a temperature shift to 36°C. Samples of cells were removed at 0 and $3^1/_2$ hours, total RNA extracted, separated on a formalhehyde gel, and transferred to GeneScreen membrane. The Northern blot was hybridized consecutively with *cdc22*+, *lacZ*+, *adh1*+ probes (Section 4d), and *cdc10*+ (a PCR fragment generated using oligonucleotides S and T - Appendix A).

ii Results

Fission yeast strains *972h⁻* (wild-type), *cdc10-129*, and *cdc10-C4* were grown to mid-exponential stage of growth at 24°C, and subjected to a temperature shift to 36°C (Section 2e.iv). Cells were harvested at 0 and $3^1/_2$ hours, and total RNA prepared (Section 2e.vi). The RNA was separated by formaldehyde gel electrophoresis, and transferred to GeneScreen membrane (Sections 2c.vii and 2c.viii). The Nothern blot was hybridized consecutively with *cdc22⁺*, *adh1⁺*, *suc1⁺*, and *cdc10⁺* probes.

The results of this experiment are shown in Figure 5G.

cdc22⁺ transcript levels were much higher in *cdc10-C4* at the permissive temperature, than at the restrictive temperature, or in *cdc10-129* and wild-type. This excludes artifactual deregulation as an explanation for *cdc22⁺* over-expression in this mutant.

The smaller transcript which hybridizes to *suc22⁺* probes is not over-expressed in *cdc10-C4*, suggesting this gene is not under MCB control. The smaller transcript is present constitutively during the cell cycle (Gordon & Fantes, 1986), consistent with the proposed role of DSP1 and MCBs in the control of expression of periodically transcribed genes. Interestingly, the larger transcript which hybridizes to *suc22⁺* probes, is present in *cdc10-C4* cells at the permissive temperature, albeit at lower levels that at the restrictive temperature. The larger transcript is usually only observed after treatment with hydroxyurea, or after certain cell cycle arrests (P. Harris, pers. comm; see lanes 5 and 6). An even lower abundance transcript, larger than the large *suc22⁺* transcript, is also present in *cdc10-C4* cells at 24°C.

Neither of the two observed *cdc10⁺* transcripts (Aves *et al.*, 1985) are affected in *cdc10-C4*, arguing that over-expression of MCB genes in this mutant is due to post-transcriptional deregulation of *cdc10⁺*.

5 g Cell cycle behaviour of *cdc22*$^+$ transcript in *cdc10-C4*

i Introduction

cdc22$^+$ transcript is periodically expressed during the normal fission yeast cell cycle, with a maximum at the G_1-S phase boundary (Gordon & Fantes, 1986). Although *cdc22*$^+$ is over-expressed in *cdc10-C4*, is the regulation of its cell cycle expression maintained? This experiment addresses this question by examining *cdc22*$^+$ transcript levels in a population of synchronously dividing *cdc10-C4* cells.

ii Results

cdc10-C4 cells were grown to mid-exponential stage of growth at 24°C, and a population of small cells selected by elutriation (Section 2e.iv). The resulting culture was incubated at 24°C, samples removed every 20 minutes, and total RNA prepared (Section 2e.vi). 10 µg of RNA from each sample was separated on a formaldehyde gel, and transferred to GeneScreen membrane (Sections 2c.vii and viii). The Northern blot was hybridized consecutively with *cdc22*$^+$, *adh1*$^+$ and histone 2A1 probes.

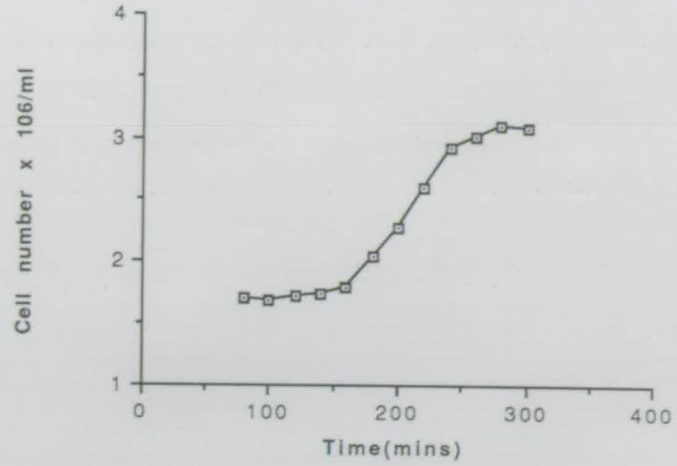
The results of this experiment are shown in Figure 5H.

cdc22$^+$ transcript is present at higher than wild-type levels throughout the cell cycle in *cdc10-C4*. Not only is *cdc22*$^+$ over-expressed in this mutant, but cell cycle regulation is lost.

Histone 2A1 is periodically expressed in *cdc10-C4*. This confirms the synchrony of the dividing cells, and also suggests that histone periodic expression during the cell cycle is independent of DSC1/MCB controls.

Figure 5 H ***cdc22*$^+$ transcript is constitutively over-expressed in *cdc10-C4*.**

cdc10-C4 cells were grown to mid-exponential stage of growth, a population of small cells selected by elutriation, and grown at 24°C. Samples were removed from the culture every 20 minutes for 4 hours, to follow one division cycle. A plot of cell number against time is shown, and the time in minutes at which samples were taken is indicated above each lane. Total RNA was prepared from each sample. The RNA was subjected to Northern blot analysis, and the blot hybridized consecutively with *cdc22*$^+$, *adh*$^+$ (Fig. 4E) and histone 2A1 (a PCR fragment using oligonucleotides S and T - Appendix A) probes. Two control lanes of RNA from asynchronous wild-type (*972h*$^-$ Asy.) and *cdc10-C4* (Asy.) cells are shown.


Cell number x 106/ml
4
3
2
1
0
100
200
300
400
Time(mins)


972h-
cdc10-C4
Asy.
Asy.
80
100
120
140
160
180
200
220
240
260
280
300
cdc22+
histone2A1
adh1+

5 i Dominance relation *cdc10* alleles

i Introduction

cdc10-C4, as are most *cdc* mutants, is a recessive loss of function mutant. Thus in the presence of wild-type copy of *cdc10*$^+$ the cdc phenotype is not observed. In the previous two experiments it has been demonstrated that a phenotype of *cdc10-C4* cells is that *cdc22*$^+$ transcript is over-expressed at 24°C. The next experiment analyses the dominance of the over-expression of *cdc22*$^+$ transcript in a variety of *cdc10-C4* diploid strains.

ii Results

Diploids of *cdc10-C4*, *cdc10-129*, and wild-type were made in the following combinations, all carrying the *mat2-102* mutation for diploid stability:

cdc10-C4 mat2-102 ade6-M216 /cdc10-C4 leu1-32;
cdc10-C4 mat2-102 ade6-M216 /cdc10-129 leu1-32;
cdc10-C4 leu1-32 / mat2-102 ade6-M216;
cdc10-129 leu1-32 /cdc10-129 lys1-131 mat2-102;
cdc10-129 leu1-32 /mat2-102 ade6-M216;
leu3-155 /mat2-102 ade6-M216.

Each strain was grown to mid-exponential stage of growth at 24°C, and subjected to a temperature shift to 36°C (Section 2e.iv). Cells were harvested at 0 and $3^1/_2$ hours, and total RNA prepared (Section 2e.vi). The RNA was separated by formaldehyde gel electrophoresis, and transferred to GeneScreen membrane (Sections 2c.vii and 2c.viii). The Nothern blot was hybridized with *cdc22*$^+$ and *adh1*$^+$ probes (Fig. 4E).

The results of this experiment are shown in Figure 5I.

In the diploid containing one copy of *cdc10*$^+$ and one copy of *cdc10-C4*, *cdc22*$^+$ transcript is present at wild-type levels at 24°C (lane 4). Thus the *cdc10-C4* phenotype of over-expression of *cdc22*$^+$ is genetically recessive. *cdc22*$^+$ transcript appears to be present at higher levels than wild-type in the *cdc10-C4 /cdc10-129* diploid at 24°C (lane 7); thus this *cdc10-C4* phenotype is possibly dominant to *cdc10-129*.

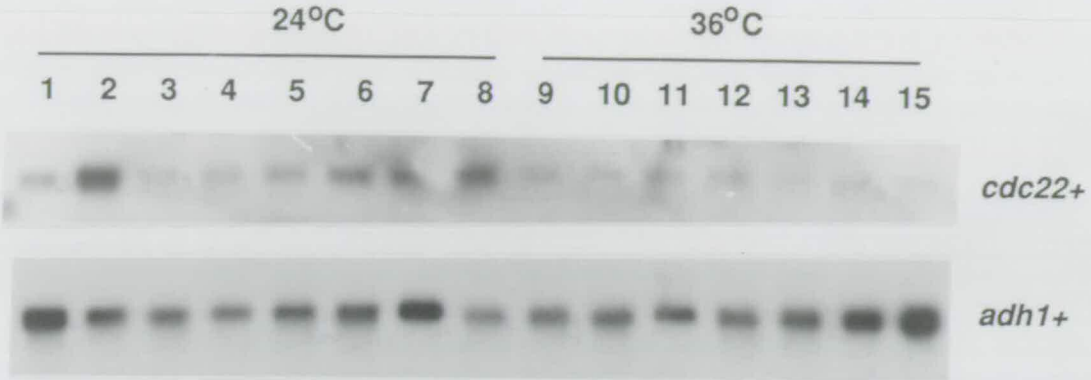


Figure 5 I **Over-expression of *cdc22*⁺ transcript in *cdc10-C4* cells is recessive to wild-type, but dominant to *cdc10-129*.**

A variety of diploids of *cdc10-C4*, *cdc10-129* and wild-type (*972h*⁻), were made, grown to mid-exponential stage of growth at 24°C, and subjected to a temperature shift to 35°C. Samples of cells were taken at 0 and $3^1/_2$ hours, and total RNA extracted. The RNA was separated on a formaldehyde gel and transferred to GeneScreen membrane. The Northern blot was hybridized with *cdc22*⁺ and *adh1*⁺ probes. (Section 4d). Lane numbers represent the following: 1, wild-type; 2 and 9, *cdc10-C4* ; 3 and 10, wild-type/wild-type; 4 and 11, wild-type/*cdc10-C4*; 5 and 12, wild-type/*cdc10-129*; 6 and 13, *cdc10-129*/*cdc10-C4*; 7 and 14, *cdc10-129*/*cdc10-129*; 8 and 15, *cdc10-C4*/*cdc10-C4*.

5j Conclusions

The *cdc10*$^{+}$ gene product, p87^{cdc10}, is a component of DSP1. The binding properties of DSP1 are specifically affected in *cdc10*^{ts} mutants after both *in vivo* and *in vitro* temperature shifts. DSP1 is supershifted in the presence of p87^{cdc10} antibody.

A mutant form of p87^{cdc10}, p87$^{cdc10\text{-}C4}$, appears to be constitutively hyperactive, resulting in *cdc22*$^{+}$ over-expression throughout the cell cycle. A reporter gene under MCB control is also over-expressed in *cdc10-C4*. This mutant phenotype is genetically recessive to wild-type, but may be dominant to *cdc10-129*.

Chapter 6: Discussion

6 a Conclusions

The main conclusions from the work presented in this thesis can be summarised as follows:

1 *cdc22*$^+$ encodes the large subunit of ribonucleotide reductase in fission yeast.

2 *cdc22*$^+$ contains MCBs (*Mlu*I cell cycle boxes) in its promoter region.

3 MCBs can confer periodic expression on a reporter gene in fission yeast, strongly implicating them in the control of *cdc22*$^+$ expression during the cell cycle.

4 A binding activity, DSP1 (DNA synthesis control in *S. pombe*), has been identified in fission yeast which specifically binds MCBs, and is probably a transcription complex.

5 Both MCBs and DSP1 are conserved through evolution, as related components are present in budding yeast.

6 DSP1 binds to MCBs constitutively during the cell cycle.

7 The product of the *cdc10*$^+$ gene, p87^{cdc10}, is a component of DSP1.

8 *cdc10*$^+$ and *cdc22*$^+$ are required for START and S-phase, respectively. Thus, this work has found a molecular link between these two cell cycle stages.

9 A mutant form of p87^{cdc10}, p87$^{cdc10\text{-}C4}$, appears to be constitutively hyper-active, resulting in *cdc22*$^+$ and reporter gene over-expression throughout the cell cycle. Paradoxically, DSP1 is not detectable in *cdc10-C4* cells.

6b Model

A model to incorporate the observations on *cdc22*+ and *cdc10*+ made in this thesis is represented in Figure 6A.

A transcription complex, DSP1, probably containing a number of components, including the gene product of *cdc10*+, p87^{cdc10}, binds MCBs in the promoter region of *cdc22*+. DSP1 binds to the MCBs constitutively during the cell cycle. At the G_1-S phase boundary DSP1 is activated by unknown processes, which cause it to stimulate transcription of *cdc22*+. For the remainder of the cell cycle DSP1 is in an inactive form, resulting in no *cdc22*+ transcription.

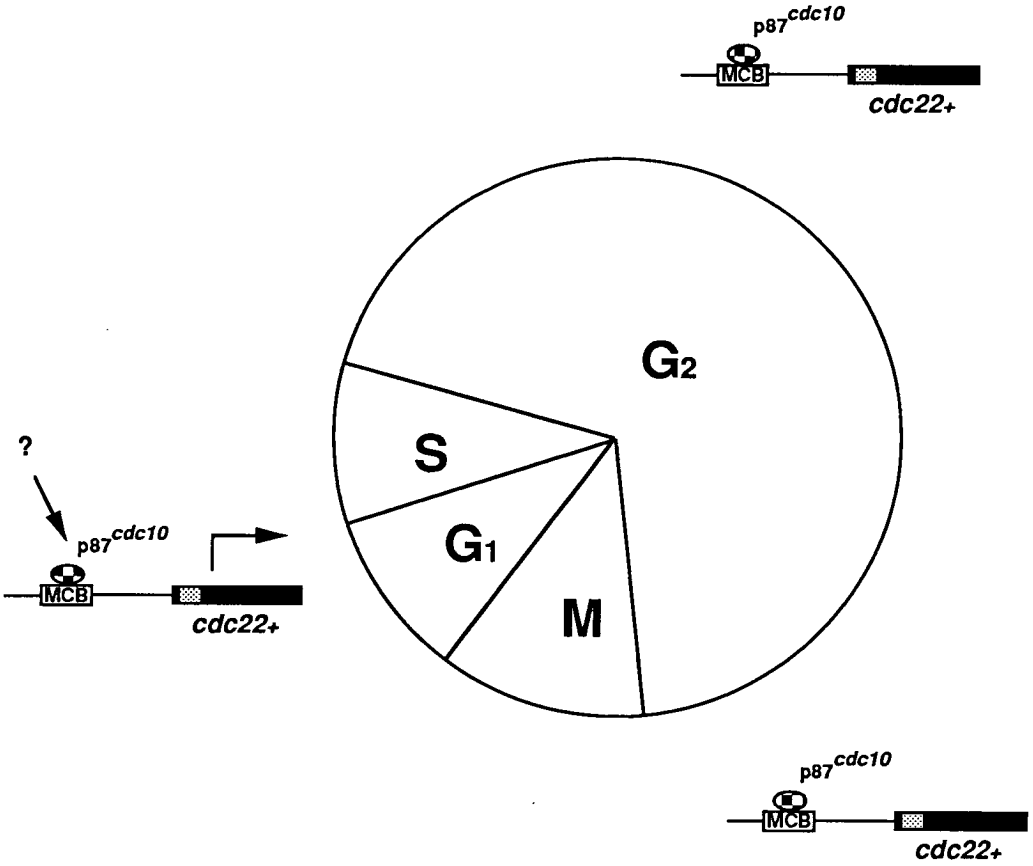


Figure 6 A **DSP1 control of *cdc22*+ expression at the G_1-S phase boundary in fission yeast.**

6 c Problems with the model

i Introduction

Although the model presented for the observations on *cdc22*$^+$ and *cdc10*$^+$ does explain the results, there are a number of unanswered questions and loose ends. In this part of the discussion I will highlight these problems, speculate a little, and suggest experiments that could resolve them.

ii What is the constitution of DSP1?

Bandshift assays using DNA substrates containing MCBs with fission yeast protein extracts have identified a specific binding activity, which we have called DSP1. A component of DSP1 is the product of the *cdc10*$^+$ gene. How representative of the 'real' DSP1 complex present *in vivo*, is this *in vitro* binding activity?

In temperature shifts with *cdc10*^{ts} mutants DSP1 is thermolabile, disappearing at the restrictive temperature, and so behaves *in vitro* as we would expect it to *in vivo*. However conditions, such as protein concentrations, are almost certainly different between the cell and the test tube. Consequently DSP1 could contain more components *in vivo*, which are not present in the complex detected by bandshift assays. Futhermore, DSP1 normally binds chromatin and not small fragments of DNA, which is likely to modify its binding properties. Interpretations of bandshift assays have to be made, therefore, with caution.

DSP1 has a very low mobility in bandshift assays and so is likely to be a large protein. Faster mobility complexes are also detected which are competed by MCBs (Figs. 4G and 4I), and so are likely to be partial forms of DSP1. Thus DSP1 is probably a protein complex containing a number of components. The faster mobility complexes are not supershifted by p87^{cdc10} antisera (Fig. 5E and Lowndes *et al.*, 1992a), arguing that there are other polypeptides beside p87^{cdc10} in DSP1.

There are other reasons for suspecting the presence of additional components in DSP1 to p87^{cdc10}. A novel type of transcription complex has been recently characterised from rat liver nuclei, called GABP (guanosine adenine binding protein; Thompson *et al.*, 1991), which may be

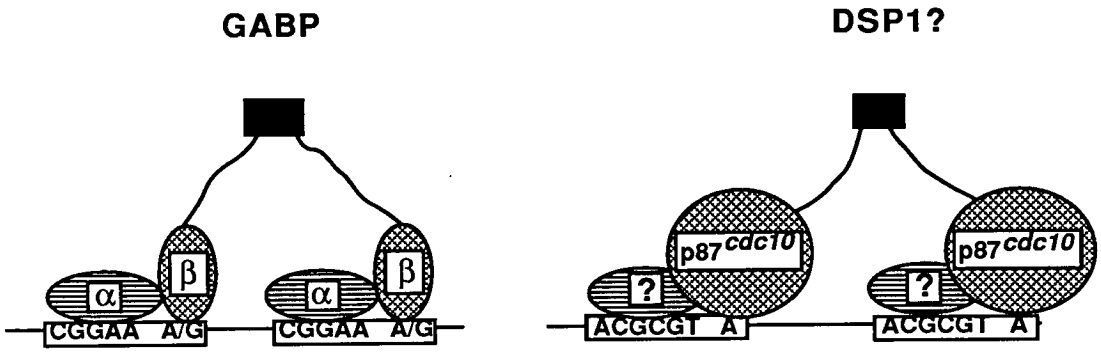


Figure 6 B **Potential similarities between GABP and DSP1.**

The GABP transcription complex is a heterodimer consisting of two α and two β-subunits. The α-subunits contact DNA and confer binding specificity. The β–subunits contain the ankyrin motif, which binds them to the α subunits and hold the the complex together. $p^{87cdc10}$ in DSP1 contains ankyrin motifs and so is possibly a β-type subunit. DSP1 requires two MCBs to bind DNA, which suggests that it may be dimeric in form. After Thompson *et al.*, 1991.

related to DSP1. GABP was originally identified as a factor that is required for VP16-mediated activation of herpes simplex virus immediate early genes (Trienzenberg *et al.*, 1989). GABP consists of a heterodimer of two α and two β-subunits (Fig. 6B). The α-subunits contact DNA and confer DNA sequence binding specificity, whereas the β-subunits bind the α subunits, and hold the two dimers together. DSP1 may be related to this type of transcription complex as p87^{cdc10} shares a motif, the '*cdc10/SWI6* 'or 'ankyrin motif', with the β-subunit of GABP. This motif is thought to important in mediating protein-protein interactions in a variety of multiprotein complexes, but specifically in GABP by binding the β-subunit to the α-subunit, as deletion of *cdc10/SWI6* motifs abolishes this binding (Thompson *et al.*, 1991). Furthermore, the predicted cdc10 polypeptide does not contain any obvious DNA binding motifs (Aves *et al.*, 1985). If DSP1 is related to this type of transcription complex, DSP1 may contain two molecules of p87^{cdc10} and two molecules of an α-type subunit (Fig. 6B).

Two genes which when mutated can suppress *cdc10* mutants have been independently characterised, and called *sct1*$^+$ and *sdc1*$^+$ (Marks *et al.*, 1992; Caligiuri & Beach, 1992). Mutant forms of both genes are able to suppress *cdc10*^{ts} mutants as well as a *cdc10*$^+$ deletion (*cdc10Δ*). *sdc1*$^+$ has been cloned and sequenced, and is essential for viability (Caligiuri & Beach, 1992). Its predicted amino acid sequence shows similarity to the predicted cdc10 polypeptide. *sdc1*$^+$ and *sct1*$^+$ are almost certainly the same gene as they map to same position on chromosome II (V. Simanis, pers. comm.).

What is the role of *sdc1*$^+$? Does it encode another component of DSP1? To prove this formally would require ts mutants or specific antibody to the gene product of *sdc1*$^+$, sdc1, to do similar experiments to those completed on *cdc10*$^+$ (Chapter 5). Perhaps wild-type DSP1 contains molecules of p87^{cdc10} and sdc1. One could then explain *sdc1*$^+$ mutants' ability to suppress *cdc10Δ* and *cdc10*^{ts} mutants, by the mutant forms of sdc1 being able to functionally replace p87^{cdc10} in DSP1. DSP1 is not, however, detected by bandshift analysis in *sct1-1 cdc10Δ* cells (V. Simanis, pers. comm.).

The genetic screens that obtained both *sdc1*$^+$ and *sct1*$^+$ used *cdc10-129* as starting strain, which would have determined the type of suppressor mutations isolated. Different suppressors, as well as *sdc1*$^+$ and

sct1$^+$, might be obtained if *cdc10-C4* was used instead as the starting strain in a similiar screen, as this mutant has strikingly different regulatory properties to *cdc10-129*.

If DSP1 is related to GABP, and p87^{cdc10} a β-type subunit, are there α-type subunits? One possibility is that sdc1 is an α-type subunit. However, considering the similarity between the predicted gene products of *sdc1*$^+$ and *cdc10*$^+$ this seems unlikely. α-type subunits contact DNA, and confer binding specificity (Thompson *et al.*, 1991): thus there are two possible experimental routes to isolate such DNA binding proteins.

DNA binding proteins have been isolated by using UV crosslinking techniques (Chodosh, 1988). This approach has already proved successful in isolating one partner to Swi6 in DSC1 (Dirick *et al.*, 1992). The *cdc22*$^+$ promoter fragment containing four MCBs (Section 4e) would be mixed with yeast cell extracts and separated by electrophoresis, as in bandshift assays. The DSP1/DNA complex would then be irradiated with UV light which covalently binds proteins bound to the DNA, the band excised after autoradiography, digested with DNAse I, and analysed by SDS-PAGE electrophoresis. Any p87^{cdc10} binding to the MCBs would be identified by specific antisera, while other components could be identified by silver staining.

Other components of DSP1 could be detected by Southwestern blot analysis (Miskimmins *et al.*, 1985) using the *cdc22*$^+$ promoter fragment as probe with total fission yeast protein extracts. If polypeptides were detected, this would suggest that the DNA binding component of DSP1 can bind DNA by itself. As a precedent for this, the α-subunit of GABP can bind DNA in the absence of the β-subunit (Thompson *et al.*, 1991). If the DNA binding part of DSP1 can bind DNA by itself this could permit its cloning, by probing a fission yeast λgt11 expression library with the *cdc22*$^+$ promoter fragment (Young & Davis, 1983).

If DSP1 is normally composed of sdc1 and p87^{cdc10}, what are their functional roles in the complex? One could be a positive regulator and the other a negative regulator of *cdc22*$^+$ transcription. Clues to the roles of these two polypeptides may be given by the following two experiments.

Is *cdc22*$^+$ transcript periodically expressed in *sdc1 cdc10Δ* cells? If *cdc22*$^+$ is periodically expressed in such cells, this would argue that mutant *sdc1* can act as an inducer of transcription.

Can *sdc1* suppress the over-expression of *cdc22*$^+$ transcript in

cdc10-C4? If *cdc22*+ transcript is not over-expressed in *sdc1 cdc10-C4*, this argues that mutant *sdc1* can act as a repressor of transcription.

The interpretations about the role of *sdc1*+ that can be made from these experiments are limited, as the effect of mutant *sdc1* would be analysed. Mutant *sdc1* may well behave differently to wild-type *sdc1*+.

iii What does DSP1 bind in the *cdc22*+ promoter?

Bandshift experiments have conclusively shown that DSP1 specifically binds multiple MCBs in DNA fragments from the promoter region of *cdc22*+ (Chapter 4). DSP1 recognizes two fragments of DNA both containing clusters of MCBs, one 5' and one 3' to transcriptional start (Figs. 4C and D). The cluster of MCBs 5' to transcriptional start is most likely to be relevant for *cdc22*+ expression, as it is in the conventional position for yeast *cis*-acting elements. However, both clusters may be required for complete and periodic expression of *cdc22*+ transcript. Key questions are: which cluster of MCBs does DSP1 bind *in vivo*; and, is one or are both clusters relevant to the control of *cdc22*+ expression?

These questions could be tested by cloning the whole *cdc22*+ promoter region into pSPΔ178, mutating single MCBs by site-directed mutagenesis, and assaying their effect on *lacZ* transcript expression. The core GC of each MCB could be changed to AT, as we know these base changes abolish binding of DSP1 to DNA, from bandshift experiments (Fig. 4I).

Promoters of yeast genes expressed by RNA polymerase II contain two types of 5' *cis*-acting elements that stimulate general transcription complexes to induce expression of genes, depending on their position relative to transcriptional start (Section 1f.i). The first type are proximally-placed and are position-dependent. The second type are distally-placed and are orientation and position-independent; these *cis*-acting elements are related to metazoan enhancers have been named UASs, for upstream activating sequences. What type of *cis*-acting element are MCBs?

The *cdc22*+ promoter region contains two clusters of MCBs, one 5' and one 3' to transcriptional start. The 5' cluster is ~160 nucleotides upstream of transcriptional start, and so could be either a proximal type *cis*-acting element or a UAS. The 3' MCB cluster, if it is relevant to transcription, cannot be defined as it is unprecedented in yeast for *cis*-

acting elements to be in such a position.

MCBs, when they are *Mlu*I restriction sites are palindromes, and so have no intrinsic orientation. The MCB trimer in pSPΔ178.3M, being palindromic, presumably stimulates *lacZ* transcription when present in either orientation. To show that MCBs are also position independent, and so are UASs, would require demonstrating that *lacZ* expression is not affected by insertion of extra DNA between the *Mlu*I trimer and the cytochrome *c* promoter.

The *cdc22*+ promoter contains seven MCBs, six of which are in two clusters. Two of the MCBs are perfect *Mlu*I restriction recognition sites and the other four are $^{5}/_{6}$ matches, and so the MCB clusters are not palindromic. To show that these MCBs can act as UASs would require cloning each into pSPΔ178 in both orientations and demonstrating that all DNAs conferred periodic expression on *lacZ*.

Although it has been established that DSP1 binds MCBs, we do not know exactly what region of DNA around the MCBs it binds. This question can be answered by DNAse I protection studies ('footprint analysis'; Galas & Schmidtz, 1978), which are currently being undertaken. Such studies determine the region of DNA protected from DNAse I by protein complexes, and so delimit the precise area of protein/DNA contact. Preliminary results have identified groups of nucleotides protected from DNase I activity by DSP1: they include all four of the MCB elements 5' to transcriptional start (Figs. 4C and 4D).

What is the relationship between DSP1/DSC1 binding and the expression levels of MCB genes, in fission and budding yeast?

DSP1 and DSC1 have remarkably similar binding specificities, binding MCBs from the promoters of both fission and budding yeast genes (Figs. 4G and I). The MCB cluster 5' of *cdc22*+ transcription start contains four MCBs, whereas the cluster 3' contains two. In budding yeast, DNA synthesis genes have been found to have only one or two MCBs (Review: Johnston & Lowndes, 1992).

Two MCBs are required for competition of DSP1 and DSC1 in bandshift assays (Fig. 4G and I), arguing that both complexes need two MCBs for binding. This observation is consistent with the proposal that DSP1 and DSC1 are related to the GABP type transcription complex which has two regions of contact (Fig. 8B). However, reporter gene experiments

in both budding and fission yeast have demonstrated that three tandem MCBs are required in for significant expression of *lacZ* transcript to occur (Lowndes *et al.*, 1991; Section 4c). These contradictory observations may be reconciled if spacing of MCBs is important for expression. Two MCBs, which are a certain distance a part, might only be required to stimulate gene expression. This hypothesis could be tested by replacing the middle MCB by random DNA of the same size in pSPΔ178.3M, and assaying its effect on *lacZ* expression.

iv *cdc10-C4*

The *cdc10-C4* mutant presents an apparent paradox. In these cells at the permissive temperature *cdc22*$^+$ transcript is constitutively over-expressed, but DSP1 is not detectable *in vitro* by bandshift assay (Sections 5b, 5c and 5f). How can DSP1 induce over-expression of *cdc22*$^+$ if it cannot bind its promoter? Bearing in mind that DSP1 *in vitro* may not be representative of DSP1 *in vivo* (Sections 6c.ii and 6c.vi), there are two possible explanations.

First, that DSP1, containing mutant p87$^{cdc10\text{-}C4}$, has altered binding affinity for MCBs *in vivo*. Such altered binding coincidentally results in over-expression of *cdc22*$^+$, but is not detectable by bandshift assay.

Second, that p87$^{cdc10\text{-}C4}$ binds MCBs by itself. In the absence of other components of DSP1 p87$^{cdc10\text{-}C4}$ causes over-expression of *cdc22*$^+$. This hypothesis would argue that p87$^{cdc10\text{-}C4}$ is an inducer of *cdc22*$^+$ transcription. Against this explanation no complexes of faster mobility, containing p87$^{cdc10\text{-}C4}$, are observed by bandshift in *cdc10-C4* cells. The hypothesis can however be tested. If the *sdc1*$^+$ gene product is another component of DSP1 (Section 6c.ii) one possibility is that p87$^{cdc10\text{-}C4}$ does not interact with this molecule at the permissive temperature. Can, therefore, mutant *sdc1* suppress *cdc22*$^+$ over-expression in *cdc10-C4*?

The phenotype of over-expression of *cdc22*$^+$ in *cdc10-C4* is recessive to wild-type, but may be dominant to *cdc10-129* (Section 5i). This difference may reflect alterations in the binding affinity to MCBs of the three forms of DSP1 present in these strains. If this interpretation is correct wild-type DSP1 would have the highest affinity and bind preferentially to the *cdc10* mutant forms, resulting in the wild-type levels of *cdc22*$^+$ transcript; and the *cdc10-C4* form of DSP1would have higher

affinity than the *cdc10-129* form, consistent with the observed over-expression of *cdc22*$^+$. If this is true, and the *cdc10-C4* form of DSP1 has higher affinity than the *cdc10-129* from, the inability to detect DSP1 in *cdc10-C4* by bandshift assays becomes even more inexplicable.

v Are there other genes under DSP1/MCB control?

In budding yeast a large group of genes required for DNA synthesis has been identified which are coordinately expressed at the G_1-S phase boundary, and are under DSC1/MCB control (Johnston & Lowndes, 1992). In fission yeast *cdc22*$^+$ is the only gene known that is periodically expressed, and under DSP1/MCB control. Are there likely to be others?

The genes encoding DNA ligase and DNA polymerase I are expressed at the G_1-S phase boundary in budding yeast, but the equivalent genes in fission yeast are not (White *et al.*, 1986; Johnston *et al.*, 1987; Bouvier *et al.*, 1992)). Thus, there is precedent for alternative forms of gene expression in the two organisms, and so one cannot predict whether other fission yeast DNA synthesis genes will be periodically expressed.

If *cdc22*$^+$ is the only gene whose transcription requires p87^{cdc10}, then expression of *cdc22*$^+$ independently of the DSP1/MCB system should bypass the requirement for *cdc10*$^+$ function. This hypothesis can be tested by introducing *cdc22*$^+$, constitutively expressed from the *nmt1* promoter, into *cdc10*^{ts} strains, and seeing if the *cdc10* cell cycle arrest is alleviated.

Both *cdc22*$^+$ and the reporter gene *lacZ*, which contain different MCB promoters, are over-expressed in *cdc10-C4*. Thus, if there are other genes under MCB control, they would be predicted to be also over-expressed in this mutant, which may permit their isolation. A differential screen (Leonard *et al.*, 1987; Lee *et al.*, 1990) could be conducted to isolate mRNA species that are selectively over-expressed in *cdc10-C4*. A cDNA library would be made from mRNA extracted from *cdc10-C4* cells grown at the permissive temperature, to enrich for over-expressed mRNAs. Replica filter lifts would be hybridized to cDNA probes made from *cdc10-C4* and wild-type cells, or *cdc10-129* cells grown at the restrictive temperature. mRNA species over-expressed in *cdc10-C4* would be identified by stronger hybridization signals when hybridized to the *cdc10-C4* probe. Isolated cDNAs would be initially characterised by restriction mapping, *cdc22*$^+$ clones discarded, and the DNA sequence

determined of the remaining clones.

Histone 2A1 transcript levels are not affected in *cdc10-C4* (Section 5g) arguing that cell cycle histone expression is controlled by other mechanisms than the DSP1/MCB system. This observation is consistent with the presence of alternative *cis*-acting repeat motifs to MCBs in the histone promoters, and the different expression pattern of histones to *cdc22*$^+$ during the cell cycle (Matsumoto *et al.*, 1987). Budding yeast histones are also expressed independently of the DSC1/MCB system, as the transcript levels are unaffected in a *SWI6* deletion (Lowndes *et al.*, 1992b).

vi How does DSP1 induce transcription of *cdc22*$^+$?

The DSP1 complex, as detected *in vitro* by bandshift, is invariant in amount and mobility during the cell cycle. This excludes the simple hypotheses that binding of DSP1, or large conformational changes in DSP1 bound to MCBs, correlates with transcriptional activation. DSP1 might induce *cdc22*$^+$ expression at the G_1-S phase boundary by subtle changes in binding to the DNA, or by changes in binding by other proteins not detected by bandshift, at this cell cycle stage. Such changes may be detectable by DNAse I protection ('footprinting') studies.

Formally we cannot exclude the possibility that DSP1 binds MCBs periodically *in vivo*. Other components of DSP1 required for periodic binding may not be present *in vitro* (Section 6c.ii). Alternatively, DSP1 may only available for binding MCBs at the appropriate cell cycle stage. As an example of the latter, the transcription factor Swi6 has been shown to be present in the nucleus only at the cell cycle stage when it induces expression of the HO endonuclease (Taba *et al.*, 1991).

How and what activates DSP1 at the G_1-S phase boundary?

p87^{cdc10} is known to be a phosphoprotein (Simanis & Nurse, 1989), and so phosphorylation is a potential form of regulation of DSP1 activity. This is quite likely, as phosphorylation is a common form of control of transcriptional regulators (Review: Jackson, 1992).

Gross phosphorylation levels of p87^{cdc10} have been shown to be invariant during the cell cycle (Simanis & Nurse, 1989). The principal phosphorylation site of p87^{cdc10} has been mapped to serine 196 (Deuschle & Beach, 1992). Permanent dephosphorylation of this site, by mutating the

serine to an alanine, reduces the ability of *cdc10-A196* to complement *cdc10-129*, and is accompanied by reduced DSP1 binding activity *in vitro* (Deuschle & Beach, 1992). Neither the phosphorylation state of serine 196 during the cell cycle, or the effect of permanent dephosphorylation of serine 196 on *cdc22*$^+$ expression patterns during the cell cycle, have been reported. Phosphorylation of serine 196 is inessential for cell cycle progress, so perhaps there are different changes than phosphorylation of p87^{cdc10}, or it is phosphorylation of other components of the complex, that cause activation of DSP1.

If phosphorylation of p87^{cdc10} is important for DSP1 transcriptional activity, what is the protein kinase responsible? The p34^{cdc2} protein kinase is required for START transition in fission yeast (Nurse & Bissett, 1981), and is an obvious candidate. However, *cdc2* mutants have been shown to have no effect on the phosphorylation state of p87^{cdc10}, so p34^{cdc2} is unlikely to be the protein kinase (Deuschle & Beach, 1992).

vii Are there alternate modes of induction of *cdc22*$^+$?

As well as being cell cycle regulated, the transcript that hybridizes to *cdc22*$^+$ probes has been shown to be induced in cells after treatment with the DNA synthesis inhibitor hydroxyurea (P. Harris & P. Fantes, pers. comm.). There are two possible explanations for this observation. First, that *cdc22*$^+$ expression, as well as being cell cycle controlled, can be induced by hydroxyurea; second, that *cdc22*$^+$ expression is only cell cycle regulated, and there is another gene that is induced by hydroxyurea whose transcript hybridizes to the *cdc22*$^+$ probe.

In budding yeast there are two genes which encode the large subunit of ribonucleotide reductase, *RNR1* and *RNR3*, which are differentially regulated (Elledge & Davis, 1990). *RNR1* expression is specifically regulated during the cell cycle by the DSC1/MCB system (Elledge & Davis, 1990). *RNR3* is coordinately induced with *RNR2*, which encodes the small subunit of ribonucleotide reductase, after treatment with drugs that damage DNA or block DNA replication by alternative *cis* and *trans* components (Elledge & Davis, 1989; 1990).

A *cdc22*$^+$ related gene could be detected by low stringency Southern blot analysis, and cloned by probing a genomic library under similar low stringency conditions.

If *cdc22*$^+$ is the only gene encoding ribonucleotide reductase in

fission yeast, is the hydroxyurea induction mediated through the DSP1/MCB system, or by different transcription factors binding MCBs or other parts of the *cdc22*$^+$ promoter? The former hypothesis could be tested by assaying the effect of hydroxyurea on *lacZ* transcript in pSPΔ178.3M carrying cells. The promoter of *lacZ* in this test plasmid only contains MCBs, so if induction was observed it would have to occur via MCBs. Such induction could occur through DSP1 or another transcription complex binding to MCBs. Induction of *cdc22*$^+$ transcript independent of the DSP1/MCB system could be tested by cloning the whole *cdc22*$^+$ promoter region 5' to *lacZ* in pSPΔ178, and then assaying *lacZ* expression after hydroxyurea treatment. The promoter region required for hydroxyurea induction outwith the MCBs could be delimitated by mutation studies.

viii What other roles might *cdc10*$^+$ have in G_1?

The *cdc10*$^+$ gene is essential for cell cycle progress, and when mutated results in cdc arrest at START. The *cdc10*$^+$ gene product, p87^{cdc10}, is a component of DSP1 which, as a transcription factor, controls the periodic expression of *cdc22*$^+$ at the G_1-S phase boundary. At the restrictive temperature *cdc10*^{ts} mutant cells cdc arrest, while there is no correlative reduction in *cdc22*$^+$ transcript (Fig. 5F). p87^{cdc10} may, therefore, have other roles to *cdc22*$^+$ induction which are required for cell cycle progress. What might these be?

The gene product with the analogous role to p87^{cdc10} as part of DSC1 in budding yeast is Swi6 (Lowndes *et al.*, 1992b; Dirick *et al.*, 1992). Swi6 is also part of the distinct transcription complex which controls expression of G_1 cyclins, *CLN*s (Fig. 1F). Expression of *CLN*s is required for cell cycle progress in budding yeast. If *cdc10*$^+$ has further analogous roles to *SWI6*, one could explain its cell cycle requirement because it controls G_1 cyclin expression in fission yeast.

One putative fission yeast G_1 cyclin, *puc1*$^+$, has been isolated (Forsburg & Nurse, 1991). One could test if *cdc10*$^+$ controls *puc1*$^+$ expression, by analysing the effect of *cdc10* mutants on *puc1*$^+$ transcript levels.

Both the predicted *cdc10* and *SWI6* polypeptides have ankyrin motifs, and so are likely to be β-type subunits in DSP1 and DSC1, respectively (Fig. 6C). Swi6 is part of three complexes which stimulate

transcription of three groups of genes: DSC1 binds MCBs to induce DNA synthesis genes; Swi6 with Swi4 bind CCBs to induce *CLN*s (Fig. 1F), and Swi4 and Swi6 have recently been shown to control expression of a chitinase gene (*CTS1*). This alternate gene induction could be conferred by different α-type subunits in the three complexes, with binding specificity to either MCBs, CCBs or the *CTS1 cis*-acting element. Potentially, $p87^{cdc10}$ could control *cdc22*$^+$ and G_1 cyclin expression by a similar mechanism.

SWI6 or *SWI4* singly are inessential for division, whereas deletion of both genes is lethal. *cdc10*$^+$ by itself is essential. The predicted *cdc10* polypeptide has modest similarities with both SW14 and SWI6 (Breeden & Nasmyth, 1987a; Andrews & Herskowitz, 1989b). These observations can perhaps be explained if *cdc10*$^+$ carries out the functions of both *SWI6* and *SWI4* in fission yeast.

6 d General considerations

i Why is *cdc22*$^+$ cell cycle regulated?

Expression of a number of genes has been found to be cell cycle regulated. Examples include the G_1 cyclins, *CLN*s, of budding yeast (Wittenberg *et al.*, 1990), *cdc25*$^+$ of fission yeast (Moreno *et al.*, 1991), and the mitotic cyclins of clam (Evan *et al.*, 1983). There are three potential reasons why gene expression is cell cycle regulated.

1 Activity of the gene product is rate limiting for cell cycle progress. Thus, expression of the gene at a particular cell cycle stage allows passage through that stage. An example of this is *cdc25*$^+$. At the end of the G_2 phase a critical amount of $p80^{cdc25}$ is required to dephosphorylate $p34^{cdc2}$ and and activate it, to permit mitotic onset (Section 1e.i).

2 A gene product is periodically expressed, because its gene product would have a deleterious effect if present at the 'wrong' time during the cell cycle. Cyclins provide an example. It has been proposed that there are G_1 and G_2 specific cyclins, in budding yeast - *CLN*s and *CLB*s, respectively - which bind and activate $p34^{cdc2}$ at different time during the cell cycle (Reed, 1991). *CLN*s are expressed only during G_1, bind to $p34^{cdc2}$

and activate it perform G_1 roles; *CLB*s are expressed only during G_2 and activate $p34^{cdc2}$ to do G_2 roles. If this hypothesis is true, inappropriate expression of cyclins would obviously have serious consequences to the cell, as $p34^{cdc2}$ would complete cell cycle functions when it shouldn't. Consistent with this hypothesis is the observation that *puc1*$^+$, a putative fission yeast G_1 cyclin, when over-expressed causes a delay in mitosis (Forsburg & Nurse, 1991).

3 Periodic expression of some genes has evolved as a form of cell economy, with expression being merely coincident with peak requirement. This explanation suggests that such periodic expression has a subsidiary role in controlling cell cycle progress.

cdc22$^+$ transcript is constitutively over-expressed in *cdc10-C4* at the permissive temperature, when the cells are able to complete apparently normal cell cycles. This argues that absolute levels of *cdc22*$^+$ transcript are not rate limiting for cell cycle progress, as S-phase onset occurs normally. Fission yeast has a very short G_1 period, so constitutive over-expression of *cdc22*$^+$ may induce a premature onset of S-phase that is undetectable. G_1 is extended in *wee1* mutants (Nurse, 1975), so an effect of abnormal *cdc22*$^+$ expression on S-phase onset may be detectable in these cells. One could do this by comparing the timing of S-phase onset in a *wee1 cdc10-C4* double mutant with the *wee1* single mutant.

As *cdc10-C4* cells complete normal cell cycles, the presence of *cdc22*$^+$ gene product at cell cycle stages other than the G_1-S phase boundary is presumably not deleterious. Thus, *cdc22*$^+$ transcript may be periodically expressed for cell economy.

ii Is the DSP1/MCB system likely to be found in higher eukaryotes?

Molecular mechanisms found to common to both budding and fission yeast have often subsequently been shown to present throughout the animal kingdom. How conserved is the DSP1/MCB system between the two yeasts, and is it present in higher eukaryotes?

The DSP1/MCB systems are, to an extent, closely related in the two yeast species. MCBs are related in DNA structure in the two yeasts. Furthermore, DSC1 and DSP1 are similar in mobility, and the binding

specificities of the complexes are extremely similar under bandshift conditions (Fig. 4G). Indeed DSC1 can bind fission yeast MCBs and DSP1 can bind budding yeast MCBs, so in terms of DNA binding specificity, as determined by bandshift, the two complexes are indistinguishable.

There are differences between DSP1/MCB systems of the two yeast species. DSP1 binds MCBs constitutively during the cell cycle (Fig. 4M) whereas DSC1 was originally reported to bind MCBs periodically (Lowndes *et al.*, 1991). Recently, however, DSC1 has been found to bind invariantly in synchronized cells after α-factor block and release (Dirick *et al.*, 1992). So the mechanism by which the two complexes induce periodic expression of their target genes may or may not be different.

p87^{cdc10} and Swi6, components of DSP1 and DSC1, respectively, are related (Breeden & Nasmyth, 1987a; Andrews & Herskowitz, 1989b). However, neither can genetically complement the other, and antibodies to p87^{cdc10} do not supershift DSC1, and *vice versa* (L. Johnston, pers. comm).

As the DSP1/MCB systems in the two yeast species are quite similar, so related components would be expected to present in higher eukaryotes. In mouse the genes encoding both subunits of ribonucleotide reductase are periodically expressed in parallel, with strong maxima in early S-phase (Engstrom *et al.*, 1985; Bjorklund *et al.*, 1990). No MCBs have been observed in the promoter region of either gene, so these two genes are likely be regulated by a different common *cis*-acting elements (L. Thelander, pers. comm.). The nearest known analogous metazoan transcription factor to DSP1/DSC1 is E2F, which has the target sequence TTTCGCGC (Mudryj *et al.*, 1990). E2F is active in late G_1 and has binding sites in the dihydrofolate reductase gene, which is expressed under cell cycle control (Farnham & Schimke, 1985). A polypeptide of similar size to p87^{cdc10} is recognized in human cells with antisera to p87^{cdc10} (Simanis & Nurse, 1989), although the significance of this observation is not yet understood. So it would seem at present that homologues of the DSP1/MCB system have not been characterised. It will be interesting to see in the future if related components are found.

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Appendix A: Oligonucleotides

This list contains all the oligonucleotides referred to in the text of the thesis.

The nucleotide sequence and annotation of each oligonucleotide is shown. The position and orientation of those oligonucleotides relative to pCDC22-1 (A-N) are shown in Figure A. Oligonucleotides O and P were used for the amplification of DNA fragments cloned into pSPΔ178. Two pairs of oligonucleotides were used for amplifying probes: Q and R, for *cdc10*$^{+}$; and S and T, for histone 2A1.

Each oligonucleotide is shown 5'- 3'; dotted underline indicates extra DNA, and underline indicates restriction sites for cloning.

All oligonucleotides were made by the Oswell DNA Service, University of Edinburgh.

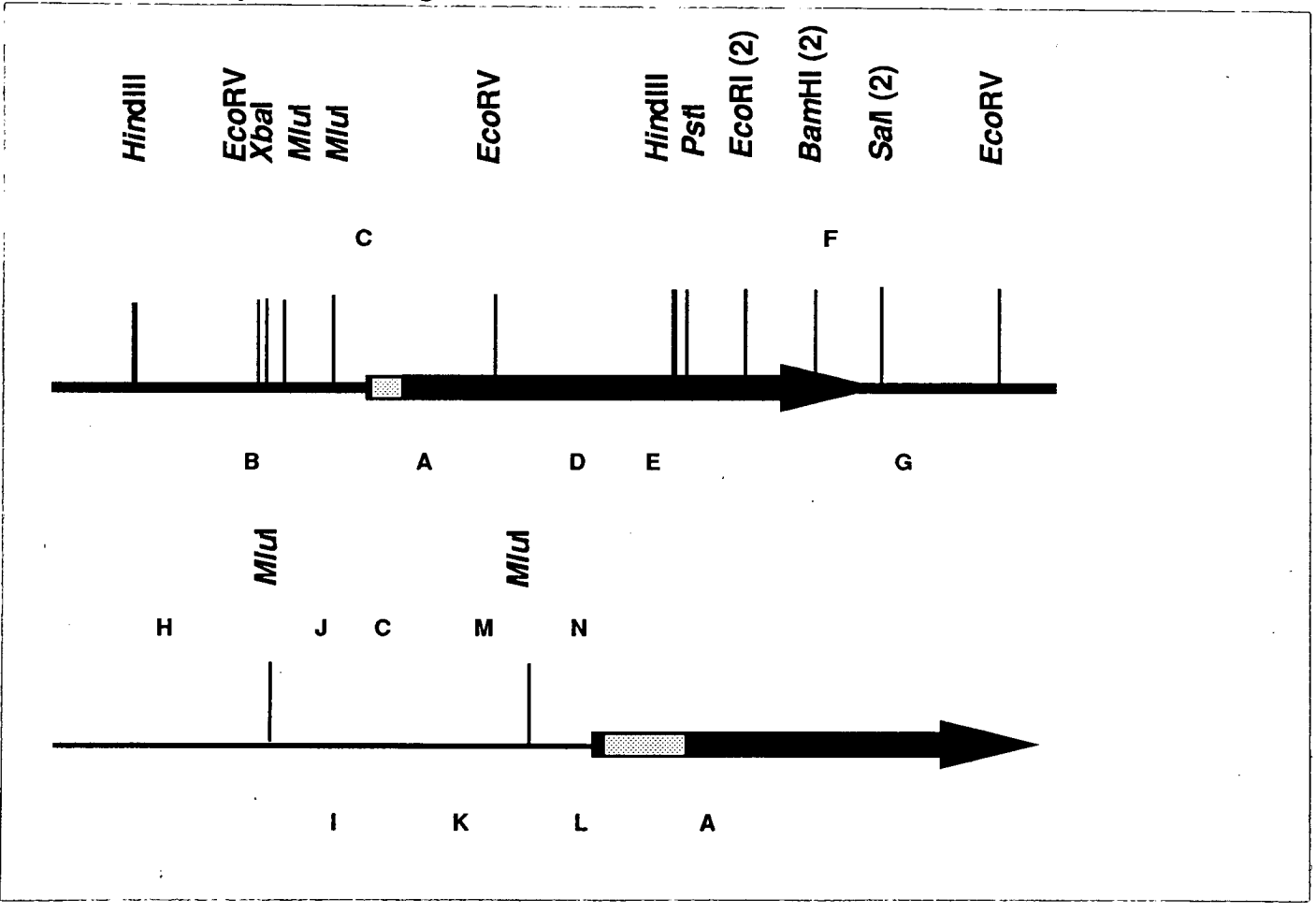

Figure A Schematic diagram of pCDC22-1, and an enlargment of the promoter region of *cdc22*$^{+}$, showing the position and orientation of oligonucleotides used in experiments. The letters refer to each oligonucletide listed below.

pCDC22-1 oligonucleotides

	5'- 3'	Tube No.
A	GATCAACATGATCGGAAT	721J
B	ACACGCGGTCCAACCGA	723J
C	AGCGGAACTTTGATGTTC	722J
D	AGATCATAGCACACACAT	720J
E	GACGACCACGGTTCTCCT	719J
F	GCGATTCCTGAAATTCCT	131H
G	CACAATTATGTATGTCGA	322L
	*Xho*I	
H	GCGCCTCGAGGTAGTTCAATCTCATAGA	716K
I	GCGCCTCGAGCTCTGTTTACGACTGAATG	832L
J	CATTCAGTCGTAAACAGAG	831L
	GCGCCTCGAGCATTCAGTCGTAAACAGAG	321M
K	CGCGCTCGAGCCGCTAAAATAAGTCCGA	715K
L	GGTGGTAAATACCGGGAA	041M
	GCGCCTCGAGGGTGGTAAATACCGGGAA	323M
M	CATTGATCAACATGACTTAAAG	040M
	GCGCCTCGAGCATTGATCAACATGACTTAAAG	322M
N	TTCCCGGTATTTACCACCTT	407T

pSPΔ178 oligonucleotides

O	CACGCCTGGCGGATCTG	839M
P	CTAAACTCACAAATTAGAGC	661N

***cdc10*⁺ oligonucleotides**

	*Bam*HI	
Q	CGCGCGGGATCCTTATGCTTGATGTTCTTT	404L
	*Xba*I	
R	GCGCTCTAGACATATGGCTTCAGCCAATTTT	405L

Fission yeast histone 2A1 oligonucleotides

S	GTCTGGAGGTAAATCTGGTG	231T
T	CAGCTCCTGACTAGGCTTTC	232T

_Mlu_I trimer

This 36 nucleotide double stranded synthetic oligonucleotide contains three *Mlu*I restriction sites separated by *Xho*I restriction sites, and *Xho*I sites at each end. It was inserted into the *Xho*I site of pSPΔ178 (Lowndes *et al.*, 1992a). One strand is shown.

*Xho*I *Mlu*I *Xho*I *Mlu*I *Xho*I *Mlu*I *Xho*I

5' - CTCGAGACGCGTCTCGAGACGCGTCTCGAGACGCGTCTCGAG - 3'

Appendix B: Publication

Control of DNA synthesis genes in fission yeast by the cell-cycle gene *cdc10*$^+$

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IN the budding yeast *Saccharomyces cerevisiae*, cell-cycle control over DNA synthesis occurs partly through the coordinate expression in late G1 phase of many, if not all, of the genes required for DNA synthesis[1–3]. A *cis*-acting hexamer element ACGCGT (an *Mlu*I restiction site) is responsible for coordinating transcriptional regulation of these genes at the G1/S phase boundary[1,4] and we have identified a binding activity, DSC1, that recognizes these sequences in a cell-cycle-dependent manner[1]. In the distantly related fission yeast[5] *Schizosaccharomyces pombe*, only one of the known DNA synthesis genes, *cdc22*$^+$, which encodes a subunit of ribonucleotide reductase, is periodically expressed in late G1 (ref. 6). The promoter region of *cdc22*$^+$ has two *Mlu*I sites and five related sequences, suggesting that similar controls over DNA synthesis genes could occur in fission yeast. We report here a binding activity in fission yeast that is very similar to DSC1 in budding yeast. We also show that the fission yeast *cdc10*$^+$ gene product, which is required for Start and entry into S phase[7,8], is a component of this binding activity.

We initially established that *Mlu*I motifs (Figs 1*a* and 2*a*) are physiologically significant in fission yeast. A plasmid containing three synthetic *Mlu*I sequences linked to the *Escherichia coli lacZ* gene was transformed into a wild-type strain and the cell-cycle expression of the *lacZ* transcript was examined in a synchronous culture (Fig. 1*b*). *LacZ* is expressed periodically. In the first cycle, expression of *lacZ* is slightly perturbed as it does not coincide precisely with *cdc22*$^+$ expression, which is similar to our results with budding yeast[1]. But in the second cycle and even at the beginnings of a third cycle, *lacZ* and *cdc22*$^+$ are expressed in the same intervals of the cell cycle. Thus *Mlu*I sequences can confer expression in late G1 to a heterologous gene in *S. pombe*, as they do in budding yeast.

To detect a binding activity in *S. pombe* that recognizes *Mlu*I motifs, we used a probe containing three tandem *Mlu*I site repeats in gel retardation assays. With this probe we detect a binding activity in fission yeast which, like the budding yeast DSC1 activity, produces a retarded complex of very low mobility (Fig. 2*b*, lane 1). Furthermore, the specificity of the fission yeast activity is essentially identical to that of DSC1 (Fig. 2*b*), hence it will be referred to as 'DSC1-like'. In both cases the uppermost retarded complex is specifically competed away by multimeric copies of the *Mlu*I sequence, ACGCGT (Fig. 2*b*, lanes 2, 3 and 17, 18), but not by multimeric copies of a mutated version of this sequene, ACtaGT (Fig. 2*b*, lanes 4, 5 and 19, 20), nor by multimeric copies of a similar oligonucleotide containing an unrelated hexamer, ATGATT (Fig. 2*b*, lanes 14, 15 and 29, 30). Therefore, both binding activities recognize the ACGCGT core itself and not other sequences found on the oligonucleotide used as a probe (see legend to Fig. 2).

The 132-base pair (bp) and 134-bp fragments from the *cdc22*$^+$ promoter containing four and two *Mlu*I elements (Figs 1*a* and 2*a*), respectively, each compete successfully for binding of both the budding yeast DSC1 activity and of the fission yeast DSC1-like activity (Fig. 2*b*; compare lanes 8–11 and 23–26). Thus both activities recognize *Mlu*I motifs in their normal sequence context

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a

GAAATTAGTAGTTCAATCTCATAGAGCAGGTTGGTAGTCGGGTTGGA<u>CCGCGT</u>GT

TTAATTTATGTAAACAG<u>TCGCGTCGCGT</u>TGCAATTGAG<u>*ACGCGT*</u>AAATAAATATTT

AATTTATTACATTCAGTCGTAAACAGAGTATTTATAAACACTTTTTTTATGTTTAAT

AAAAGATAAATGTAACAGTTGAATGTATTGTATCAGGTCAGACCACTTCAACATG

TTTAA<u>TCGCGT</u>TTTTTTAAAAAAATATTTTTTTAATTTTTAAAAGTCGGACTTATTTT

AGCGGAACTTTGATGTTCAGAAGTGAAAAAGATAAATCTATTTAGCAAGTCTTAATT

AACGTCTTTTAGATATAGTAGAGCTACAAAATGATCCGGTTTCCACTCTTAGCTT

TATTTACA*TT*GATCAACATGACTTAAAGTTCGGATG<u>ACGCGA</u>CGGGCCACATCTCA

ACCGGTTTAATTAATTAGCGTG<u>*ACGCGT*</u>CTGAACGCTTTTTCATTTACTATAAATAT

TCCCGGTATTTACCACCTTACAAACTAGAACAAACACG**ATG**

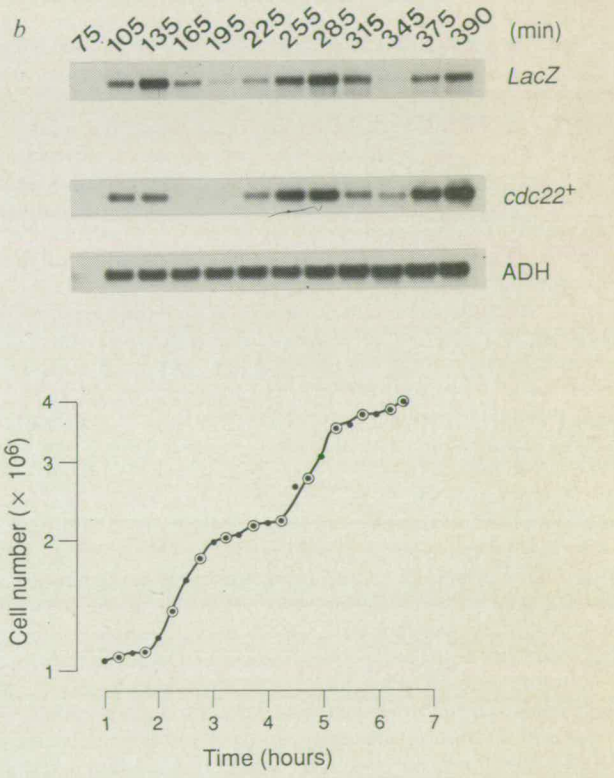


FIG. 1 *a*, The DNA sequence of the *S. pombe cdc22*$^+$ promoter region. The likely ATG at the start of translation is shown in bold and the asterisk marks the start of transcription (C.J.McI. and P.A.F., manuscript in preparation). The two *Mlu*I sequences, ACGCGT, are italicized and underlined and the near-match sequences that are likely to be significant[2] are also underlined. *b*, *Mlu*I motifs confer periodic expression on a reporter gene in *S. pombe*. Northern blot analysis of RNA was carried out on cells synchronized by elutriation and containing a test plasmid with three ACGCGT sequences linked to *lacZ*. The transcript levels of *lacZ* and *cdc22*$^+$ are shown together with alcohol dehydrogenase (ADH), which acted as a control to show that the gel loading was even. The times in minutes at which samples were taken are shown above each lane. Note that the RNA in lane 1 was degraded during preparation. The lower panel shows the increase in cell numbers during the experiment.

METHODS. The budding yeast test plasmid pLGΔ178.3M[1] containing three repeats of the *Mlu*I sequence makes use of the *S. cerevisiae* cytochrome *c* minimal promoter which would be expected to function in *S. pombe*[9]. As it also carries the *S. cerevisiae URA3* gene which can be selected in a *S. pombe ura4* mutant, the plasmid was adapted for use in fission yeast. The *S. cerevisiae* 2 μm origin of replication was removed by digestion with *Eco*RI and replaced by ARS6, a *S. pombe* chromosomal origin of replication. The resulting vector, pSPΔ178.3M, was transformed into *S. pombe* h$^-$ *ura4* and synchronized by elutriation[6]. Cell numbers were monitored using a Coulter counter and samples were removed at intervals (circled points in the lower panel). RNA extraction, northern blotting and probing have been described[10].

as they occur in the *cdc22*$^+$ promoter. Similarly, the fission yeast binding activity recognized the *Mlu*I sites in the promoter region of the periodically expressed DNA synthesis gene *CDC9* (DNA ligase) from budding yeast[10]. A 55-bp fragment from this promoter[1], which contains a single *Mlu*I site and a near-match (Fig. 2*a*), competes successfully for binding of both DSC1 and the DSC1-like activity from fission yeast. (Fig. 2*b*, lanes 12, 13 and 27, 28). In contrast, a 166-bp fragment from the *cdc22*$^+$ upstream containing only a single *Mlu*I near-match (Fig. 2*a*) did not compete for binding of either the fission or the budding yeast activities (Fig. 2*b*, lanes 6, 7 and 21, 22). These experiments have been repeated using the 132-bp fragment from the *cdc22*$^+$ upstream as a labelled substrate, which gives essentially identical results (data not shown). In conclusion, the identical competition profiles for both binding activities, particularly when using promoter fragments from periodically expressed DNA synthesis genes from these two highly divergent organisms[5], strongly argues that the two binding activities are functionally conserved.

Mutants defective in the DSC1 and DSC1-like binding activities would greatly facilitate their further characterization. As these activities control genes essential for DNA synthesis, such mutants might themselves be cell-cycle mutants with execution points in late G1. In the fission yeast, only *cdc2*$^+$ and *cdc10*$^+$ are known to act in late G1 and prevent DNA synthesis[7,8,11]. Significantly, the *cdc10*$^+$ gene product is homologous with the budding yeast SWI4 and SWI6 gene products[12,13], which are believed to be transcriptional activators[13,14]. Furthermore, they actually regulate the cell-cycle specific expression of the HO endonuclease (involved in mating type switching) which is periodically transcribed in late G1 (refs. [13-16]). Thus the *cdc10*$^+$ gene may have a similar function. Consistant with this, *cdc10*$^-$ mutants blocked at the restrictive temperature have substantially lower *cdc22*$^+$ transcript levels than in wild-type cells (M.J.F-S. and P.A.F., unpublished results). We have now found that *cdc10*$^-$ mutants are also defective in their DSC1-like activity (Fig. 3). When *cdc10-129* mutant cells are shifted to the restrictive temperature there is a rapid loss of DSC1-like binding activity which is not observed in similarly treated wild-type cells (Fig. 3*a*; compare lanes 1-6 with 7-12). This loss was evident after only 5 min at 37 °C and there was no binding activity after 30 min, which is well within one cell cycle, so the loss cannot be a secondary consequence of a cell-cycle block.

To confirm this result, we prepared crude extracts from cells containing one of three *cdc10*$^-$ mutant alleles grown at the permissive temperature. Extracts were incubated at 28 °C for 0, 5, 10 and 15 min before assaying their binding activities (Fig. 3*b*). In one allele, *cdc10-C4*, no DSC1-like binding activity was detectable even before the incubation at 28 °C (Fig. 3*b*, lanes 13-16), which presumably indicates that the protein product is extremely unstable. The other two alleles, *cdc10-129* and *cdc10-K28*, produced much reduced DSC1-like activity, which was rapidly inactivated at 28 °C *in vitro* (Fig. 3*b*, lanes 5-8 and 9-12). This loss of activity is not simply due to inhibition of binding in the mutant cells, because mixing extracts from mutant and wild-type cells has no effect on wild-type activity (Fig. 3*c*). Furthermore, three revertants of *cdc10-129* that are able to grow at 37 °C contained binding activity that was insensitive to the temperature shift (Fig. 3*d*, lanes 1-6). Finally, introduction of a plasmid carrying the *cdc10*$^+$ gene into the *cdc10-129* mutant strain fully restores the binding activity at the restrictive temperature (Fig. 3*d*, lanes 7 and 8). Thus the temperature-dependent loss of DSC1-like activity in *cdc10*$^-$ cells seems to be due to a temperature-sensitive gene product and suggests strongly that the *cdc10*$^+$ protein is directly involved with the binding activity.

To determine whether the *cdc10*$^+$ gene product itself is a component of the fission yeast DSC1-like activity, we used anti-*cdc10*$^+$ polyclonal serum, which when added to a binding reaction caused a super-shift of the DSC1-like retarded complex (Fig. 4*a*, lanes 3-7), an effect not seen with preimmune serum (Fig. 4*a*, lane 2). When high concentrations of the anti-*cdc10*$^+$ serum were used, the super-shifted complex did not even enter a polyacrylamide gel (Fig. 4*a*, lanes 3, 4), but in agarose its electrophoretic mobility increased when the serum was diluted (Fig. 4*b*, lanes 3-7), presumably because fewer of the available epitopes are bound. This shows that the *cdc10*$^+$ gene product is itself part of the fission yeast DSC1-like activity.

Given the homology of *cdc10*$^+$ with *SWI4* and *SWI6*, the *cdc10*$^+$ protein is very likely to be a transcription factor. Also, the occurrence of the '*SWI6-cdc10*$^+$' motif (also known as the 'ankyrin' repeat) in these three proteins[12,13,17] suggests that they may be analogous to the β-subunit of the recently characterized heteromeric GABP transcription factor[18]. The *cdc10*$^+$ protein is probably not the only component of the fission yeast *Mlu*I binding activity as several of the retarded species shown in Fig. 2*b* are competed out by *cdc22*$^+$ and *CDC9* upstream fragments. Whereas the faster migrating species are partly due to proteolysis of the uppermost complex (see legend to Fig. 2), it could be significant that only this uppermost species is absent in *cdc10*$^-$ mutants and it alone is affected by the *cdc10*$^+$ antiserum.

The striking similarity between the mobility and specificity of the budding and fission yeast DSC1 activities argues that these binding activities have been functionally conserved in both organisms. But much of the molecular detail has to be established, for instance the similarity between the proteins in the retarded complexes (the *cdc10*$^+$ antiserum we used did not bind to the buddng yeast complex) and the regulation of the binding activities. As the *cdc10*$^+$ gene product is a phosphoprotein[19], the *cdc2*$^+$ protein kinase might be involved in its phosphorylation. When *cdc10*$^+$ is present on a high-copy-number plasmid, the level of the DSC1-like activity is not increased significantly above the wild-type level (Fig. 3*d*, lanes 7, 8), which is consistent with some form of post-translational control. Alternatively, assembly of the complex could be limited by a component other than the *cdc10*$^+$ protein.

It is nevertheless clear that the mechanisms for regulating DNA synthesis genes are similar in both organisms and, therefore, that this particular aspect of DNA synthesis control is conserved. The system is more limited in fission yeast, for example its DNA ligase gene *cdc17*$^+$ is not cell-cycle regulated, unlike *CDC9* in budding yeast[10]. Indeed, of the DNA synthesis genes known in the fission yeast only ribonucleotide reductase (*cdc22*$^+$) seems to be cell-cycle regulated[6]. Interestingly, this enzyme is cell-cycle regulated in many organisms (for a review, see ref. 20), which suggests that it could be rate-limiting and therefore have a widespread role in controlling DNA synthesis. Whether it is regulated in these other organisms by a system of *Mlu*I sites, or related sequences, together with a DSC1-like binding activity is not yet clear. However, budding and fission yeasts are widely divergent evolutionarily[5] so that systems conserved in both organisms are frequently widespread in nature. □

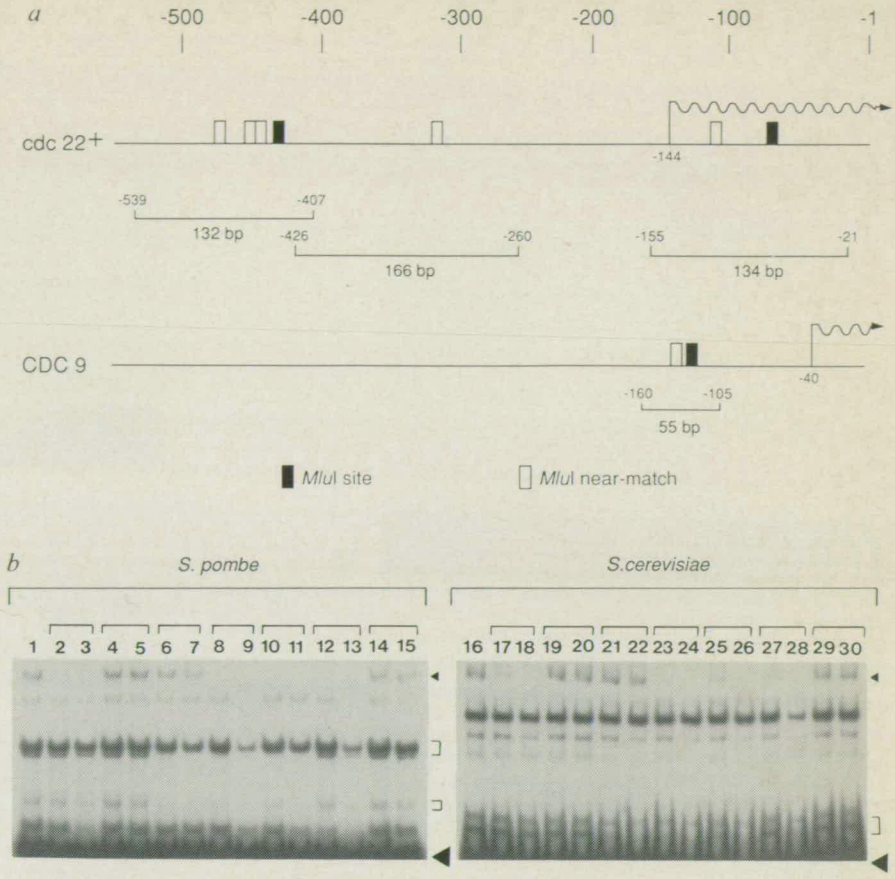


FIG. 2 Gel retardation analysis showing binding of specific proteins from *S. pombe* and *S. cerevisiae* to the ACGCGT sequence element. *a*, The promoter regions of the periodically expressed *cdc22*$^+$ gene from *S. pombe* and *CDC9* gene from *S. cerevisiae* and the relative positions of the DNA fragments used in competition experiments. The scale is in base pairs and numbered relative to the ATG codon. Filled boxes represent perfect matches to the ACGCGT sequence element, whereas open boxes represent near-matches where either the A or the T are altered from the *Mlu*I consensus[2]. The wavy arrows represent the position of the transcripts for both genes. The 132 bp, 166 bp and 134 bp DNA fragments were generated by the polymerase chain reaction, whereas the 55-bp fragment was generated by digestion with *Mnl*I and *Alu*I restriction endonucleases. *b*, Clarified crude extracts were prepared from the *S. cerevisiae* strain CG378 and from *S. pombe* wild-type cells and 20 μg or 5 μg aliquots, respectively, were used with a 73-bp *Alu*I/*Sau*3A fragment from pLGΔ178.3M as previously described[1]. The competitor DNAs used were as follows (in each case a 10-fold and a 100-fold molar excess was used): lanes 1 and 16, no specific competitor; lanes 2, 3 and 17, 18 a tetrameric form of the *Mlu*I site oligonucleotide, 5′-TCGAGACGCGTC-3′; lanes 4, 5 and 19, 20 a tetrameric form of the mutated *Mlu*I site oligonucleotide, 5′-TCGAGACtaGTC-3′; lanes 6, 7 and 21, 22, the 166-bp fragment from the *cdc22*$^+$ upstream; lanes 8, 9 and 23, 24, the 132-bp fragment; lanes 10, 11 and 25, 26, the 134-bp fragment; lanes 12, 13 and 27, 28, the 55-bp fragment from the *CDC9* upstream; lanes 14, 15 and 29, 30, multimeric form of an oligonucleotide containing an unrelated hexamer, 5′-TCGAGATGATTC-3′. The multimeric forms of the competitor oligonucleotides were generated by ligation and either total ligation products were used or the tetrameric form was purified by gel electrophoresis. The small arrowhead indicates the specific DSC1 and DSC1-like retarded products, the larger arrowhead indicates the unbound probe. Brackets indicate specific retarded species, which may correspond to proteolytic products of the uppermost retarded complexes as gradual loss of these due to proteolysis is accompanied by a concomitant increase in the faster-migrating species (unpublished observations). The fission yeast activity is more readily detectable in crude extracts then the budding yeast activity, so to give comparable autoradiographic times and reveal all the retarded species, only 5 μg *S. pombe* crude extract was used in this experiment.

METHODS. All binding reactions were performed with 0.5 ng γ-^{32}P-5′ end-labelled 73-bp *Alu*I/*Sau*3A fragments at ~1×10^8 c.p.m. μg^{-1}. The preparation of clarified crude extracts, protein estimations, binding buffer and gel running conditions were as before[1], except that electrophoresis was through nondenaturing 4% (40:1) polyacrylamide gels.

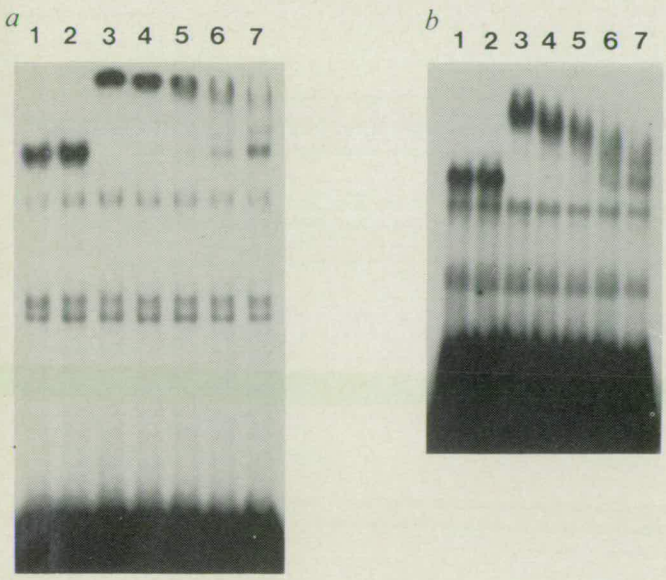
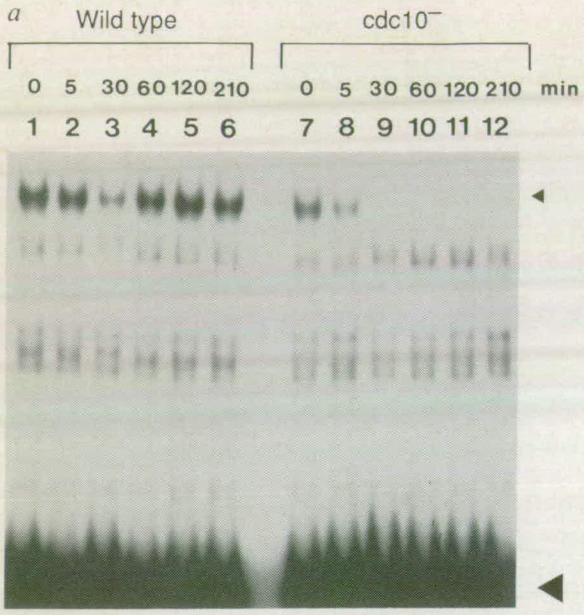
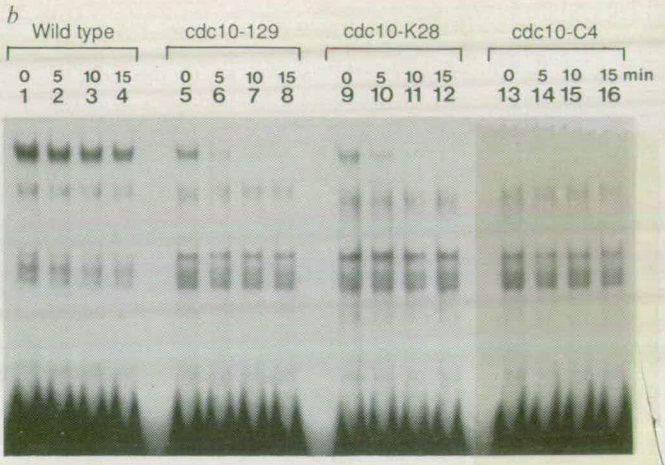


FIG. 4. The DSC1-like activity of *S. pombe* contains the *cdc10*$^+$ gene product. Binding reactions were allowed to equilibrate as before[1] (see also legend to Fig. 2) and then further additions were as follows: lane 1, binding buffer; lane 2, 1/12.5 dilution of pre-immune serum; lanes 3–7, a dilution series of anti-cdc10$^+$ serum, 1/12.5, 1/25, 1/50, 1/100, 1/200. Equal volumes of each reaction were then loaded onto either *a*, 4% (40:1) non-denaturing polyacrylamide or *b*, 2% Nusieve: 1% agarose gels.

METHODS. All binding reactions were performed as described previously[1] and in Fig. 2 legend, except that once equilibrated on ice a further 1 μl of either binding buffer or the appropriate dilution of serum was added and incubated for a further 5 min on ice before loading onto gels. Gels were run as before[1]. Both the preimmune and immune sera were from the same rabbit.





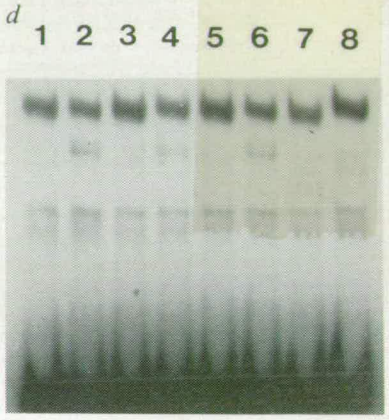


FIG. 3 Gel retardation analysis of the 'DSC1-like' activity in *S. pombe cdc10*$^-$ mutants. *a*, *In vivo* temperature shift. Mid-log cultures of *S. pombe* wild type (lanes 1–6) and *cdc10–129* cells (lanes 7–12), grown at 25 °C, were shifted to 37 °C and samples taken at 0, 5, 30, 60, 120 and 210 min. Clarified crude extracts were prepared and 10 μg wild type or 20 μg *cdc10–129* aliquots were assayed for DSC1-like activity. The small arrowhead indicates the specific DSC1-like retarded product and the larger arrowhead indicates the unbound probe. *b*, *In vitro* temperature shifts. Clarified crude extracts were prepared from wild type (lanes 1–4), *cdc10–129* (lanes 5–8), *cdc10-K28* (lanes 9–12) and *cdc10-C4* (lanes 13–16) cells grown at 25 °C to mid-log phase. These clarified crude extracts were then incubated at 28 °C for 0, 5, 10 and 15 min respectively, as indicated, immediately before use in binding assays. Note that 10 μg wild-type extract and 20 μg *cdc10*$^-$ mutant extract was used for each time point. *c*, Mixing experiment. Clarified crude extracts from wild-type cells (10 μg; lane 1) or *cdc10–129* mutant cells held at the restrictive temperature for 1 h (20 μg; lane 2) were assayed either individually or mixed together (10 μg wild type + 20 μg *cdc10–129*; lane 3). *d*, Analysis of revertants and plasmid rescue of the mutant phenotype. Three spontaneously occurring intragenic revertants (confirmed by genetic analysis) of the *cdc10–129* allele were isolated, grown to mid-log phase at 25 °C, sampled (lanes 1, 3 and 5), shifted to 37 °C for 1 h and then sampled once more (lanes 2, 4 and 6). Lanes 1, 2, revertant 1; lanes 3, 4 revertant 2; lanes 5, 6 revertant 3. The *cdc10–129* mutant strain was also transformed with a plasmid carrying the wild type *cdc10*$^+$ gene, grown at 25 °C to mid-log phase, sampled (lane 7), then shifted to 37 °C for 1 h and sampled again (lane 8).

METHODS. All binding reactions were performed as described before[1] and in the legend to Fig. 2, using 10 μg clarified crude extract unless otherwise stated.

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