CELL CYCLE CONTROL GENES AND THE CHARACTERIZATION OF *CLN3*$^{+}$

SEARCH FOR CELL CYCLE CONTROL GENES

AND THE CHARACTERIZATION OF *CLN3*$^{+}$ IN *SACCHAROMYCES CEREVISIAE*

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (1990)
(Biochemistry)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Search for Cell Cycle Control Genes and the Characterization of *CLN3*$^{+}$ of *Saccharomyces cerevisiae*

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NUMBER OF PAGES: xi, 84

ABSTRACT

Cell cycle control genes in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been studied in detail in the past few years. The *cdc25*$^+$ and *wee1*$^+$ genes of *S. pombe* play key roles in the commitment to division. Genes homologous to the mitotic inducer *cdc25*$^+$ and the mitotic inhibitor *wee1*$^+$ of *Schizosaccharomyces pombe* were searched for in *Saccharomyces cerevisiae* using DNA cross-hybridization as the method of detection. Such homologs were not found in *Saccharomyces cerevisiae* by this method.

Attention was therefore turned towards sequencing and partially characterizing a previously cloned gene, *WHI1*$^+$, and its mutant form *WHI1-1* (now call *CLN3*$^+$ and *CLN3-1* respectively). Sequence analysis showed that CLN3$^+$ is a cyclin homolog. Cyclins are probably present in all eukaryotes and play an important role in controlling the onset of mitosis. However, unlike these mitotic cyclins, CLN3$^+$ functions in G1.

CLN3-1 cells enter a new round of the cell cycle at an aberrantly small cell size and are α-factor resistant. Sequence analysis showed that the CLN3-1 protein was a truncated form of CLN3$^+$ caused by a nonsense mutation in the *CLN3*$^+$ gene. Cells over-expressing *CLN3*$^+$ had the same phenotype as *CLN3-1* cells, suggesting that the truncated CLN3-1 protein was a hyperactive form of the wild-type protein.

CLN3$^+$ and *CLN3-1* were placed downstream of the yeast *GAL1* promoter in a shuttle plasmid. Cells transformed with these plasmids and grown in the presence of galactose and the absence of glucose produced CLN3$^+$ or CLN3-1 in large amounts. Cell size was reduced in such cells. These cells were also α-factor resistant.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Bruce Futcher for his guidance, helpful discussions and for creating an enjoyable environment to work in. In addition, I am grateful for the support that my family, friends and co-workers have bestowed upon me for my work and time spent away.

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ILLUSTRATIONS

ABBREVIATIONS

α	alpha
δ	delta
γ	gamma
μ	micro
mM	millimolar
M	moles per liter
g	gram
μg	microgram
μl	microliter
ml	milliliter
l	liter
nm	nanometer
μm^3	cubic micrometer
cm^3	cubic centimeter
bp	base pair
kb	kilobase
kDa	kiloDalton
Ci	Curie
%	percentage
°C	degree Celcius

Introduction

As eukaryotic cells grow and divide, they progress through the cell cycle. The cell cycle generally consists of four phases, G1, S (DNA synthesis), G2, and M (mitosis). Progress through the cell cycle is affected by a variety of conditions such as nutrient availability and external stimuli, e.g. mating pheromones in yeast (reviewed by Pringle and Hartwell, 1981). These factors may affect one or more of the cell cycle phases. Cell cycle control mechanisms are responsible for coordinating growth with division and for maintaining the correct order of events during a cell cycle. In coordinating growth with division, cells maintain some mean size over many generations; this implies the existence of mechanisms that control the timing of division with respect to cell size.

The correct ordering of events from G1 to S to G2 to M is also important for viability. Some events cannot begin without the completion of a previous event; for instance, initiation of mitosis depends on completion of DNA replication. Checkpoints exist that maintain the correct orderly set of events (reviewed by Hartwell and Weinert, 1989). The existence of these checkpoints is demonstrated by the existence of mutants that lack them. For instance, a temperature-sensitive mutant of the DNA ligase gene *cdc9* arrests in S phase at the restrictive temperature (Hartwell and Weinert, 1989). However, a *rad9 cdc9* double mutant at the restrictive temperature allows cells to enter mitosis without completion of DNA synthesis. The *rad9* mutant is defective in the checkpoint responsible for monitoring the completion of DNA synthesis before the onset of mitosis.

Of fundamental importance in understanding the cell cycle is to investigate the control mechanisms. These control mechanisms ensure coordination of growth with division and maintain the correct orderly set of events from G1 to S to G2 to M.

Much of the work in investigating the control mechanisms of the cell cycle in the eukaryotes has come from the study of the yeasts *Saccharomyces cerevisiae* (budding yeast) (Hartwell, 1974) and *Schizosaccharomyces pombe* (fission yeast) (Nurse *et al.*, 1976). Their progression through distinct stages of growth during the cell cycle, their short generation times and existence as both stable haploids and diploids, ideally suit them for study of the cell cycle. More importantly, the isolation of various mutations particularly the cell division cycle mutations or *cdc* mutants have helped define greater than 50 genes involved in the combined cell cycles (Pringle and Hartwell, 1981; Nurse *et al.*, 1976; Kohli, 1987). These mutant *cdc* genes confer a temperature-sensitive lethality at the restrictive temperature of 37°C, but are viable at the permissive temperature of 23°C. At their restrictive temperature, each individual mutation gives rise to a characteristic stage specific arrest in the cell cycle (Hartwell *et al.*, 1974).

1.1 Control Points in the Cell Cycle

In this large set of cell cycle genes, only a few have identified regulators of progression through the cell cycle. One control point called Start is found in G1 near the G1 to S boundary and is the major control point in *S. cerevisiae* (Hartwell *et al.*, 1974). Start is a point at which the decision to proceed into a new round of division is made. If cells pass Start, they are committed to complete a new round of the cell cycle. *CDC28, CDC36, CDC37* and *CDC39* in *S. cerevisiae* (Reed, 1980; Nasmyth and Reed, 1980; Breter *et al.*, 1983) are required for completion of Start. Cells containing temperature-sensitive mutations in these genes arrest at Start at their restrictive temperature (Hartwell *et al.*, 1974). In *S. pombe*, $cdc2^+$ and $cdc10^+$ function at Start (Nurse *et al.*, 1976; Nurse and Thuriaux, 1980; Nurse and Bissett, 1981; Aves *et al.*, 1985). Certain *cdc2* and *cdc10* temperature-sensitive mutants arrest at Start.

Start is not only defined as the point of arrest in G1 by certain *cdc* mutants, but by arrest due to nutrient limitations, and by arrest through exposure to mating pheromones (reviewed by Pringle and Hartwell, 1981). Cells arrested at Start have not yet begun DNA synthesis and in the case of *S. cerevisiae* lack the initial formation of a bud. However, cells arrested at Start can still progress through alternative pathways of development such as conjugation. There is evidence of a similar Start control point in higher eukaryotes. Mammalian cells under poor growth conditions arrest in G1 (reviewed by Pardee *et al.*, 1978).

Two important *cdc* genes involved in control of the yeast cell cycles are the *CDC28* gene of *S. cerevisiae* and *cdc2*$^+$ of *S. pombe*. Investigation of these genes is warranted by their essential roles in controlling progression through their respective cell cycles at two different places, Start and the G2/M boundary (discussed later). *CDC28* has been cloned (Nasmyth and Reed, 1980) and sequenced (Lorincz and Reed, 1984) and encodes a 36 kDa phosphorylated protein kinase (Reed *et al.*, 1985). *cdc2*$^+$ has also been cloned (Beach *et al.*, 1982) and sequenced (Hindley and Phear, 1984) and encodes a 34 kDa phosphorylated protein kinase (Simanis and Nurse, 1986). Cdc28 and cdc2 share 62% overall amino acid identity (Lorincz and Reed, 1984; Hindley and Phear, 1984) and can rescue temperature-sensitive mutants of each other (Beach *et al.*, 1982; Booher and Beach, 1986). Considering the distant relationship between the two yeasts and the similarity of structure and function of the *CDC28* and *cdc2*$^+$ genes, it is appropriate to believe that some general control mechanism of the cell cycle exists in all eukaryotes (Note the nomenclature: In *S. cerevisiae* genes are italicized and proteins are not. Capital letters indicate dominance and small letters recessiveness. *S. pombe* genes are italicized and proteins are not. The "+" indicate the wild-type and the "-" the mutant. Proteins have no "+" or "-").

1.2 *cdc2⁺/CDC28* Homologs

In fact a general mechanism of cell cycle control in eukaryotes probably does exist since there are homologous cell cycle genes in a variety of eukaryotes (see Table 1). Interest has grown rapidly in identifying some unifying eukaryotic cell cycle control process due to the discovery of a human homolog to the *cdc2⁺* gene of *S. pombe*. Human *cdc2⁺* cDNA clones expressed in *S. pombe* complement a *cdc2* mutation (Lee and Nurse, 1987). The human *cdc2⁺* gene product was detected directly (Draetta *et al.*, 1987) using an antibody made to the highly conserved polypeptide sequence EGVPSTAIREISLLKE found in both cdc2 and Cdc28 (Lee and Nurse, 1987). An immune complex containing the human $p34^{cdc2}$ homolog has protein kinase activity *in vitro* (Draetta *et al.*, 1987).

Likewise, a cdc2 homolog is found in starfish oocytes. The oocytes contain an "M-phase specific" histone H1 kinase (H1K), which is active during meiosis or mitosis (reviewed in Wu *et al.*, 1986). A protein complex that copurifies with the kinase activity has been found to contain a 34 kDa protein that is recognized by the "PSTAIRE" antibodies (Lee and Nurse, 1987; Labbe *et al.*, 1988; Arion *et al.*, 1988).

Finally, a cdc2 homolog is found as a component of the maturation promotion factor or MPF. MPF was first described as a cytoplasmic agent that induced premature meiosis in the absence of protein synthesis when injected into *Xenopus* oocytes (Masui and Markert, 1971; Smith and Ecker, 1971; Gerhart *et al.*, 1984). MPF activity has since been found in a variety of eukaryotes and is believed to be a general inducer of mitosis (reviewed by Hunt, 1989). MPF is activated before mitosis and deactivated shortly after. Lohka *et al.* (1988) have purified an MPF complex consisting of a 32 kDa and 45 kDa protein. Immunoprecipitation using the conserved "PSTAIRE" antibody (Lee and Nurse, 1987) recognized the $p32^{cdc2}$ protein in an immune complex with the 45 kDa protein from the purified MPF (Gautier *et al.*, 1988). The $p32^{cdc2}$ in the immunoprecipitate is able to

Table 1. Homologous Cell Cycle Components in Eukaryotes I

	S.c.*	S.p.**	Humans	*Xenopus*	Clams/ Urchins	Starfish	*Drosophila*
Cyclin		cdc13	p62	B1,B2, A-type	A,B/B	-	A,B
cdc2/CDC28	CDC28	cdc2	$p34^{cdc2}$	MPF-$p32^{cdc2}$	MPF-$p32^{cdc2}$	$p34^{cdc2}$-H1K	-
cdc2/CDC28 Substrates	p40	cdc13 (cyclin)	p62 (cyclin)	p45 (B1,B2) (cyclin)	p62 (cyclin)	-	-
Associated Components	CKS1 (=p13)	suc1 (=p13)	p13	(p13?)	-	p13	-
	-	nim1	-	-	-	-	-
	MIH1 (=cdc25)	cdc25	(cdc25?)	-	-	-	stg
	(WEE1?)	wee1	-	-	-	-	-

* *S. cerevisiae*
** *S. pombe*
\- not done

phosphorylate the 45 kDa protein as well exogenous histone H1 *in vitro* (Gautier *et al.*, 1988).

1.3 Cdc28 and cdc2 Function at Start

Cdc28 of *S. cerevisiae* is associated with a complex of approximately 160 kDa that is formed during the cell cycle (Wittenberg and Reed, 1988). Maximum kinase activity is found with the formation of the 160 kDa complex. The kinase activity of Cdc28 probably controls entry of the cells into a new round of the cell cycle. Temperature-sensitive mutants of *cdc28* arrest at Start at 37°C since the kinase is defective (Reed, 1980; Nasmyth and Reed, 1980).

Other proteins are associated with Cdc28 at Start. Immunoprecipitation of Cdc28 from *S. cerevisiae* cell extracts using antibodies to a LacZ-Cdc28 fusion protein (Reed, 1982) co-precipitate a complex of 160 kDa (Reed *et al.*, 1985). *In vitro* studies with the Cdc28 protein kinase demonstrate that a co-precipitated 40 kDa protein is phosphorylated by the Cdc28 protein kinase (Reed *et al.*, 1985; Mendenhall *et al.*, 1987). This experiment was repeated using temperature-sensitive mutants of *cdc28* which fail to show protein kinase activity at the restrictive temperature. These mutants are unable to phosphorylate the 40 kDa protein. The 40 kDa protein co-precipitated with Cdc28 using antibodies specific for the N-terminus of the Cdc28 protein kinase. Both exogenous histone H1 and the 40 kDa protein are phosphorylated by the Cdc28 protein kinase *in vitro* (Wittenberg and Reed, 1988).

Three other gene products may interact with Cdc28. They come from *CLN1*, *CLN2* and *CKS1*. *CLN1* and *CLN2* were cloned by their ability to suppress in high copy number certain temperature-sensitive alleles of *cdc28* (Hadwiger *et al.*, 1989b). This

suggests some physical interaction with Cdc28. *CKS1* is discussed later (Hadwiger *et al.*, 1989a).

Not much is known about the interactions of the products of the *S. pombe* genes *cdc2*$^+$ and *cdc10*$^+$ at Start. The only indication that *cdc2*$^+$ and *cdc10*$^+$ are required at Start is from the work done on temperature-sensitive mutants as discussed earlier. Substrates of the cdc2 kinase that function at Start have yet to be identified. However, it is known that the phosphorylation state of cdc2 changes during nitrogen starvation (Simanis and Nurse, 1986). Cells arrest in G1 prior to Start under conditions of nitrogen starvation. The cdc2 kinase becomes dephosphorylated and protein kinase activity is lost (reviewed by Lee and Nurse, 1988).

Cdc10$^+$ has been cloned, sequenced and characterized (Aves *et al.*, 1985). Like *cdc2*$^+$'s function at Start, nothing is known about *cdc10*$^+$ function. The gene product has no similarity to any other known proteins. Transcription of the *cdc10*$^+$ gene does not change during the cell cycle.

1.4 Passage Through Start

How does the cell decide when it is appropriate to traverse Start under conditions of normal growth? Evidence from a variety of experiments indicates that some critical cell size must be achieved as a prerequisite for the cell to pass through Start and enter a new round of the cell cycle. In *S. cerevisiae* and *S. pombe*, cells enter a new round of the cell cycle at a constant size (Johnston *et al.*, 1977; Fantes, 1977; Johnston *et al.*, 1979; Lorincz and Carter, 1979). In *S. cerevisiae*, smaller daughter cells arise under conditions of slow growth (such as a poor carbon source). These cells take longer than the mother cell to pass through Start since they must grow more before they reach the critical size (Hartwell and Unger, 1977; Carter and Jagadish, 1978). In *S. cerevisiae* and *S. pombe*, larger than

normal cells (made by a blockage in S and then a release) divide more quickly and have shorter G1 phases. This shortens their cell cycles (Fantes, 1977; Singer and Johnston, 1981).

This critical size observed for cells to pass through Start implies that the cells must monitor their size. This raises the question of how the cells do so. One possibility is that the accumulation of some factor to a critical concentration acts as a positive activator for Start. Inversely, some inhibitory factor may play a negative role. As a consequence of growth and increased cell volume, this inhibitor might be diluted out enabling the cells to pass through Start. However, cells are not identical in their rates of entry into the S phase from, for example, a block in G1, and so some 'random factor' must be considered in explaining how cells initiate Start. Wheals (1982), introduced the "sloppy size" model in order to take into consideration the randomness associated with entry into the S phase. In this model cells monitor their size in a sloppy manner, with no absolute restriction on an exact size. After this sloppy size has been achieved, the cells can randomly enter a new round of the cell cycle.

1.5 Mutants with Aberrant Size Control

It seems logical that by looking for cells with aberrant size control, mechanisms for cell cycle regulation at Start can be identified. Mutants exist in both *S. cerevisiae* and *S. pombe* that allow the cells to enter a new round of the cell cycle at a reduced cell size.

In *S. cerevisiae* four such mutations are known. These mutations define four genes, *CLN3*$^+$ (previously called *WHI1*$^+$ or *DAF1*$^+$)(Carter and Sudbury, 1980), *whi3* (R. Nash and B. Futcher, personal communication), *WHI2* (Sudbery *et al.*, 1980) and *CLN2* (Hadwiger *et al.*, 1989b).

CLN3-1 mutants (the mutation is dominant) were first identified by their reduced cell size at division (Carter and Sudbury, 1980). This non-lethal mutation affects the timing of Start and allows cells to enter a new round of the cell cycle at about half the size of wild-type cells. *whi3* was also identified by its ability to divide at a reduced cell size (R, Nash and B. Futcher, personal communication). *whi3* cells are intermediate in size compared to *CLN3-1* and wild-type cells.

WHI2 has been cloned (Saul and Sudbery, 1985) and sequenced (Kelly *et al.*, 1988) and is involved in coordinating cell proliferation with the availability of nutrients. *whi2* mutants continue to divide even when the carbon source is depleted. Normal cells grown under conditions of nutrient depletion arrest in G1 and take on properties characteristic of stationary cells. *whi2* mutants are defective in this control and resemble exponentially growing cells.

CLN2 was cloned by its ability to rescue certain temperature-sensitive strains of *cdc28* when over-expressed on multicopy number plasmids (Hadwiger *et al.*, 1989b). *CLN2-1* (dominant) cells are phenotypically similar to *CLN3-1* cells.

1.6 The G2/M Control Point

A second control point in *S. cerevisiae* and *S. pombe* is identified by certain *cdc* genes. In addition to the G1 control point Start, recessive temperature-sensitive lethals of *cdc2* (Nurse *et al.*, 1976) and dominant *cdc2* mutations *cdc2-1w* and *cdc2-3w* (Thuriaux *et al.*, 1978), define a second control point in the cell cycle of *S. pombe*. This second control point exists at the G2 to M boundary and is the major control point in *S. pombe*. Much is known about the genetic and biochemical interactions of genes that function at the G2/M boundary in *S. pombe*. The temperature-sensitive lethals of *cdc2* are unable to enter mitosis at the restrictive temperature and the dominant *cdc2* mutations do so but at a

reduced size ("wee phenotype"). Another *cdc* gene identified that functions at the second control point in *S. pombe* is *cdc13*$^+$. *cdc13*$^+$ is essential for the initiation of mitosis and plays no role at the Start control point (Nurse *et al.*, 1976; Booher and Beach, 1987; Booher and Beach, 1988). This second control point is not as easily seen in *S. cerevisiae*. However, there is increasing indirect evidence that *CDC28* may indeed function at a second control point in *S. cerevisiae*. Much of this evidence comes from the existence of homologous genes to those of *S. pombe* (discussed later). The *S. pombe* genes function at the G2/M boundary. The *S. cerevisiae* homologs probably play analogous roles at the G2/M boundary.

1.7 cdc2 Complex at the G2/M Boundary

cdc2 of *S. pombe* is associated with a complex of proteins that forms at the G2/M boundary with the corresponding maximal kinase activity (Draetta *et al.*, 1988; Booher *et al.*, 1989). cdc2 is active as a kinase only if dephosphorylated at tyrosine15 (Gould and Nurse, 1989). Phosphorylation of the tyrosine residue inactivates the kinase. Products from two genes, *suc1*$^+$ and *cdc13*$^+$ have been shown to interact in the complex with cdc2 of *S. pombe*. *suc1*$^+$, an essential gene, was first described by its ability to rescue certain *cdc2* temperature-sensitive mutants when over-expressed (Hayles *et al.*, 1986). *suc1*$^+$ is involved in the control of division, since over-expression of *suc1*$^+$ delays division and the cells enter mitosis at twice the size of wild-type cells (Hindley *et al.*, 1987). A physical interaction with the cdc2 protein kinase has been demonstrated *in vitro*. Anti-suc1 antibodies co-immunoprecipitate cdc2 from *S. pombe* lysates (Brizuela *et al.*, 1987). In addition, anti-cdc2 or anti-cdc13 antibodies co-immunoprecipitate suc1 (Booher *et al.*, 1989). However, the cdc2 protein kinase does not phosphorylate suc1 *in vitro* (Brizuela *et al.*, 1987). Approximately 5% of total cdc2 protein kinase is co-precipitated with suc1 (Brizuela *et al.*, 1987; Booher *et al.*, 1989).

1.8 Isolation of *suc1*$^+$ Homologs

suc1$^+$ homologs have been found in other eukaryotes. A human suc1 homolog has been found using antibodies raised to the human p34^{cdc2}. These immunoprecipitate a complex from HeLa cells containing a 13 kDa (suc1 homolog) and 62 kDa protein (Draetta and Beach, 1988) as well as p34^{cdc2}. Furthermore, the human complex phosphorylates casein and the human 62 kDa protein *in vitro* (Draetta and Beach, 1988). The 62 kDa protein is associated with cdc2 and is necessary for Histone H1 specific kinase activity (Brizuela *et al.*, 1989). A cDNA clone of the 62 kDa has been cloned and sequenced and its product is homologous to B-type cyclins (Pines and Hunter, 1989).

Using *S. pombe* suc1 coupled to Sepharose beads Arion *et al.* (1988) have demonstrated the existence of a starfish suc1 homolog. A suc1 Sepharose bead column binds the starfish p34^{cdc2} homolog using extracts from starfish oocytes.

Dunphy *et al.* (1988) have demonstrated the existence of a *suc1*$^+$ homolog in *Xenopus*. Cell free extracts (that mimic the MPF-dependent entry into mitosis), passed through a suc1-Sepharose column lose their MPF activity. *S. pombe* suc1 inhibits MPF activation. This inhibition is due to the *S. pombe* suc1 binding to the *Xenopus* p34^{cdc2} homolog. In addition, the *S. pombe suc1*$^+$ product, p13, blocks tyrosine dephosphorylation in *Xenopus* and thus blocks activation of the kinase (Dunphy *et al.*, 1988). This suggests that a *S. pombe suc1*$^+$ homolog exists in *Xenopus* that interacts with its p34^{cdc2} homolog.

Finally, a *suc1*$^+$ homolog, *CKS1*, is present in *S. cerevisiae*. *CKS1* was identified by its ability to suppress a temperature-sensitive *cdc28* mutation (Hadwiger *et al.*, 1989a). This is evidence that like the cdc2-suc1 interactions of *S. pombe* a similar complex exists in *S. cerevisiae*. A unifying cell cycle control mechanism for progression into M from G2 seems more likely with the discovery of these homologous genes in different organisms.

1.9 cdc13 and its Association with cdc2

cdc13$^+$ was first described as a gene necessary for the initiation of mitosis. It has no role in the G1 Start control (Nurse *et al.*, 1976). There is direct evidence that cdc13 interacts with cdc2. A cold-sensitive mutant of *cdc2*, defective only at the G2 control point, is rescued by an allele of *cdc13* which simultaneously confers a temperature-sensitive *cdc* phenotype (Booher and Beach, 1987). The *cdc13*$^+$ gene product p63, in the cdc2 complex is a substrate of the cdc2 protein kinase (Booher *et al.*, 1989). The cdc2 protein kinase phosphorylates cdc13 (Booher *et al.*, 1989). Phosphorylation of cdc13 by the cdc2 kinase occurs simultaneously with cdc2 dephosphorylation (Booher *et al.*, 1989; Moreno *et al.*, 1989).

There is some evidence that cdc13 regulates cdc2 function in a cell cycle dependent manner, by the recent finding that cdc13 is homologous to cyclins (Solomon *et al.*, 1988; Goebel and Byers, 1988). cdc2 kinase activity oscillates together with cdc13 abundance, reaching a maximum at mid-mitosis (Booher *et al.*, 1989; Moreno *et al.*, 1989). Studies using immunofluorescence with either anti-cdc13 or anti-cdc2 antibodies show an increase in fluorescence in the nucleus up to the end of G2. A sudden loss of signal at nuclear division is then observed (Booher *et al.*, 1989). These results are consistent with cdc13 being a cyclin interacting with cdc2.

1.10 Cyclins

Cyclins are proteins whose levels rise and fall with each successive cell cycle. They were first discovered in marine invertebrate eggs and oocytes (Evans *et al.*, 1983) and are believed to regulate entry and exit from mitosis. Cyclins have been cloned and

sequenced and include the clam cyclin A and B (Swenson *et al.*, 1986), the B-type sea urchin cyclin (Pines and Hunt, 1987), cdc13 the B-type cyclin in *S. pombe* (Booher and Beach, 1988; Solomon *et al.*, 1988; Goebel and Byers, 1988), two B cyclins from *Xenopus* (Minshull *et al*,. 1989), an A-type and a B-type cyclin from *Drosophila* (Whitfield *et al.*, 1989; Lehner and O'Farrell, 1989) , a B-type cyclin of humans (Pines and Hunter, 1989) and an A-type cyclin of humans (Wang *et al.*, 1990). The two different classes of cyclins (A and B) are defined by their amino acid sequences.

Only a few of the cyclins to date have been shown to mirror the initial observation of a rise in abundance prior to mitosis followed by a sudden decline. The other cyclins have been identified by their homology to other known cyclins and are still under investigation. Clam A and the sea urchin cyclins are translated rapidly after fertilization from maternal mRNAs (Rosenthal *et al.*, 1980; Evans *et al.*, 1983). At the end of each mitosis (or meiosis), the clam A and sea urchin cyclins are proteolytically destroyed (Evans *et al.*, 1983). *S. pombe* cyclin, the product of the *cdc13*$^{+}$ gene, has also been shown to accumulate to its highest levels just prior to mitosis followed by a rapid drop (Booher *et al.*, 1989).

The cyclin appearance and disappearance in the cell cycle closely resembles that of MPF. It also appears that cyclin can act like MPF and induce mitosis. *Xenopus* oocytes are naturally arrested at the G2/M border in meiosis I and continue meiosis only after fertilization (reviewed by Maller, 1985). Clam cyclin A mRNA (Swenson *et al.*, 1986) and sea urchin cyclin mRNA (Pines and Hunt, 1987) when injected into *Xenopus* oocytes are able to induce meiosis. Recently, cyclins have been identified in the MPF complex. The 45 kDa proteins of the MPF complex is in fact a mixture of two different proteins, the B-type cyclins (Gautier *et al.*, 1990). Minshull *et al.* (1989) have shown that in *Xenopus*, the synthesis of cyclins is necessary in the ability to enter mitosis. Murray *et al.* (1989) have shown that without the destruction of cyclin, exit from mitosis does not occur.

The mitotic cyclins are absolutely essential for progression through the cell cycle. They regulate the entry of cells into mitosis by acting directly with their cdc2-like protein kinase (reviewed by Nurse, 1990). Destruction of the cyclin is necessary for the loss of kinase activity and subsequent exit from mitosis.

The conserved cyclins in different organisms and their association with the highly conserved $p34^{cdc2}$ homologs further support the idea of some universal control mechanism involved in progression through the cell cycle. Cyclins have recently been found in *S. cerevisiae* as well; these will be discussed later.

1.11 Other Genes Associated with *cdc2*$^+$ at G2/M

Three other genes *wee1*$^+$, *cdc25*$^+$ and *nim1*$^+$, also appear to work as part of the regulatory mechanism controlling the onset of mitosis. The *wee1*$^+$ gene product is an inhibitor of mitosis. *wee1*$^-$ mutants initiate mitosis at approximately half the size of wild-type cells (Fantes, 1979; Russell and Nurse, 1987a). Over-expression of *wee1*$^+$ in *S. pombe* lengthens G2, resulting in cells entering mitosis at an increased size (Russell and Nurse, 1987a). A constant generation time in these mutants is maintained by lengthening G1. Conversely, over-expressed *cdc25*$^+$ results in a decrease in G2 with cells entering mitosis at a reduced size compared to wild-type (Fantes, 1979; Russell and Nurse, 1986). Generation time remains the same as in wild-type cells. cdc25 may be the rate-limiting activator of mitosis. Over-expression of *cdc25*$^+$ in a *wee1*$^-$ background results in mitosis at drastically reduced sizes and cell death (Russell and Nurse, 1986). The additive result suggests that wee1 and cdc25 work in independent pathways, and antagonistically regulate cdc2.

The function of *nim1*$^+$ is phenotypically very similar to that of *cdc25*$^+$ and was first identified by its ability when over-expressed to rescue a *cdc25* temperature-sensitive mutant (Russell and Nurse, 1987b). *nim1*$^+$ function is not necessary in a *wee1*$^-$ background

indicating that it probably works with cdc25 to antagonize wee1 (Russell and Nurse, 1987b). *wee1*$^+$, *cdc25*$^+$ and *nim1*$^+$ have been cloned and sequenced, and each appears to act as a dosage dependent regulator of *cdc2*$^+$. wee1 and nim1 share homology to serine/threonine protein kinases (Russell and Nurse, 1987a; Russell and Nurse, 1987b).

Since the structure and function of Cdc28 and cdc2 are highly conserved, their regulators may also be highly conserved. This is true for suc1/CKS1. The discovery of *cdc25*$^+$, *wee1*$^+$ and *nim1*$^+$ in *S. pombe* suggests that *cdc25*$^+$, *wee1*$^+$ or *nim1*$^+$ homologs may exist in *S. cerevisiae*. The discovery of these homologs in *S. cerevisiae* would help to understand the regulation of the Cdc28 protein kinase and its role in controlling entry into a new round of the cell cycle at the G2/M boundary.

1.12 Activation of cdc2 at the G2/M Boundary

How does cdc2 interact with wee1, cdc25 and nim1 and control the onset of mitosis? "Wee" alleles of cdc2 can be divided into two types based on their genetic interactions with *cdc25*$^+$ and *wee1*$^+$. Russell and Nurse (1986, 1987a) found that *cdc2-3w* and *cdc2-4w* mutants do not require *cdc25*$^+$ (they suppress the lethality of *cdc25*$^-$) but are sensitive to the inhibitory affects of *wee1*$^+$. These mutations form one "wee" type. *cdc2-1w* mutants are insensitive to the inhibitory affects of *wee1*$^+$ but require *cdc25*$^+$ for viability. They are not able to suppress *cdc25* mutations. This is the second "wee" type. The existence of different "wee" type *cdc2* mutations that are affected differently by *cdc25*$^+$ and *wee1*$^+$ indicates that cdc25 and wee1 act independently.

nim1 interacts with cdc2 indirectly. This is based on two lines of evidence. First, over-expression of *nim1*$^+$ does not suppress *cdc2* temperature-sensitive mutants (Russell and Nurse, 1987b). Secondly, *nim1*$^+$ is not required in *wee1*$^-$ mutants (Russell and Nurse, 1987b). nim1 probably acts on wee1 and not directly on cdc2.

Activation of the cdc2 kinase is dependent on two independent events. One involves the activation of cdc2 by the mitotic activator cdc25. The other event involves removing the inhibitory affect of wee1 by nim1. A temperature-sensitive mutation in *cdc25* (*cdc25-22*) causes cells to arrest just prior to mitosis at the restrictive temperature. cdc2 kinase activity is absent at the restrictive temperature but rapidly increases after the shift to the permissive temperature (Moreno *et al.*, 1989). cdc2 is activated specifically by tyrosine15 dephosphorylation (Gould and Nurse, 1989). Perhaps cdc25 regulates cdc2 through dephosphorylation at tyrosine15. However, this might be indirect since cdc25 has no similarity to protein phosphatases. Replacement of tyrosine15 to phenylalanine15 in cdc2 results in cells prematurely entering mitosis. It may be that the cdc2 phe15 kinase is constitutively active and does not require cdc25. The cdc2 phe15 mutation suppresses the *cdc25-22* temperature-sensitive mutant (Gould and Nurse, 1989).

Since cdc2 is a protein kinase that is itself phosphorylated (Hindley and Phear, 1984; Simanis and Nurse, 1986; Gould and Nurse, 1989), wee1 and nim1 (acting through wee1) might regulate cdc2 through phosphorylation. However, since wee1 is a serine/threonine kinase, and the most important phosphorylation site on cdc2 is a tyrosine, the interaction between cdc2 and wee1 probably involves other intermediates. Phosphorylation of wee1 by nim1 likely inhibits the activity of wee1. There is no biochemical evidence to indicate whether wee1, cdc25, nim1 and cdc2 work directly on each other or through some other intermediates.

1.13 Mitotic Control Homologs in *S. cerevisiae*

Evidence has been presented suggesting a common mechanism in eukaryotic cell cycle control, particularly at Start in G1 and at the initiation of mitosis. However, investigation has been concentrated in *S. cerevisiae* at the dominant control point Start and

in *S. pombe* at the G2/M control point. Some common mechanism seems to exist in *S. cerevisiae* and *S. pombe* that is responsible for the two control points. This is evident by the strong homology in structure and function of the two essential protein kinase genes *CDC28* and *cdc2*$^+$ involved in cell cycle control. This raises the possibility of finding other homologous genes between the two organisms that are directly involved with *cdc2*$^+$ and *CDC28*. Therefore, the initial aim of the project was to attempt to isolate a *cdc25*$^+$ or *wee1*$^+$ homolog in *S. cerevisiae*. If found, the gene(s) would be cloned and characterized using the *wee1*$^+$, *cdc25*$^+$ and *cdc2*$^+$ interactions of *S. pombe* as a model. Homologous genes were looked for using DNA cross-hybridization. ^{32}P-dATP labelled *cdc25*$^+$ DNA sequences or ^{32}P-ATP labelled synthetic *wee1*$^+$ oligonucleotides and ^{32}P-dATP labelled *wee1*$^+$ DNA sequences were used to search *S. cerevisiae* genomic banks for their respective homologs. This approach was used since the project was in partial collaboration with Paul Russell who i) was the source of the *cdc25*$^+$ and *wee1*$^+$ genes and who ii) took the approach of using complementation experiments to look for *wee1*$^+$ and *cdc25*$^+$ homologs in *S. cerevisiae*.

1.14 Project Goals

S. cerevisiae DNA clones hybridizing to the probes used were sequenced. The sequence revealed only slight similarities at the DNA level, and more importantly, no similarities at the protein level. Thus, neither *cdc25*$^+$ or *wee1*$^+$ homologs were found in *S. cerevisiae* using DNA cross-hybridization. The main focus of the project was then directed towards sequencing and characterizing an *S. cerevisiae* size control gene previously cloned, *CLN3*$^+$ (Nash *et al.*, 1988; Cross, 1988). Although CLN3$^+$ acts in the G1 phase it was found that CLN3$^+$ was homologous to cyclins (Nash *et al.*, 1988). Cyclins have been implicated with some aspect of controlling the onset of mitosis in a wide range of eukaryotes (reviewed by Murray and Kirshner, 1989). It is possible that two sets of cyclins may exist that

interact with Cdc28/cdc2. One set may function at the G2/M boundary and the other at the G1 control point or Start. Common to the two sets of cyclins is their interaction with their respective $p34^{cdc2}$ homologs. G2/M cyclins have already been identified. Included among these cyclins are cdc13 from *S. pombe* (Booher and Beach, 1988; Solomon *et al.*, 1988; Goebel and Byers, 1988), p62 from human (Pines and Hunter, 1989) and the p45 cyclins from *Xenopus* (Gautier *et al.*, 1990). Since *CLN3-1* mutants allow passage of Start at a reduced cell size, $CLN3^{+}$ plays a role in the G1/S transition (Nash *et al.*, 1988), and is the first G1 cyclin to be identified. This raises the exciting possibility that $CLN3^{+}$ may have a direct role in regulating the Cdc28-p40 complex of *S. cerevisiae*.

Materials and Methods

2.1 Media

LB 10.0 g Difco-Bacto Tryptone

5.0 g Bacto-yeast extract

10.0 g NaCl

1.0 g Glucose

H_2O to 1 litre

When appropriate, antibiotics were added after autoclaving.

2xYT 16.0 g Bacto-tryptone

10.0 g Bacto-yeast extract

10.0 g NaCl

H_2O to 1 litre,

pH to 7.4; sterilized by autoclaving.

YEPD 20.0 g Bacto-peptone

10.0 g Bacto-yeast extract

900 ml H_2O

autoclaved

20.0 g glucose in 100ml H_2O autoclaved separately was added.

For plates 20.0 g/l agar was added.

For YEPGal, galactose (<.01% glucose) was used in place of glucose.

For α-factor plates, α-factor was added to autoclaved media to $5x10^{-6}$ M.

YNB -(ura) 6.7 g Bacto-yeast nitrogen base w/o amino acids

2.0 g an all amino acid mix (at least 50 μg/ml of each)

Distilled H_20 to 1 litre, autoclaved, followed by the addition of the appropriate sugar or other energy source.

2.2 Strains

(i) Bacteria

Escherichia coli A, B, C *CEN* banks (M. Rose), *LEU2* banks and the I, II, III and IV banks (B. Futcher)

JA226 C600 hsdR⁻M⁺ recBC⁻ lop^{II} *thi⁻ leuB6 st*^{R}

MV1193 Δ(*lac⁻proAB*) *thi supE* Δ(*sr1⁻recA*)*306::Tn10*(*tet*^{R}; F': *traD*36 *proAB lacI*^{q}ZΔm15)

M13K07 phage

(ii) Yeast

S. cerevisiae

*LL20 MAT*α *leu2-3 leu2-112 his3-11 his3-15* [psi⁺] [cir⁺]

*GFR88 MAT*α *his4-39* (*S288C* background)

BF310-4b MATa CLN3-1 leu2 his3 lys2

BF338-3b MATa CLN3⁺ ade1 leu2 ura3 his3 G418^{R}

*BF350-12c MAT*α *CLN3⁺ leu2 ura3 his G418*^{R}

2.3 Preparation of DNA

(i) Synthetic Oligonucleotides

Oligonucleotides AB258, AB259, AB260, and AB374 were synthesized at the Institute for Molecular Biology and Biotechnology at McMaster University; other oligonucleotides were made at Cold Spring Harbor Laboratory.

(ii) Isolation of DNA

Small scale and large scale preparations of *E. coli* plasmid DNA were carried out using the alkali lysis method as described by Maniatis, (1982) with an additional phenol/chloroform, chloroform extraction and 70% ethanol wash.

CsCl gradients were run at 55K rpm, room temperature in a TV-865 Sorval rotor. Bands were identified by ethidium bromide fluorescence using long wave U.V. light. The lower band (corresponding to closed circular DNA) was extracted with a syringe, the ethidium bromide removed by repeated extractions with a butanol mix (iso-amyl alcohol, chloroform and NaCl saturated water), and the DNA dialysed in 0.5x SSC to remove most of the CsCl. NaCl exchange reactions (exchanged Cs^{+1} ions with Na^{+1} ions) were performed to remove the remaining CsCl and the DNA was ethanol precipitated and dissolved in TE.

(iii) Measurement of Plasmid DNA

Plasmid DNA was mixed in a non-denaturing buffer (20 mM KH_2PO_4, 20 mM K_2HPO_4, 0.5 mM EDTA) at pH 11.8 with 0.25 μg/ml of ethidium bromide. The non-denaturing buffer maintains the integrity of the double stranded DNA. The ethidium bromide intercalates in double-stranded DNA and under the proper wavelength of light, fluoresces. The ability of the ethidium bromide to intercalate depends on the double-stranded nature of the DNA. Ethidium bromide can intercalate less in supercoiled DNA than linear DNA. The amount of fluorescence is proportional to the amount of ethidium bromide present in the DNA. The samples were then heated at 100°C for 2 minutes then cooled for 2 minutes in water to room temperature. Linear DNA and nicked circular DNA lose their associated ethidium bromide when denatured, whereas closed circular double-stranded DNA snaps back together allowing ethidium bromide to once again intercalate. The amount of fluorescence after heating was used to quantitate the amount of closed circular DNA in the sample. The machine was calibrated with 0.5 μg of DNA prior to use.

(iv) Restriction Digests

All restriction digests were carried out in accordance with the manufacturers specifications. Enzymes were purchased from Bethesda Research Laboratory (BRL), Pharmacia, and New England Biolabs.

(v) Ligations

Ligations were performed in the presence of 0.1 mM ATP, 100 μg/ml BSA, 1 unit T4 DNA Ligase (BRL) under the manufacturers buffer specifications and at room temperature.

2.4 Gel Electrophoresis

(i) Gels

0.8% agarose gels were placed into MAX submarine trays (Hoefer) and run at constant voltage. 1x Tris Borate EDTA (gives high resolution of DNA fragments and a high buffering capacity) was used as the running buffer and in the gels. 20 μl samples (typically 0.1-1 μg of DNA) were added with loading dye and placed into preformed slots. The 1 kb ladder (BRL) was used as a marker.

(ii) Purification of DNA Fragments from 0.8% Agarose Gels

10-20 μg of cut DNA was loaded into large slots (0.5 cm^3) and run for the appropriate period of time depending on the size of the fragments. DNA that had migrated through the gel was made visible by brief staining in ethidium bromide. DEAE paper (Schleicher and Schuell) was inserted at the front of the band, and the DNA was allowed to bind the DEAE (this was monitored by the EtBr fluorescence on the paper under U.V. light). DNA was extracted from the DEAE using a high salt buffer. Alternatively, bands were excised from the gels and the DNA recovered using a GENECLEAN kit (BIO101 Inc.).

2.5 Radioisotopes

(i) Sources

Deoxyadenosine 5'-[α-thio][^{35}S]triphosphate, 1200 Ci/mmol (NEN), adenosine 5'-[γ-^{32}P]triphosphate 3000 Ci/mmol (Amersham) and deoxyadenosine 5'-[α-^{32}P]triphosphate 3000 Ci/mmol (Amersham) were used in sequencing and labelling of DNA.

(ii) Labelling of DNA

α-^{32}P dATP was used in the random primer labelling technique of Feinberg *et al.* (1983). A mixture of hexameric DNA molecules when mixed with single-stranded DNA of interest, hybridize randomly along the DNA. These act as primers, and are lengthened by the addition of unlabelled dCTP, dGTP, dTTP, α-^{32}P dATP and *Klenow* (BRL) under the appropriate buffer conditions. The newly synthesized radioactive double-stranded DNA was cleaned up by phenol/chloroform extractions and ethanol precipitated. Prior to hybridization, the probe was heat denatured.

End-labelling of oligonucleotides was by the forward reaction of *T4 polynucleotide kinase* (BRL). Standard reactions contained 3.0 pmol of synthetic oligonucleotide, 3.0 pmol of γ-^{32}P ATP, and 0.5 unit *T4 polynucleotide kinase* under the appropriate buffer conditions.

(iii) Plasmid Probe Constructions

Plasmid pUCSS and pWEE1-12 (Figures, 1 & 8) containing *cdc25*$^{+}$ and *wee1*$^{+}$ of *S. pombe* respectively, were used to construct ^{32}P-dATP probes. pUCSS was cut at the unique *Bam*HI and *Xba*I sites, liberating a 2.0 kb fragment. This fragment was gel purified and used to make ^{32}P-dATP labelled probes by the random primer technique of Feinberg *et al.* (1983). Plasmid pWEE1-12 was cut with *Hind*III, *Eco*RI and *Hind*III and *Hind*III and *Xho*I. These yielded fragments of 0.8 kb, 2.3 kb and 2.6 kb with respect to the enzymes used (Figure 8). Similarly, these fragments were gel purified and used to make

^{32}P-dATP probes. These probes were used to look for homologs of *cdc25*$^{+}$ and *wee1*$^{+}$ in the A, B, and C *CEN* banks and the *LEU2* bank. Banks A, B and C were constructed by the digestion of *GRF88* yeast genomic DNA with *Sau*3A followed by ligation into the *Bam*HI site of the yeast shuttle vector YCp50. Average size fragments of A are 10-15 kb, 15-20 kb in B and 20-30 kb in C. The *LEU2* bank was constructed by *Sau*3A digestion of *LL20* yeast genomic DNA followed by ligation into the *Bam*HI site of plasmid pGT46. The I, II, III and IV banks were made in the same manner.

2.6 Sequencing

CLN3^{+} and *CLN3-1* cloned into the single-strand producing vector pUC118 (to give pUC118*CLN3*$^{+}$, pUC118*CLN3-1*) were cut at the unique *Pst*I and *Xba*I sites dropping out a 1.5 kb yeast genomic fragment. This created the linear DNA molecules pRD and pBK respectively. *CLN3*$^{+}$ and *CLN3-1* cloned into the single-stranded producing vector, pUC119 (to give pUC119*CLN3*$^{+}$, pUC119*CLN3-1*) were cut at the unique *Kpn*I and *Hpa*I sites dropping out a 0.5 kb yeast genomic fragment. This created the linear DNA molecules pBL and pGR. pRD, pBK, pBL and pGR were used to make nested deletions including RD84, RD87 and BK84 by the method of Henikoff, (1984). With the use of *M13K07* as a helper phage, single-stranded DNA was recovered, essentially as described by Vieira and Messing, (1987). NH_4OAC was used instead of NaCl in the precipitation of the phage. Sequencing was carried out by the dideoxy chain termination method of Sanger *et al.* (1977) and a Sequenase kit (U.S. Biochemicals). Samples were heated to 75-80°C for 2 minutes in a heat block then loaded on a 6% denaturing acrylamide gel. Gels were run at 55W for 2-9 hours, dried at 80°C under vacuum and put on film (Kodak XAR) overnight.

2.7 Construction of pGal1(RD84 or BK84) and pGal1(RD87T_4 or BK84T_4)

To control the expression of *CLN3*$^{+}$ and *CLN3-1* both genes were put into

plasmid pCGS110 (*ampicillin, URA3*, 2 μm, *GAL1* and *10*) downstream of the *GAL1* promoter of *S. cerevisiae*. Two approaches were taken in the construction of the pGal1(RD84 or BK84) and pGal1(RD87T_4 or BK84T_4) plasmids. Both approaches involved linearizing pCGS110 by cutting at the unique *Bam*HI and *Nru*I sites. Deletions RD87 and BK84 were digested at their unique *Sph*I and *Hpa*I sites generating fragments of approximately 1.9 kb which contained the entire coding region of either *CLN3*$^+$ (RD87) or *CLN3-1* (BK84). Digestion of RD84 by *Sph*I and *Sma*I generated a fragment of approximately 2.4 kb containing the entire coding region of *CLN3*$^+$. This gave an additional 500 bp at the 3' end of *CLN3*$^+$ compared to using *Hpa*I.

In the first approach, *Bam*HI-*Sph*I oligonucleotide adapters were used. Plasmids pGal1RD84 and pGal1BK84 were created by simply ligating cut pCGS110 with the cut RD84 and cut BK84 respectively. These were transformed into *JA226* and clones were selected by their ability to grow on LB plates containing ampicillin.

Alternatively, the 5' overhangs produced by *Bam*HI in pCGS110 were filled in using *Klenow* creating blunt ends. The 3' overhangs produced by *Sph*I in RD87 and BK84 were made blunt with *T4 DNA polymerase I* (BRL). *Hpa*I created blunt ends and no further manipulations were necessary. The blunt-ended pCGS110 and RD87 or BK84 were ligated together. Plasmids pGal1RD87T_4 and pGal1BK84T_4 were created. These were transformed into *JA226* and clones were selected by their ability to grow on LB plates containing ampicillin.

Restriction analysis and subsequent sequence analysis of the junctions confirmed that the proper constructs were made (Figures, 12 A, B & 13 A, B).

2.8 Transformations

(i) Yeast

Yeast were transformed by a modified method of Hinnen *et al.* (1978) and

Futcher, (1981).

(ii) Bacteria

Transformations were carried out as described by Hannahan, (1983). *recA*$^-$ cells were grown to an O.D. of 0.4. Cells were made competent with the use of K^{2+}, Mn^{2+}, Ca^{2+} and cobalt in TFB solution. Cells were allowed to grow in rich media (SOC) and spread on plates with the appropriate antibiotics.

2.9 Measurement of Cell Density

(i) Yeast

Yeast cell density during logarithmic growth was calculated using a Hemacytometer (Reichert). 10 µl of cell culture was placed on the hemacytometer and covered with a cover slip. Using the light microscope at 400x magnification, cell numbers were counted on a grid of 25 squares (x10^4 cells/ml). Alternatively, cells were measured using the Coulter Counter ZM.

(ii) Bacteria

Cell density was determined using the Du-50 Spectrophotometer (Beckman). Measurements depended on the wave lengths (550-660 nm) and cell type (*recA*$^+$ or *recA*$^-$) used. Calibration of the instrument was carried out using the media of interest.

2.10 Colony Hybridizations

(i) General Procedure

Plates containing approximately 300-500 colonies of bacteria were replica-plated, grown overnight to restore colonies and transferred to circular nitrocellulose filters (Schleicher and Schuell). The cells were lysed by the sequential transfer of the nitrocellulose filters (colonies faced-up) onto 3MM Whatman paper (in pyrex trays) saturated with: i) 10% SDS for 3 minutes, ii) 20 mM HCl for 5 minutes, iii) DNA

denaturing solution containing NaOH for 5 minutes, iv) twice with neutralizing solution for 5 minutes and v) 2xSSC, 20 mM Tris for 5 minutes. The filters were air dried for a few hours then baked at 80°C for 2 hours. (this is a modified procedure of Maniatis, 1982).

(ii) Hybridization solutions

Conditions were set up depending on the melting temperature (Tm) and desired stringency of the hybridization (the conditions referred to in Results I were as follows, hybridization in 22% formamide at 42°C overnight, washed twice in 3xSSC, 0.1% SDS at room temperature then 3 times at 42°C). The hybridization solution used included 5xDenhardts solution, deionized formamide (variable), 0.1% sodium dodecylsulphate, 2.5 mM $NaHPO_4$, 5X SSC, and 10 μg/ml of sonicated denatured calf thymus DNA. Tms for oligonucleotides were calculated according to the following formula Tm= (A-T)*2 + (G-C)*4. All hybridizations were carried out overnight 20°C below the Tm value. Washing conditions were variable, depending on the sensitivity required. After hybridization and washing, filters were placed in saran wrap and exposed to Kodak XAR film for various periods of time and at varying temperatures (room temperature, -70°C).

2.11 Southern Analysis

Transfer of DNA to nitrocellulose (Schleicher and Schuell) from agarose gels (Sigma) was performed as described by Maniatis, (1982). The filters were air dried then baked at 80°C in a vacuum oven for 2 hours.

2.12 Sizing of Cells Containing pGal1 Constructs

Wild-type cells, cells containing the pGal1RD84 or pGal1BK84T_4 constructs and cells containing pCGS110 were grown in YNB (-ura) media in the presence of 2% raffinose overnight until mid-log phase. The cells were then aliquoted and diluted down into fresh media. Cells were split into two and raffinose to 2% was added to one half. To

the other half galactose and raffinose to final concentrations of 2% each were added. After 8-10 hours at 30°C cell size was determined using the Coulter Channelyzer 256.

2.13 Analysis of α-factor Resistant Cells

Wild-type cells, cells containing the pGal1RD84 or pGal1BK84T_4 constructs, cells containing pCGS110 and cells which had lost either of the *GAL1* constructs were analysed for their sensitivity to the mating pheromone α-factor. All cells were grown to mid-log phase in YNB (-ura) lacking galactose but in the presence of 2% raffinose. 5 µl (equal numbers of cells from each sample, approximately 1000) of logarithmically growing cells were then spotted onto various plates containing glucose +/- α-factor and plates containing galactose +/- α-factor. Cells were allowed to grow for 2-4 days at 30°C until colonies appeared. Resistance to α-factor was scored by the ability of cells to grow on plates in the presence of $5x10^{-6}$M α-factor.

2.14 Sterilization

All heat stable material and media were autoclaved under high temperature (120°C) and pressure ($1.41x10^4$ kg/m^2)

2.15 Computer analysis

Sequence entry, editing, merging and manipulations were carried out using the Intelligenetics Suite at the Cold Spring Harbor Laboratory. Homology searches using either DNA or protein sequences against the PIR (Protein Identification Resource), EMBL (European Molecular Biology Laboratories) and NIH (GenBank DNA Sequence Library) were carried out using the FastA program.

RESULTS I Search for *cdc25*$^+$ and *wee1*$^+$ Homologs

3.1 Initial Screen for a *cdc25*$^+$ Homolog in *S. cerevisiae*

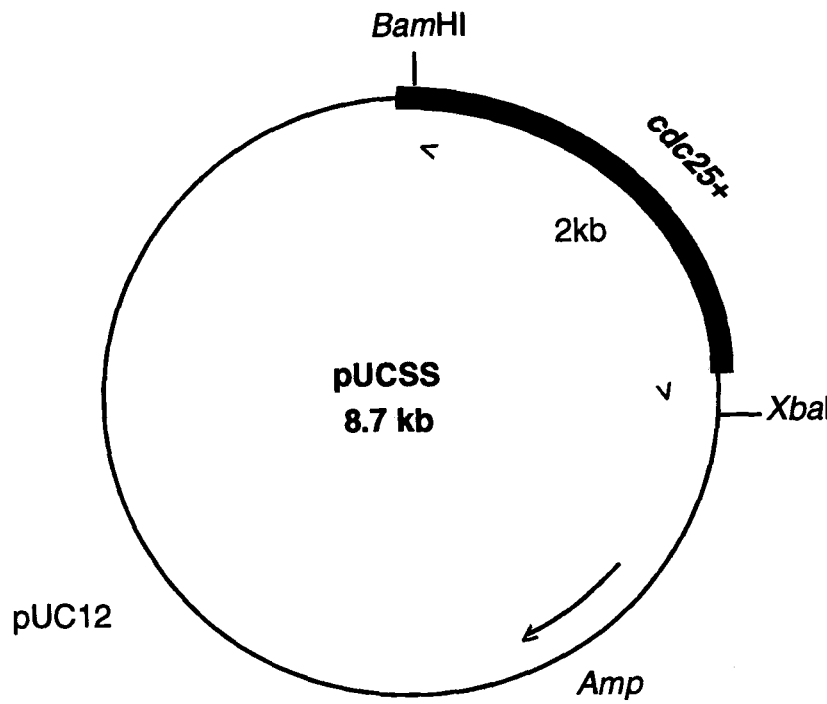
The existence of a *cdc25*$^+$ homolog in *S. cerevisiae* was investigated. An *S. pombe Bam*HI-*Xba*I *cdc25*$^+$ ^{32}P-dATP labelled fragment (Figure 1) was used to probe total *S. cerevisiae LL20* DNA cut with *Eco*RI. Southern analysis using low stringency hybridization and washes (see Materials and Methods) revealed a hybridizing 3.5 kb fragment on an autoradiogram. To clone this potential *S. pombe cdc25*$^+$ homolog, colony hybridizations were carried out on bacteria containing yeast genomic DNA. The yeast *CEN* banks A, B, C and the *LEU2* and the I, II, III and IV banks were used. Adequate numbers of cells (10,000) were plated to ensure that the *S. cerevisiae* genome was repeatedly represented.

Colony hybridizations were carried out under the same conditions used in the initial Southern analysis. However, these did not produce any recognizable signals when the experiment was repeated several times, when longer exposures were used and even when less stringent hybridization and wash conditions were used. The main problem was high background signals, possibly due to small amounts of vector sequence contaminating the probe.

3.2 Extended Use of Southern Analysis

An alternative approach to clone the potential *cdc25*$^+$ homolog by DNA cross-hybridization involved the use of Southern analysis. Since the original Southern analysis

Figure 1. Map of pUCSS containing the *cdc25*$^{+}$ gene of *S. pombe*. pUCSS contains the entire ORF of the *S. pombe cdc25*$^{+}$ gene. The 2.0 kb *Bam*HI-*Xba*I fragment shown was gel purified and used as a random primed ^{32}P-dATP labelled probe to search *S. cerevisiae* banks for a *cdc25*$^{+}$ homolog by DNA cross-hybridization.


BamHI
cdc25+
2kb
pUCSS
8.7 kb
XbaI
pUC12
Amp

showed a potential *cdc25*$^+$ homolog in *S. cerevisiae*, it seemed plausible, though more tedius to detect such DNA cross-hybridization by Southerns and not by colony hybridizations. Mixtures of plasmid DNAs from large numbers of *E. coli* clones (approx. 10,000 from each of the A, B, C and *LEU2* banks separately) containing *S. cerevisiae* DNA were prepared and cut with *Eco*RI (Figure 2). *S. cerevisiae* strain *BF310-4b* (a derivative of *LL20*) was used as a control and was cut with *Pst*I, *Hind*III, *Eco*RI and *Bam*HI (Figure 2, lanes 8-11). Figure 2, lanes 3 and 4, show that DNA from only two mixtures, C2 and L, hybridized to the *S. pombe cdc25*$^+$ *Bam*HI-*Xba*I ^{32}P-dATP labelled probe. This is evident by the appearance of a 3.5 kb *Eco*RI fragment which was identical to that of the control *Eco*RI cut (Figure 2, lane 10). Subsequent screening of DNA from these two banks eventually led to the isolation of four mixtures (#2, #3, #14 and #18) of bacterial colonies (2,000 each) which contained *S. cerevisiae* DNA plasmids that cross-hybridized to the *S. pombe cdc25*$^+$ probe.

3.3 Isolation of the Potential Homolog *GTcdc25*$^+$

Plasmids from mixtures #2, #3, #14, #18 and *BF310-4b* yeast DNA were digested with a second restriction enzyme, *Pst*I. The rational for using an additional restriction enzyme was the following: if the bacterial clones contained the same yeast DNA fragment originally seen by Southern analysis, then digestion using different restriction enzymes should yield identical hybridization patterns to that of the control yeast genomic DNA. Upon further screening of the four plasmid mixtures by Southern analysis, it was apparent that only mixtures #2 and #3 had hybridizing bands corresponding to bands in the appropriate lanes of the control (Figure 3, lanes 1, 2, 6 and 7). Eventually, one single clone *GTcdc25*$^+$ was isolated which cross-hybridized to the *S. pombe cdc25*$^+$ probe.

Figure 2. Autoradiogram showing different banks containing *S. cerevisiae* DNA probed with the *Bam*HI-*Xba*I *S. pombe cdc25*$^{+}$ ^{32}P-dATP labelled fragment. Each lane contains the combined plasmid DNA of 10,000 individual bacteria colonies. A6 to A4, B4 to B1, C4 to C2 and L correspond to different *E. coli* banks of *S. cerevisiae* DNA. Plasmid DNA from each one of these banks were cut with *Eco*RI. Control *BF310-4b S. cerevisiae* DNA was cut with *Pst*I, *Hind*III, *Eco*RI or *Bam*HI and 2 μg of each digest were loaded. Lanes 1-3 various C bank plasmid DNA (C4, C3 and C2); lane 4 L bank plasmid DNA; lane 5 empty; lane 6 1 kb ladder; lane 7 empty; lanes 8-11 *BF310-4b* cut with the enzymes indicated; lanes 12-15 various B bank plasmid DNA (B4, B3, B2 and B1); and lanes 16-18 various A bank plasmid DNA (A6, A5 and A4). Notice the presence of a common 3.5 kb band in lane 3 (C2), lane 4 (L) and lane 10 (*BF310-4b* cut with *Eco*RI) as indicated by the arrow.

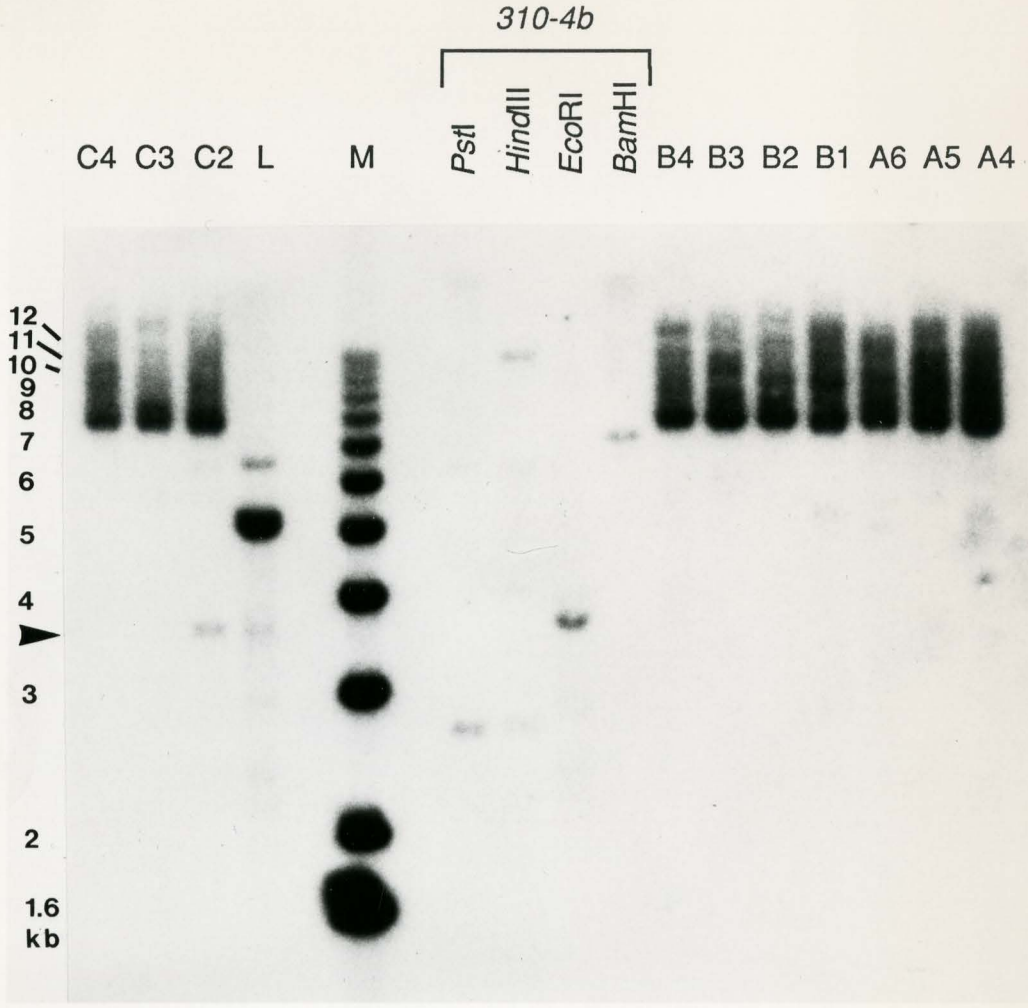
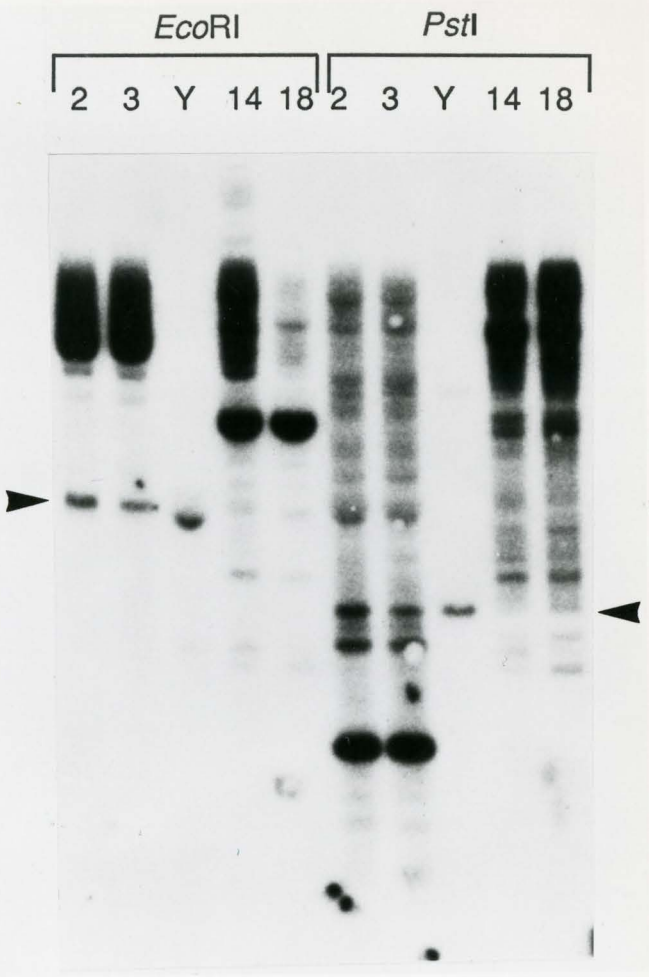

310-4b
C4 C3 C2 L M PstI HindIII EcoRI BamHI B4 B3 B2 B1 A6 A5 A4
12
11
10
9
8
7
6
5
4
3
2
1.6
kb

Figure 3. Autoradiogram showing potential *S. cerevisiae cdc25*$^{+}$ homologs in the C2 and L banks. The initial 10,000 individual bacteria colonies that contained potential *S. cerevisiae cdc25*$^{+}$ homologs from the C2 and the L banks discussed in Figure 2 were replica-plated. Plasmid DNAs from approximately 2,000 individual colonies from each of the initial 10,000 colonies (C2 and L banks) were isolated. The lanes marked #2 and #3 each represent different combined plasmid DNA from 2,000 individual bacteria colonies from the C2 bank. The lanes marked #14 and #18 each represent different combined plasmid DNA from 2,000 individual bacteria colonies from the L bank. Lanes 1-5 are cut with *Eco*RI, lanes 6-10 are cut with *Pst*I. Lanes 3 and 8 (Y) are *BF310-4b* yeast control DNA cut with *Eco*RI and *Pst*I respectively. Identical bands are found in both the *Eco*RI and *Pst*I digests of DNA from *BF310-4b* and the C2 (#2 and #3) bank and not in the L bank (#14 and #18). This is indicated by the arrows. Therefore, within 4,000 individual bacteria colonies (2,000 from #2 and 2,000 from #3) were at least two containing the *S. cerevisiae* genomic DNA that cross-hybridized to the *S. pombe* probe.


EcoRI
PstI
2 3 Y 14 18 2 3 Y 14 18

3.4 Restriction Mapping and Sequencing of *GTcdc25*$^+$

Restriction mapping and Southern analysis yielded a 2.8 kb *Pst*I fragment, the smallest fragment of *GTcdc25*$^+$ that hybridized to the *S. pombe cdc25*$^+$ probe. A larger 3.5 kb *Eco*RI fragment containing the entire 2.8 kb *Pst*I fragment was subcloned into pUC119 for the production of nested deletions and sequencing. Partial sequencing of the 3.5 kb *Eco*RI fragment revealed an open reading frame. Homology searches using the Intelligenetics Suite at CSHL found no homology to cdc25 of *S. pombe* or to any other protein in the combined data banks of the Protein Identification Resource (PIR) and the Genetic Sequence Data Bank (GenBank).

The best region of cross-hybridization was found from nucleotides 171-279 of *GTcdc25*$^+$ to nucleotides 1492-1590 of *S. pombe cdc25*$^+$ (Russell and Nurse, 1986) (Figure 4). Southern analysis on *Bgl*I digests of the nested deletions revealed that the ability of the *S. pombe cdc25*$^+$ probe to cross-hybridize to the cloned DNA was lost when this region was removed (Figure 5, deletion plasmid #102). The region of cross-hybridization was present in deletion plasmid #93 but was removed in deletion plasmid #102. This was evident when comparing the DNA sequences of deletion plasmids #93 and #102 (Figure 4) Therefore, the DNA cross-hybridization between *GTcdc25*$^+$ and *cdc25*$^+$ of *S. pombe* is limited to a region of 67 out of 108 nucleotide base pairs. Translation in all frames shows that this region cannot encode a protein similar to cdc25. Therefore, the putative gene *GTcdc25*$^+$ is not a *S. cerevisiae cdc25*$^+$ homolog and is very unlikely to be a cell cycle gene.

3.5 Similar Approaches Used in Looking for a *wee1*$^+$ Homolog in *S. cerevisiae*

Three degenerate synthetic oligonucleotides to the *wee1*$^+$ gene were constructed, AB258, AB259 and AB260. The three synthetic oligonucleotides were end-

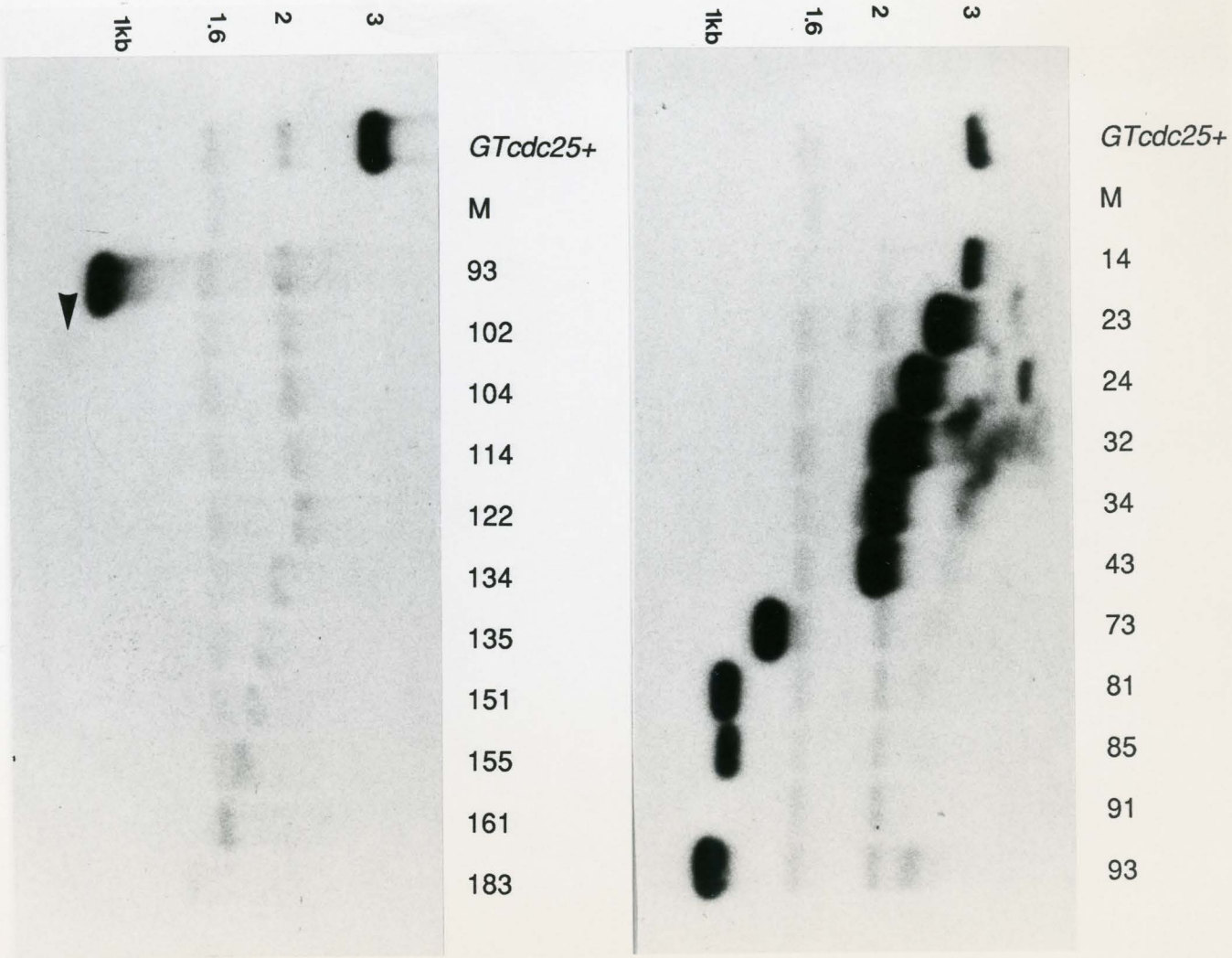
Figure 4. Region of DNA cross-hybridization between *GTcdc25*$^+$ of *S. cerevisiae* and *cdc25*$^+$ of *S. pombe*. DNA sequences are aligned for comparison with *GTcdc25*$^+$ (nucleotides 141-320) as the upper strand and *S. pombe cdc25*$^+$ as the lower strand (nucleotides 1462-1631). The DNA sequences shown are both found in their respective open reading frames. 63.3% identity (as indicated by the dashes) in a 108 nucleotide overlap was found by computer analysis. A region of strong cross-hybridization is indicated by the region between the Xs. Nested deletions that were created in *GTcdc25*$^+$ proceed from left to right. Deletion plasmid #93 contains all the DNA sequences shown. Deletion plasmid #102 lacks the sequences shown, and does not hybridize to the *S. pombe* probe.

```
                150       160       170       180       190       200
GTcdc25+ GACATGGATGATACGGCCGGGTCAAACGGTTCCGATTGCAGTTGGAATGCAAATGAGAGT
                                    :::::: ::: : :  :::::  :: ::
cdc25+   CTTTCCTAGCTTGAAAGTTAGGTCCCCTTCTCCGATGGCATTCGCTATGCAA--GA-AGA
              1470      1480      1490      1500      1510

                210       220       230       240       250       260
GTcdc25+ AGCAGATCATTGTTGGGCAGCGGCTTCAACACCAGTGCTTCGTCGTACGGATACCATAAA
          :: ::  : :: :: :::   : :   X:::::::  :::::::::X  : : :::
cdc25+   TGCGGAATA-TGATGAGCA--AGAT---ACACCAGTCGTTCGTCGTACCCAAAGCAT-GT
                  1530        1540         1550      1560       1570

                270       280       290       300       310       320
GTcdc25+ CTGTCATTTATACCAATCTGCGACACCGTGTCTTCTGCGAACACACCCAGTAGTAGAGTG
          : ::: ::  :: :  ::
cdc25+   TTCTCAATTCCACAAGACTAGGGCTTTTCAAAAGCCAAGATCTTGTGTGCGTTACGCCAA
              1580      1590      1600      1610      1620      1630
```

Figure 5. Autoradiogram showing regions of cross-hybridization to the *S. pombe cdc25*$^+$ probe on *GTcdc25*$^+$ nested deletions. Nested deletions of *GTcdc25*$^+$ were created for the purpose of sequencing and were found useful for this Southern. The faint bands of approximately 3.0, 2.0 and 1.6 kb (see bottom panel) are the *Bgl*I restriction digest fragments of *GTcdc25*$^+$. The largest deletions are indicated by the larger numbers moving from left to right and from the top panel to the bottom panel. The first fragment to decrease in size is the 3.0 kb *Bgl*I fragment followed by the 2.0 kb *Bgl*I fragment. Cross-hybridization to the 3.0 kb fragment is seen in the first lane of both panels of *GTcdc25*$^+$ prior to deletion; the lanes indicated by the M represent the 1kb ladder. Cross-hybridization to the *S. pombe Bam*HI-*Xba*I *cdc25*$^+$ ^{32}P-dATP labelled fragment to the deleted 3.0 kb fragment is last seen in deletion plasmid #93. Deletion plasmid #102, in which about 250 more bases have been deleted, no longer cross-hybridizes to the *S. pombe* probe as indicated by the arrow.


1kb
1.6
2
3
GTcdc25+
M
93
102
104
114
122
134
135
151
155
161
183
1kb
1.6
2
3
GTcdc25+
M
14
23
24
32
34
43
73
81
85
91
93

labelled with ^{32}P-ATP and used in colony hybridizations and Southern analysis of *S. cerevisiae* DNA. These oligonucleotides were used to probe yeast banks identical to those used in searching for a *cdc25*$^{+}$ homolog. In addition four other banks I, II, III and IV were used. Using these ^{32}P-ATP labelled oligonucleotides to probe a Southern with *LL20 S. cerevisiae* total yeast DNA, signals were present on the autoradiograms.

3.6 Colony Hybridization Analysis on Potential *wee1*$^{+}$ Homologs

DNA from two colonies, #19 and #24 were found to cross-hybridize to both oligo AB259 and oligo AB260 (Figure 6, column A row 4 & column C row 4). However, upon restriction enzyme analysis and subsequent Southern analysis of clones #19 and #24, oligos AB259 and AB260 were found to be specific for different sized DNA fragments generated by digestion with a variety of different enzymes (data shown for only #24 Figure 7). This result suggested that many of the restriction enzyme sites had to lie between the two areas homologous to the AB259 and AB260 oligo probes. Since the oligos came from regions close together (approximately 500 base pairs apart, see Figure 8) in the *wee1*$^{+}$ gene of *S. pombe*, this suggested that the *S. cerevisiae* clone was not a *wee1*$^{+}$ homolog. Also, i) the synthetic oligonucleotides made were actually mixtures of oligonucleotides to account for the redundancy of the codons used by a number of amino acids (AB259 GCCATTCCPAAPTCTCCTATYTT, AB260 CPTCTGCYTCYTCYTCCATTGCPAA), ii) ^{32}P-dATP labelled probes made from different fragments covering the entire coding region of the *wee1*$^{+}$ gene (Figure 8), did not cross-hybridize to total *S. cerevisiae* DNA. Therefore, clones #19 and #24 probably cross-hybridized by chance to the redundant synthetic oligonucleotides. If a homolog to *wee1*$^{+}$ does exist in *S. cerevisiae*, it is possible that any similarity between the two may be found only in their function and not in their DNA sequences. Complementation experiments seem like logical alternatives to hybridization.

Figure 6. Autoradiogram of a colony hybridization. This is a secondary screen of plasmid DNA from a small number of individual bacteria colonies probed with the ^{32}P-ATP labelled synthetic oligonucleotides AB259 (top) and AB260 (bottom) made to the *wee1*$^+$ gene of *S. pombe*. The synthetic oligonucleotides AB259 and AB260 were made to regions of *wee1*$^+$ as indicated in Figure 8. A larger scale colony hybridization was first carried out and bacteria colonies containing plasmid DNA with potential *wee1*$^+$ homologs were used in this secondary screen. These bacteria colonies contain DNA from *S. cerevisiae* yeast genomic banks A, B, C, I and IV. Colonies #19 (bank A, row 4) and #24 (bank C, row 4) contained *S. cerevisiae* DNA that cross-hybridized to both oligo AB259 and oligo AB260. Plasmid DNA from bacteria colony #24 was used in subsequent studies. Negative controls (bacteria cells containing the plasmid YCp50 with no yeast genomic DNA insert) are indicated by the arrows. Oligo AB260 does hybridize weakly to the negative control.

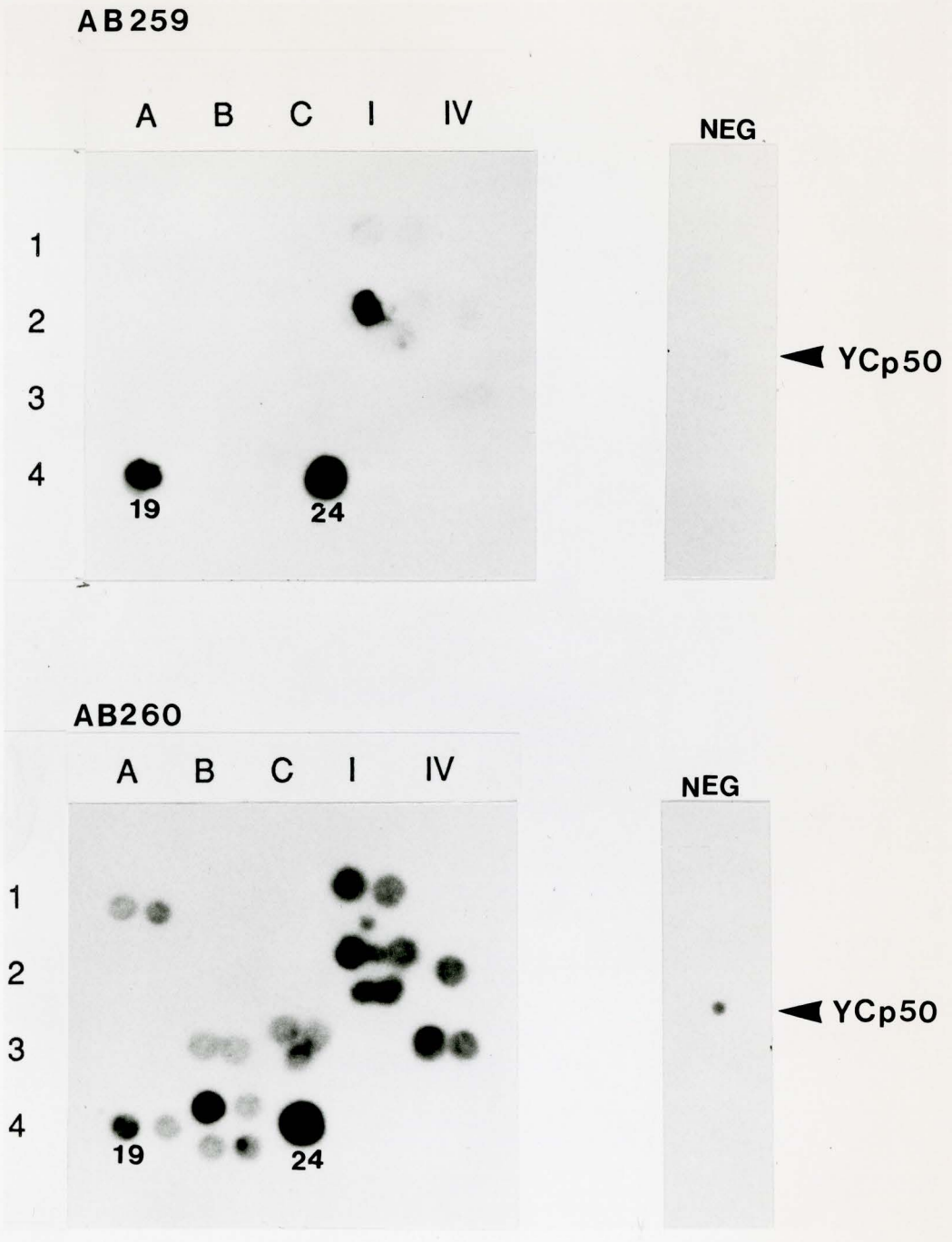
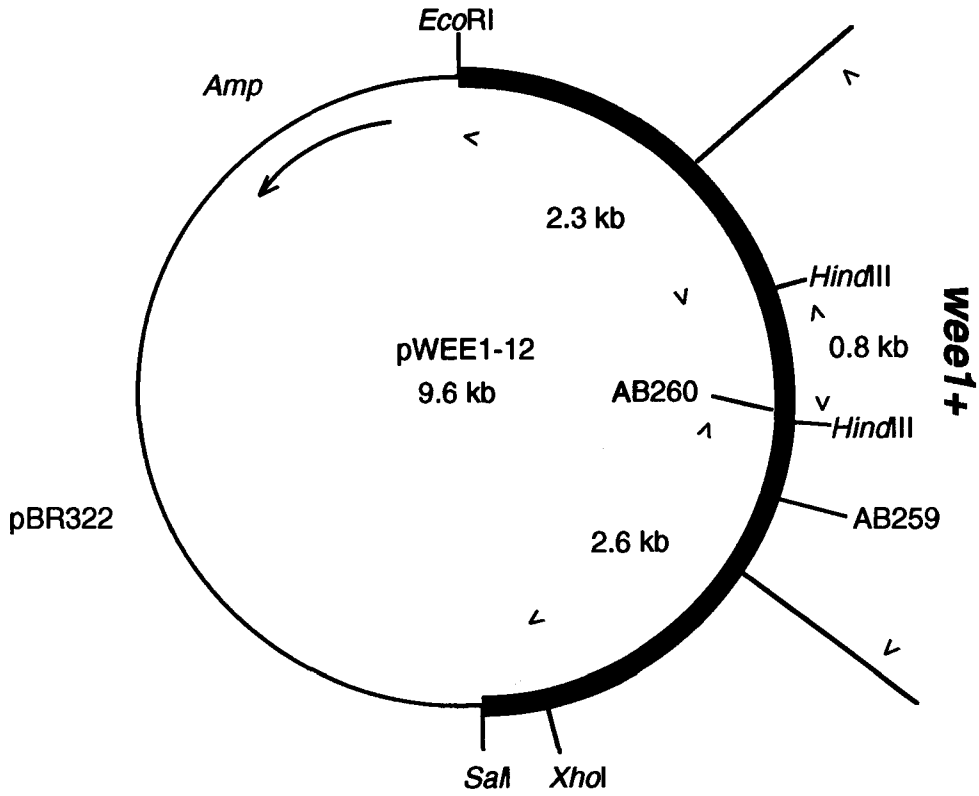

AB259
A
B
C
I
IV
1
2
3
4
19
24
NEG
YCp50
AB260
A
B
C
I
IV
1
2
3
4
19
24
NEG
YCp50

Figure 7. Autoradiogram of restriction digests of a potential *wee1*$^+$ homolog. Plasmid DNA from bacteria colony #24 (the results for #19 were not reproducible) containing *S. cerevisiae* bank C DNA was cut with different restriction enzymes and subjected to Southern analysis. The blot was probed with either the ^{32}P-ATP labelled oligonucleotide AB259 (dark bands in each lane) or similarly labelled AB260 (indicated by the arrows in each lane). In any one lane, the two probes almost always hybridized to different fragments. This suggested that the two oligos hybridized far apart on the plasmid in #24. Since the two oligos are only 500 bp apart in *wee1*$^+$, it was unlikely that clone #24 contained a *wee1*$^+$ homolog. The lane marked YCp50 was the vector used in making the *S. cerevisiae* banks and did not cross-hybridize to either probe used. The smallest fragment that hybridized to both probes was about 4.0 kb (seen in the *Pvu*II lane).

BamHI
BamHI + PvuII
PvuII
ClaI + PvuII
ClaI
PstI
YCp50
L
BamHI + BglII
BglII
EcoRI
EcoRI + HindIII
HindIII
XbaI
12
11
10
9
8
7
6
5
4
3
2
1.6
1kb

Figure 8. Map of pWEE1-12 containing the *wee1*$^+$ gene of *S. pombe*. pWEE1-12 contains the entire ORF of the *S. pombe wee1*$^+$ gene and flanking yeast DNA (indicated by the heavy line). Indicated by the arrows are the fragments that were gel purified, and used as ^{32}P-dATP labelled probes. These fragments were used to search *S. cerevisiae* banks for a *wee1*$^+$ homolog by DNA cross-hybridization. Also indicated are the regions from which oligos AB259 and AB260 were constructed.


EcoRI
Amp
2.3 kb
HindIII
0.8 kb
wee1+
pWEE1-12
9.6 kb
AB260
HindIII
pBR322
AB259
2.6 kb
SalI
XhoI

RESULTS II Analysis of CLN3+

4.1 Sequencing of *CLN3+* and *CLN3-1*

Since attempts to isolate homologs of the *cdc25+* or *wee1+* genes of *S. pombe* by DNA cross-hybridization were unsuccessful, work was focused on characterizing the *CLN3+* gene of *S. cerevisiae* (then known as *WHI1+*). The *CLN3+* gene was cloned in the lab by S. Anand and B. Futcher. The first step in characterizing the gene was to sequence it. This sequencing was done in collaboration with R. Nash.

CLN3+ and *CLN3-1* were inserted into the single-stranded producing vectors pUC118 and pUC119. These created plasmids pRD and pBL (*CLN3+* in pUC118 and pUC119 respectively) and plasmids pBK and pGR (*CLN3-1* in pUC118 and pUC119 respectively). Nested deletions in all four plasmids made by the method of Henikoff, (1984) were used to sequence both strands of *CLN3+* and *CLN3-1* (Figures 9 & 10). The *CLN3+* gene is located in a 1.74 kb open reading frame (Figure 11). It begins approximately 410 nucleotides downstream of the *Xho*I site and ends approximately 60 nucleotides upstream of the *Hpa*I site. This ORF codes for a potential protein of 580 amino acids with a molecular weight of 65 kDa. The 5' end of the mRNA has not been mapped but by the following observations it is predicted that the first methionine indicated is the correct one: the first codon for methionine in the largest ORF begins at nucleotide +1 (Figure 11) just downstream of a stop codon at position -18; there are 105 nucleotides before the next methionine in the ORF coding for 35 amino acids, which by random chance is improbable. Cross, (1988) sequenced the same gene at about the same time (called *DAF1+*) and found the same sequence; immunoprecipitations of *CLN3+* and *CLN3-1* give proteins of the

Figure 9. Creation of nested deletions of plasmids pRD (*CLN3*$^+$) and pBK (*CLN3-1*) by the method of Henikoff (1984). Nested deletions were made after cutting out a 1.5 kb *Pst*I-*Xba*I fragment from pUC118*CLN3*$^+$ and pUC118*CLN3-1*. The 3' overhangs produced by digestion with *Pst*I were resistant to *exonuclease* III treatment and the 5' overhangs produced by *Xba*I digestion were sensitive. This removed the *CYC3* gene and directed the nested deletions from the 5' end towards the 3' end of *CLN3*$^+$ (pRDs) or *CLN3-1* (pBKs). After *exonuclease* III treatment, *S1* and *Klenow* were used to create blunt ends which were ligated together. Deletions were made approximately every 250 bp to ensure overlapping sequences. The different length inserts were then sequenced using the universal primer which hybridizes to the primer binding site (PBS). pRD84 and pRD87 were plasmids created by deletions of pUC118*CLN3*$^+$ that ended just 5' of the first ATG of the *CLN3*$^+$ gene. pBK84 was a plasmid created by deletion of pUC118*CLN3-1* that ended just 5' of the first ATG of the *CLN3-1* gene. The 1.5 kb *Pst*I-*Xba*I fragment was degraded by the *exonuclease*III.

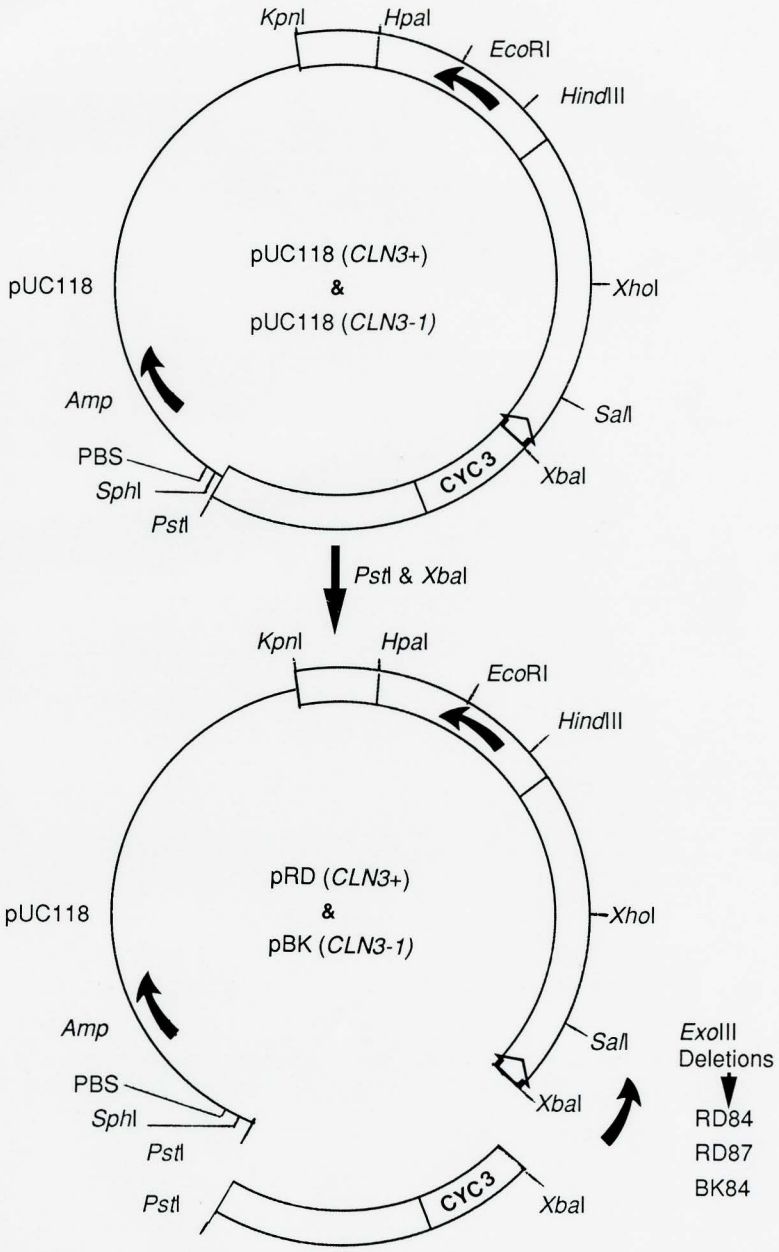

KpnI
HpaI
EcoRI
HindIII
pUC118 (CLN3+)
&
pUC118 (CLN3-1)
pUC118
XhoI
Amp
SalI
PBS
SphI
PstI
CYC3
XbaI
PstI & XbaI
KpnI
HpaI
EcoRI
HindIII
pRD (CLN3+)
&
pBK (CLN3-1)
pUC118
XhoI
Amp
SalI
PBS
SphI
PstI
PstI
CYC3
XbaI
ExoIII
Deletions
RD84
RD87
BK84

Figure 10. Creation of nested deletions of plasmids pBL (*CLN3*$^+$) and pGR (*CLN3-1*) by the method of Henikoff (1984). Nested deletions were made after cutting out a 0.5 kb *Kpn*I-*Hpa*I fragment from pUC119*CLN3*$^+$ and pUC119*CLN3-1*. The 3' overhangs produced by digestion with *Kpn*I were resistant to *exonuclease* III treatment and the blunt ends produced by *Hpa*I digestion were sensitive. This directed the nested deletions from the 3' end towards the 5' end of *CLN3*$^+$ (pBLs) or *CLN3-1* (pGRs). After *exonuclease* III treatment, *S1* and *Klenow* were used to create blunt ends, which were ligated together. Deletions were made approximately every 250 bp, to ensure the overlapping of sequences. The different length inserts were then sequenced using the universal primer which hybridizes to the primer binding site (PBS). The 0.5 kb *Kpn*I-*Hpa*I fragment is degraded by the *exonuclease* III.

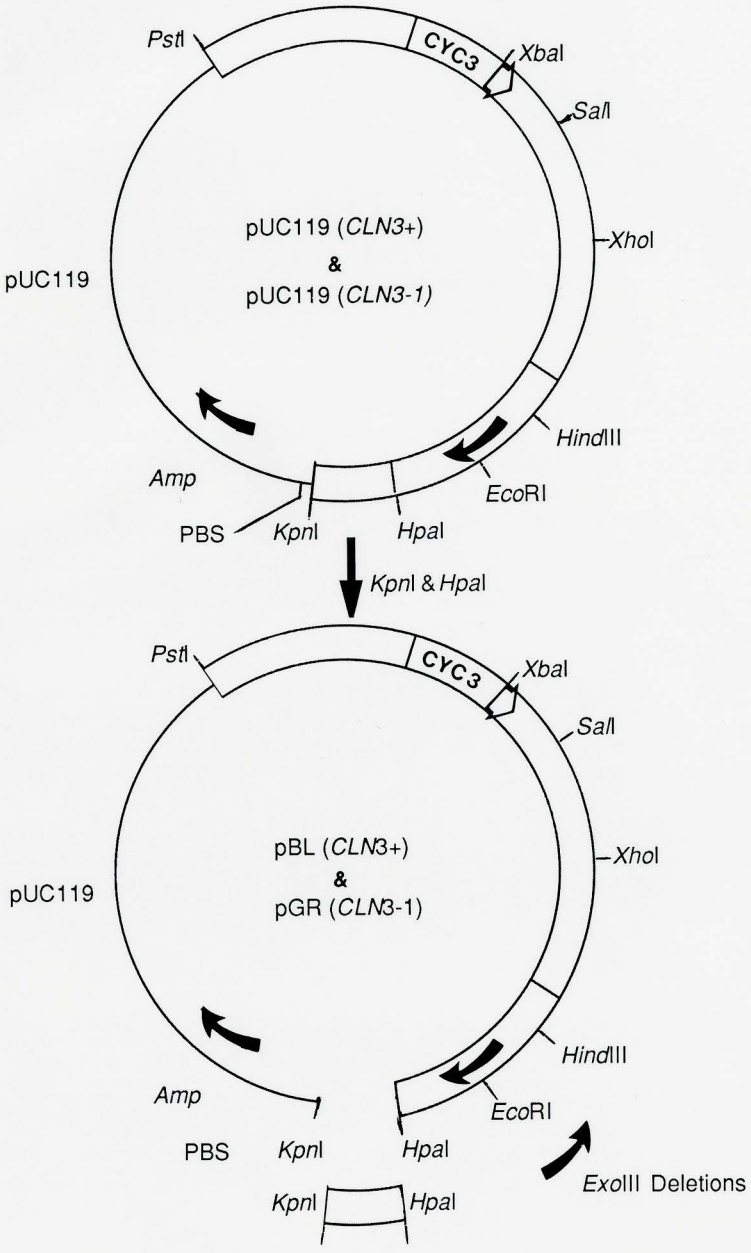

PstI
CYC3
XbaI
SalI
XhoI
pUC119 (CLN3+)
&
pUC119 (CLN3-1)
pUC119
HindIII
EcoRI
Amp
PBS
KpnI
HpaI
KpnI & HpaI
PstI
CYC3
XbaI
SalI
XhoI
pBL (CLN3+)
&
pGR (CLN3-1)
pUC119
HindIII
EcoRI
Amp
PBS
KpnI
HpaI
ExoIII Deletions
KpnI
HpaI

Figure 11. Sequence of *CLN3*$^{+}$. The nucleotide and amino acid sequences are shown. The point mutation is indicated by the "T". This stop codon in place of a glutamine results in a truncated protein of 403 amino acids (CLN3-1).

```
CTGACAGAGA CACCCGTAGA GGCTACATTA CTGATTTGGG AAATTTCCCA AATTGGAAAT ATCACCTCGT  -931
SalI
CGACGTGCTG CGGTGCATGG CTGTTTTACC CGTTTAGGAA AAAACTCGGC GGGTTTTCTT GACGGGCAAA  -861

TGTCGCCATT CGTCTCGTTT GAACGCTTGG CCTGTAAAAC AGCTAACTCA TTCACTATCT CTATCCTGCG  -791

ACCGTGATGA TACCGAAAGT ATGCTGATAC AGATCGGTCC CTCATATCGC GATAGGGCTT TCTGAGCTCT  -721

CCTCCCCCTT CTTCCCTCCC CAGCCAAAGA GGCCGGTTTT CCTTCTGGGG AAGTGTCGCA ACCAAACGCT  -651

GTGCTCAAAC CCTAAATTGT GAACTTTCAA GAAAAAAAAA AAGAAAAAGT GAAAAATTAT CAGGCAAGAA  -581

AAAGAAATTA CCAAGCCTGC TCTCACTGTA ATGATCAAGT TACATAAATT TACTATCGGA TTAGTGTGTC  -511

CCTCTGCCAC ATTTCCATAT TTGGCCTTGG GTTTTTGCCC TCATCTTTTT TTTTTCTTCC TCCTACCTAT  -441
                    XhoI
TTATAATTTG TATATCTGTA CTTTCCTCGA GCTTTTAATC TTCTTCTTAA ACATTTTCTT GTGTAGTATA  -371

CTTTCACACA ATTTCTTTCT TGATTTTTTC TTCTACTACT GAGTCTGCCA GTCAAATGGA TTTCTGAGGA  -301

AAGAGGACTA TACCCATTAG GAAACGAATT GCCCGAGTAG TCTCCTCTGC CGACTTAAAC CAACCTTTTT  -231

CTATTTCTCT TTTCTTTTCT CCCTCTTTTT TCTCTGTACT AGCATCCAAA AGCAAGCATC CATCCGAGTC  -161

CCAGTCGCAA TCTCACATCT CCAATTTAAC GTATCCATTG CATTTCCTCA TTCGGTTTAA CTCCTCTGCA  -91

TTTCTTTTCT GACCCATAGC ATTTCTTACA TTCCATTGCA TCTCCCTTTT ACTCTCGTTC AAGACACTGA  -21

TTTGATACGC TTTCTGTACG ATG GCC ATA TTG AAG GAT ACC ATA ATT AGA TAC GCT AAT GCA   42
                  MET Ala Ile Leu Lys Asp Thr Ile Ile Arg Tyr Ala Asn Ala

AGG TAT GCT ACC GCT AGT GGC ACT TCC ACC GCC ACT GCC GCC TCT GTC AGC GCT GCC    99
Arg Tyr Ala Thr Ala Ser Gly Thr Ser Thr Ala Thr Ala Ala Ser Val Ser Ala Ala

TCA TGT CCT AAT TTG CCC TTG CTC TTG CAA AAG AGG CGG GCC ATT GCT AGT GCA AAG   156
Ser Cys Pro Asn Leu Pro Leu Leu Leu Gln Lys Arg Arg Ala Ile Ala Ser Ala Lys

TCT AAA AAC CCT AAT CTC GTT AAA AGA GAA TTG CAA GCA CAT CAC TCA GCG ATC AGC   213
Ser Lys Asn Pro Asn Leu Val Lys Arg Glu Leu Gln Ala His His Ser Ala Ile Ser

GAA TAC AAT AAT GAT CAA TTG GAC CAC TAT TTC CGT CTT TCC CAC ACA GAA AGG CCG   270
Glu Tyr Asn Asn Asp Gln Leu Asp His Tyr Phe Arg Leu Ser His Thr Glu Arg Pro

CTG TAC AAC CTG ACT AAC TTC AAC TCT CAG CCA CAA GTT AAT CCG AAG ATG CGT TTC   327
Leu Tyr Asn Leu Thr Asn Phe Asn Ser Gln Pro Gln Val Asn Pro Lys MET Arg Phe

TTG ATC TTT GAC TTC ATC ATG TAC TGT CAC ACA AGA CTC AAT CTA TCG ACC TCG ACT   384
Leu Ile Phe Asp Phe Ile MET Tyr Cys His Thr Arg Leu Asn Leu Ser Thr Ser Thr

TTG TTC CTT ACT TTC ACT ATC TTG GAC AAG TAT TCC TCG CGG TTC ATT ATC AAG AGT   441
Leu Phe Leu Thr Phe Thr Ile Leu Asp Lys Tyr Ser Ser Arg Phe Ile Ile Lys Ser

TAC AAC TAC CAG CTC TTG TCC TTG ACC GCG CTT TGG ATT TCG TCC AAA TTT TGG GAC   498
Tyr Asn Tyr Gln Leu Leu Ser Leu Thr Ala Leu Trp Ile Ser Ser Lys Phe Trp Asp

TCC AAG AAT AGA ATG GCC ACT TTG AAA GTC TTG CAA AAC TTG TGT TGC AAT CAA TAT   555
Ser Lys Asn Arg MET Ala Thr Leu Lys Val Leu Gln Asn Leu Cys Cys Asn Gln Tyr

TCT ATA AAG CAA TTC ACG ACT ATG GAA ATG CAT CTT TTC AAA TCA CTC GAT TGG TCC   612
Ser Ile Lys Gln Phe Thr Thr MET Glu MET His Leu Phe Lys Ser Leu Asp Trp Ser

ATC TGT CAG TCG GCA ACA TTC GAC TCC TAC ATC GAC ATC TTC TTG TTC CAA TCT ACG   669
Ile Cys Gln Ser Ala Thr Phe Asp Ser Tyr Ile Asp Ile Phe Leu Phe Gln Ser Thr
                                                HindIII
TCC CCG TTA TCG CCT GGC GTT GTC CTT TCT GCC CCT TTG GAA GCT TTC ATT CAA CAG   726
Ser Pro Leu Ser Pro Gly Val Val Leu Ser Ala Pro Leu Glu Ala Phe Ile Gln Gln

AAA CTG GCC TTA TTA AAT AAC GCT GCT GGT ACT GCT ATT AAT AAA TCG TCC TCT TCT   783
Lys Leu Ala Leu Leu Asn Asn Ala Ala Gly Thr Ala Ile Asn Lys Ser Ser Ser

CAA GGC CCC TCT TTG AAC ATC AAC GAG ATC AAA TTG GGT GCC ATT ATG TTG TGC GAG   840
Gln Gly Pro Ser Leu Asn Ile Asn Glu Ile Lys Leu Gly Ala Ile MET Leu Cys Glu

TTA GCT TCC TTC AAT CTC GAA TTA TCA TTT AAA TAT GAT CGT TCA CTA ATT GCG CTG   897
Leu Ala Ser Phe Asn Leu Glu Leu Ser Phe Lys Tyr Asp Arg Ser Leu Ile Ala Leu

GGT GCA ATT AAC CTC ATC AAA TTA TCT TTG AAC TAC TAT AAT TCA AAC CTT TGG GAA    954
Gly Ala Ile Asn Leu Ile Lys Leu Ser Leu Asn Tyr Tyr Asn Ser Asn Leu Trp Glu

AAT ATC AAT CTG GCT TTG GAG GAA AAC TGC CAA GAC CTA GAT ATT AAA TTG TCA GAA   1011
Asn Ile Asn Leu Ala Leu Glu Glu Asn Cys Gln Asp Leu Asp Ile Lys Leu Ser Glu

ATC TCT AAT ACT TTA TTG GAT ATA GCA ATG GAC CAA AAT TCT TTC CCC TCC AGT TTC   1068
Ile Ser Asn Thr Leu Leu Asp Ile Ala MET Asp Gln Asn Ser Phe Pro Ser Ser Phe

AAA TCA AAA TAT TTG AAT AGC AAT AAG ACA TCT TTA GCA AAA TCT CTC TTA GAC GCA   1125
Lys Ser Lys Tyr Leu Asn Ser Asn Lys Thr Ser Leu Ala Lys Ser Leu Leu Asp Ala
                                                EcoRI
TTA CAA AAC TAT TGT ATT CAA TTG AAA CTG GAA GAA TTC TAC CGT TCA CAA GAA TTG   1182
Leu Gln Asn Tyr Cys Ile Gln Leu Lys Leu Glu Glu Phe Tyr Arg Ser Gln Glu Leu
                                T

GAA ACC ATG TAC AAT ACT ATC TTT GCT CAG TCC TTT GAC AGC GAT TCA TTG ACT TGT   1239
Glu Thr MET Tyr Asn Thr Ile Phe Ala Gln Ser Phe Asp Ser Asp Ser Leu Thr Cys

GTT TAC TCA AAT GCT ACT ACT CCA AAG AGC GCT ACG GTT TCA TCT GCG GCC ACA GAC   1296
Val Tyr Ser Asn Ala Thr Thr Pro Lys Ser Ala Thr Val Ser Ser Ala Ala Thr Asp

TAT TTC TCG GAT CAC ACT CAT TTA AGA AGG TTG ACC AAA GAT AGC ATT TCT CCA CCA   1353
Tyr Phe Ser Asp His Thr His Leu Arg Arg Leu Thr Lys Asp Ser Ile Ser Pro Pro

TTT GCC TTC ACT CCA ACC TCA TCT TCA TCC TCT CCA TCT CCA TTC AAT TCC CCT TAC   1410
Phe Ala Phe Thr Pro Thr Ser Ser Ser Ser Pro Ser Pro Phe Asn Ser Pro Tyr

AAG ACT TCA AGT TCA ATG ACG ACC CCA GAC TCT GCA TCA CAC CAT TCA CAT TCA GGT   1467
Lys Thr Ser Ser Ser MET Thr Thr Pro Asp Ser Ala Ser His His Ser His Ser Gly

TCG TTC TCT TCT ACC CAA AAT TCT TTT AAA AGG TCA CTG AGC ATC CCA CAA AAT TCA   1524
Ser Phe Ser Ser Thr Gln Asn Ser Phe Lys Arg Ser Leu Ser Ile Pro Gln Asn Ser

AGC ATC TTT TGG CCA AGC CCA CTA ACT CCC ACC ACC CCA TCT CTA ATG TCA AAT AGA   1581
Ser Ile Phe Trp Pro Ser Pro Leu Thr Pro Thr Thr Pro Ser Leu MET Ser Asn Arg
                                                                    NcoI
AAA TTA TTA CAA AAT TTA TCT GTG CGT TCA AAA AGA TTA TTT CCT GTT AGA CCC ATG   1638
Lys Leu Leu Gln Asn Leu Ser Val Arg Ser Lys Arg Leu Phe Pro Val Arg Pro MET

GCC ACT GCT CAC CCA TGC TCT GCC CCC ACC CAA CTG AAA AAG AGA TCA ACT TCC TCT   1695
Ala Thr Ala His Pro Cys Ser Ala Pro Thr Gln Leu Lys Lys Arg Ser Thr Ser Ser

GTG GAT TGT GAT TTT AAT GAT AGT AGC AAC CTC AAG AAA ACT CGC TGA AACGACAAAA    1753
Val Asp Cys Asp Phe Asn Asp Ser Ser Asn Leu Lys Lys Thr Arg  .
                                                HpaI
AAAAAATGCA TTTAACAACA AATAAATTAA AATTTGCAAA GCAAATACGT TAACATACAT TAATGATCTT  1823

TTTTTCTTTT TGTTTTCACA GGACTCATTA TTTCTTTAAA TATACTTATA TTACATTTGC ATAGAATTAC  1893

AAAAAAAAAA AAATTATAAA AACGCACAAC CTAAAATACG ATTACTATCG TTTTTTCTCC TTACTTCCTC  1963

TAGTCACATT ATACATTTTT TTTCATCCTT CACAGACGTA TCTTAATACT AAGTGTGGAT CAATATAATG  2033

GGTATAGCTT GAACATCTGC CCCTCTCTAT CTAATGTTTT TTTCTTGAAT TATTTAGTAT TTGCTGTATA  2103

CTAGTTTTAT TTATCAATAC AGACGAATGT TCAAGAATTT CTCATTGTTT TCCGCCAACA TTTCTTTAGC  2173

AACATTCCAA TCTTCATCGC TACCTAACAC AACAAAATCC CCATCTTCGT CCTGATATTT GATCTTGGTG  2243

ATTGGTGAAA TGTTGTTATT ATGTGTATTC GAAATTTTAG AATTGATCGC CATTATCAAG TCGTCAAAAT  2313
                                  BglII
TCCAAACTTT TTCTACCAAA AGTGTGAAGA TC/                                           2345
```

expected sizes (66-75 kDa and 45 kDa respectively) for the ORF which begins at nucleotide +1 on acrylamide gels; the first ATG is bordered by nucleotides at both the 5' and 3' ends constituting a sequence very similar to the consensus sequence for translation initiation for yeast mRNAs (Hamilton *et al.*, 1987) and higher eukaryotes (Kozak, 1986).

The region sequenced (Figure 11), overlaps with two previously sequenced genes on chromosome I of *S. cerevisiae*. The 5' non-coding sequence of *CLN3*$^+$ overlaps with the *CYC3* gene (Dumont *et al.*, 1987) and the 3' non-coding sequence overlaps with the *CDC24* gene (Miyamoto *et al.*, 1987).

The codon bias of *CLN3*$^+$ was calculated by the method of Bennetzen and Hall, (1982) to be 0.27. The low codon bias suggests that CLN3$^+$ is not an abundant protein.

The sequence of the mutant *CLN3-1* gene differs from the wild type *CLN3*$^+$ gene by one base pair change at position 1210 (Figure 11). This C to T transition introduces a stop codon in place of glutamine. This results in a truncated mutant protein of 403 amino acids instead of the 580 amino acids coded for by *CLN3*$^+$. *CLN3*$^+$ cells were transformed with *CLN3-1* plasmid DNA from which the *Eco*RI-*Hpa*I fragment containing the stop codon had been removed (see Figure 11 for locations of restriction sites). By recombination and gap repair (Rothstein, 1983), the transformed cells replaced the missing DNA sequence with wild-type sequence. These cells now contained two copies of *CLN3*$^+$ and had mode volumes of 35 μm^3 (data not shown). This was the expected mode volume for two copies of *CLN3*$^+$ (Nash *et al.*, 1988) and showed that the point mutation at nucleotide 1210 was responsible for the *CLN3-1* mutation.

Computer analysis using the FastA program found homology to the clam cyclin A (Swenson *et al.*, 1986) the sea urchin cyclin (Pines and Hunt, 1987) and *S. pombe* cdc13 (Booher and Beach, 1988) in the Genbank and EMBL data bases. The best region of similarity was restricted to a section of approximately 100 amino acids in CLN3$^+$. This covered amino acids 106 to 206 and was 50% similar to the cyclin consensus sequence (data

not shown). The similarity between CLN3$^+$ and the mitotic cyclins implies the possible role of CLN3$^+$ in cell cycle control. The similarity led us and others to change the name of the gene from *WHI1*$^+$ (Carter and Sudbury, 1980) or *DAF1*$^+$ (Cross, 1988) to *CLN3*$^+$.

4.2 pGal1(*CLN3*$^+$) and pGal1(*CLN3-1*) Constructs

In order to further characterize the *CLN3*$^+$ gene, it was necessary to control its expression. The promoter of the *GAL1* gene was used since it provided a natural means by which to precisely control expression of the inserted gene(s) in *S. cerevisiae* (Johnston and Davis, 1984). In the absence of glucose but in the presence of galactose, expression of *CLN3*$^+$ or *CLN3-1* would be turned on at high levels. However, with glucose present in the media, expression of either gene would be entirely off.

Plasmids were constructed with *CLN3*$^+$ (pGal1RD84 and pGal1RD87T$_4$) and *CLN3-1* (pGal1BK84 and pGal1BK84T$_4$) downstream of the *GAL1* promoter in plasmid pCGS110 (Figures 12 & 13). Constructs were made for both *CLN3*$^+$ and *CLN3-1* ensuring that the lengths of the 5' untranslated regions were conserved for proper transcription initiation using the *GAL1* promoter. Furthermore, no intervening ATG translation initiation codons were present prior to the first ATG of *CLN3*$^+$ or *CLN3-1* in either the pGal1RD84, RD87T$_4$ or pGal1BK84, BK84T$_4$ constructs respectively. Restriction analysis and subsequent sequencing of the junctions between the *GAL1* promoter and either *CLN3*$^+$ or *CLN3-1* confirmed that the proper constructs were made. Two different approaches were carried out (Figures 12 & 13) to ensure that the constructs would be made. Only the constructs in Figure 12 A (pGal1RD84) and 13 B (pGal1BK84T$_4$) were used in subsequent experiments.

Figure 12. Maps of the pGal1RD84 (*CLN3*$^+$) and pGal1BK84 (*CLN3-1*) constructs. In **A**, a 2.4 kb *Sph*I-*Sma*I fragment containing the entire *CLN3*$^+$ gene was taken from nested deletion pRD84 (see Figure 9) and inserted into pCGS110 digested with *Bam*HI and *Nru*I. The *Bam*HI and *Sph*I ends were made compatible with the use of a *Bam*HI *Sph*I adaptor as indicated in the expanded junction region. The blunt ends generated by *Sma*I and *Nru*I were simply ligated together. The directed insertion insured that the *CLN3*$^+$ gene was in the same orientation as the *GAL1* promoter. This created pGal1RD84. The construct was made with i) conservation of the proper distance for transcription initiation (prior to the *CLN3*$^+$ ORF) required by the *GAL1* promoter of *S. cerevisiae*, and ii) ensuring that no other initiation codon was present prior to that in the *CLN3*$^+$ ORF.
In **B**, pGal1BK84 was constructed essentially the same way as pGal1RD84 except a shorter 1.9 kb *Sph*I-*Hpa*I fragment from nested deletion pBK84 (see Figure 9) was inserted into pCGS110.

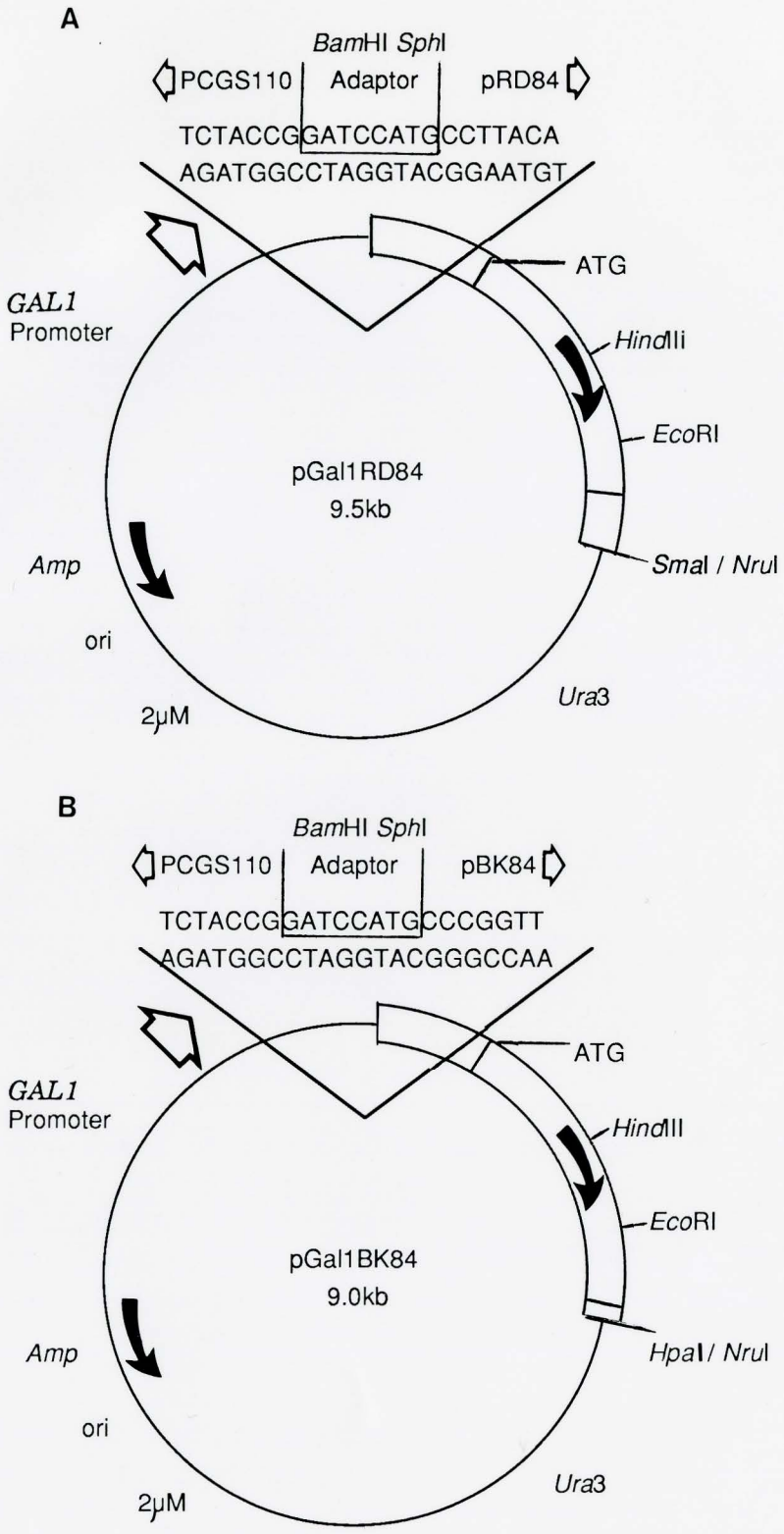
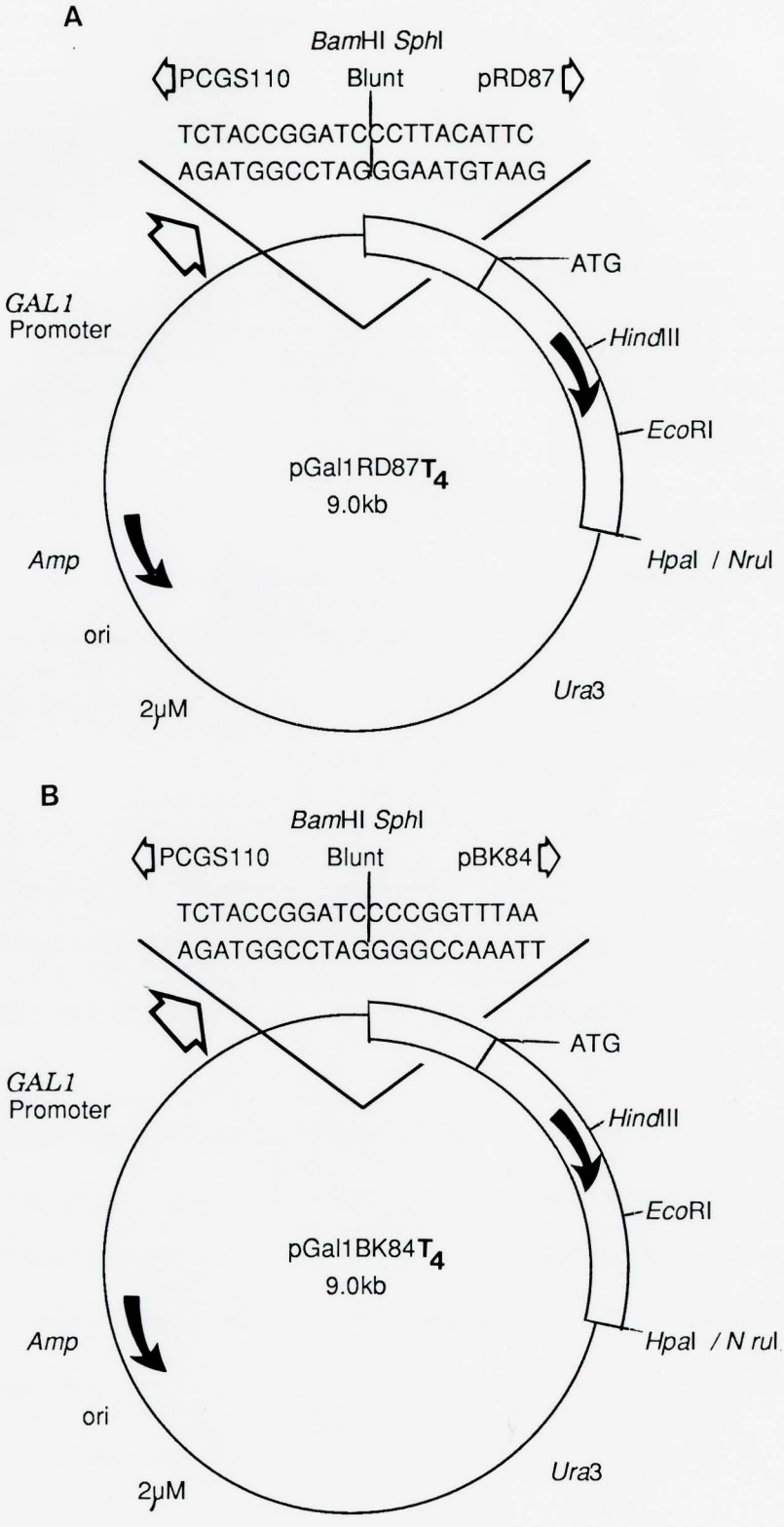

A
BamHI SphI
PCGS110
Adaptor
pRD84
TCTACCGGATCCATGCCTTACA
AGATGGCCTAGGTACGGAATGT
ATG
GAL1
Promoter
HindIII
EcoRI
pGal1RD84
9.5kb
Amp
SmaI / NruI
ori
Ura3
2μM
B
BamHI SphI
PCGS110
Adaptor
pBK84
TCTACCGGATCCATGCCCGGTT
AGATGGCCTAGGTACGGGCCAA
ATG
GAL1
Promoter
HindIII
EcoRI
pGal1BK84
9.0kb
Amp
HpaI / NruI
ori
Ura3
2μM

Figure 13. Maps of the pGal1RD87T_4 (*CLN3*$^+$) and pGal1BK84T_4 (*CLN3-1*) constructs. In **A**, a 1.9 kb *Sph*I-*Hpa*I fragment containing the entire *CLN3*$^+$ gene was taken from nested deletion pRD87 (see Figure 9) and inserted into pCGS110 digested with *Bam*HI and *Nru*I. The *Bam*HI and *Sph*I ends were made compatible by blunt-ending the *Bam*HI 5' overhang using *Klenow* and by blunt-ending the *Sph*I 3' overhang using *T4 DNA polymerase* I. The blunt ends generated by *Hpa*I and *Nru*I were simply ligated together. This created pGal1RD87T_4. The construct was made with i) conservation of the proper distance for transcription initiation (prior to the *CLN3*$^+$ ORF) required by the *GAL1* promoter of *S. cerevisiae*, and ii) ensuring that no other initiation codon was present prior to that in the *CLN3*$^+$ ORF.
In **B**, pGal1BK84T_4 was constructed essentially the same way as pGal1RD87T_4 except a 1.9 kb *Sph*I-*Hpa*I fragment from nested deletion pBK84 (see Figure 9) was inserted into pCGS110.


A
BamHI SphI
PCGS110
Blunt
pRD87
TCTACCGGATCCCTTACATTC
AGATGGCCTAGGGAATGTAAG
ATG
GAL1
Promoter
HindIII
EcoRI
pGal1RD87T4
9.0kb
Amp
HpaI / NruI
ori
Ura3
2μM
B
BamHI SphI
PCGS110
Blunt
pBK84
TCTACCGGATCCCCGGTTTAA
AGATGGCCTAGGGGCCAAATT
ATG
GAL1
Promoter
HindIII
EcoRI
pGal1BK84T4
9.0kb
Amp
HpaI / N rul
ori
Ura3
2μM

4.3 Expression Using the *GAL1* Promoter

Plasmids pCGS110, pGal1RD84 and pGal1BK84T_4 were transformed separately into the yeast strain *BF338-3b* (*MATa CLN3⁺ ura3*). *Ura⁺* transformants were selected by their ability to grow on plates lacking uracil. Colonies were picked and streaked for single clones and for subsequent plasmid loss experiments. Transformants containing these *GAL1* constructs were tested for transcription of *CLN3⁺* or *CLN3-1*, reduced cell volume and for α-factor resistance in the presence of 2% raffinose and 2% galactose (raffinose does not repress the *GAL1* promoter). Over-expression of either *CLN3⁺* or *CLN3-1* using the strong *GAL1* promoter was compared to expression using only a few copies of the gene by integration.

4.4 *CLN3⁺* Messenger RNA Levels Increase in the Presence of Galactose

Cells grown in the presence or absence of galactose containing pGal1RD84 or pGal1BK84T_4 or no plasmid were harvested and total RNA was extracted. The RNA was prepared and run on a denaturing agarose gel. Probes consisting of an end-labelled oligo internal to the gene (*CLN3⁺* and *CLN3-1*) and random primed sequences from the yeast *ADH1* gene (control) were used to probe the northern. Messenger RNAs of approximately 2.0 kb were more abundant (approximately 50x) in the presence of galactose than the endogenous levels of the control (data not shown). Cells lacking the *GAL1* constructs or cells containing the constructs but grown on glucose had normal endogenous levels of the 2.0 kb *CLN3⁺* mRNA.

4.5 Cell Size and α-factor Resistance

Two further criteria by which to confirm the operation of the *GAL1* constructs were to test for the reduction in cell volume and resistance to α-factor when grown in the absence of glucose and in the presence of galactose.

CLN3-1 cells were first identified by their ability to enter a new round of the cell cycle at a reduced cell size (Carter and Sudbury, 1980), which was attributed to the presence of a truncated form of the wild-type CLN3$^+$ protein (Cross, 1988; Nash *et al.*, 1988). Over-expression of either the wild-type or mutant form of the *CLN3*$^+$ gene when expressed from integrated tandem copies also resulted in a reduced cell volume when compared to wild-type sizes. *BF338-3b* cells containing either pGal1RD84 or pGal1BK84T$_4$ when grown in the presence of 2% galactose and 2% raffinose had mode cell volumes of approximately 16 and 17 μm^3 respectively (Figure 14, panels C & D). The same cells grown in the presence of 2% raffinose alone or cells containing pCGS110 with no insert, were virtually identical to wild-type cell size of approximately 26 μm^3 (Figure 14, panel A). Cells which had lost the plasmids also were found to have a size similar to those of wild-type cells. Thus, the *GAL1* constructs do reduce cell size.

Previously, it was demonstrated that *CLN3-1* cells were α-factor resistant (Cross, 1988; Nash *et al.*, 1988). The ability of the *GAL1* constructs to confer α-factor resistance on cells was tested. Plates containing either 2% glucose or 2% raffinose and 2% galactose were made either with or without α-factor (5x10^{-6} M). Approximately 1000 individual cells were spotted onto plates and allowed to grow for 2-4 days at 30°C. α-factor resistant cells were scored by their ability to grow into colonies in the presence of α-factor on the plate. Cells sensitive to α-factor were unable to grow. As shown in Figure 15, Panel D (5 & 7), *BF338-3b* cells containing either of the two plasmids (pGal1RD84 or pGal1BK84T$_4$) when grown with galactose as the carbon source formed colonies even in the

Figure 14. Coulter Channelyzer plots of cell volume distributions. Cells were grown to mid-log phase in YNB (-ura) media in the presence of 2% raffinose. Cells were then diluted down and grown in the presence of 2% raffinose or 2% raffinose and 2% galactose. Cell volumes were then looked at using the Coulter Channelyzer. Graphs for +/- 2% galactose were overlapped. Cell numbers are plotted along the y-axis and mode cell volumes are plotted along the x-axis. Mode cell volumes are shown. Panel **A** represents control wild-type cells *BF338-3b* (*MATa CLN3*$^+$ *ade1 leu2 ura3 his3 G418*^{R}) grown in the presence or absence of 2% galactose (only one graph is shown since under the two different conditions, the graphs were identical), panel **B** are the same cells transformed with pCGS110 (*URA*$^+$) alone with no insert, panel **C** are cells (clone 3(1)) which were transformed with pGal1RD84 (*CLN3*$^+$) and panel **D** are cells (clone 9(1)) transformed with pGal1BK84T$_4$ (*CLN3-1*). These experiments were carried out numerous times on different clones yielding the same results.

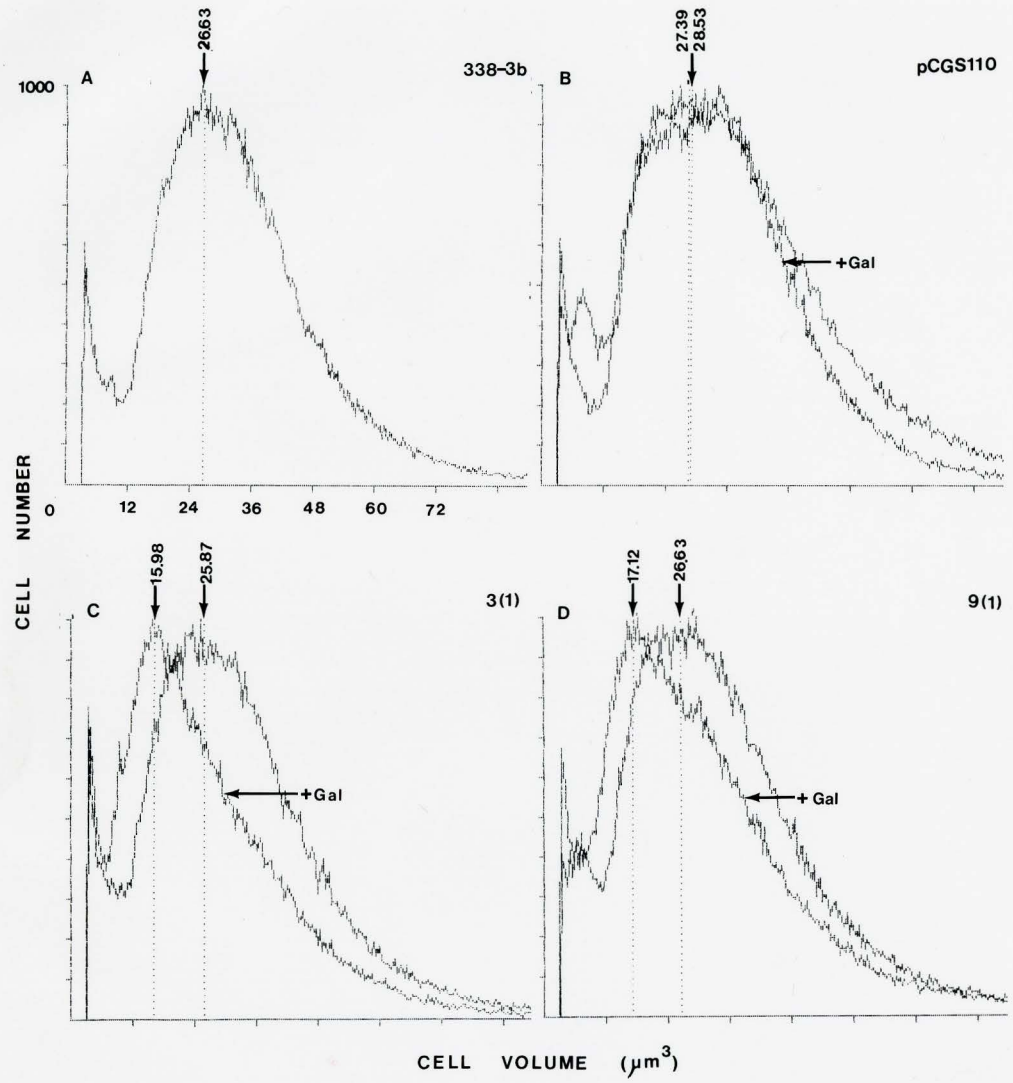
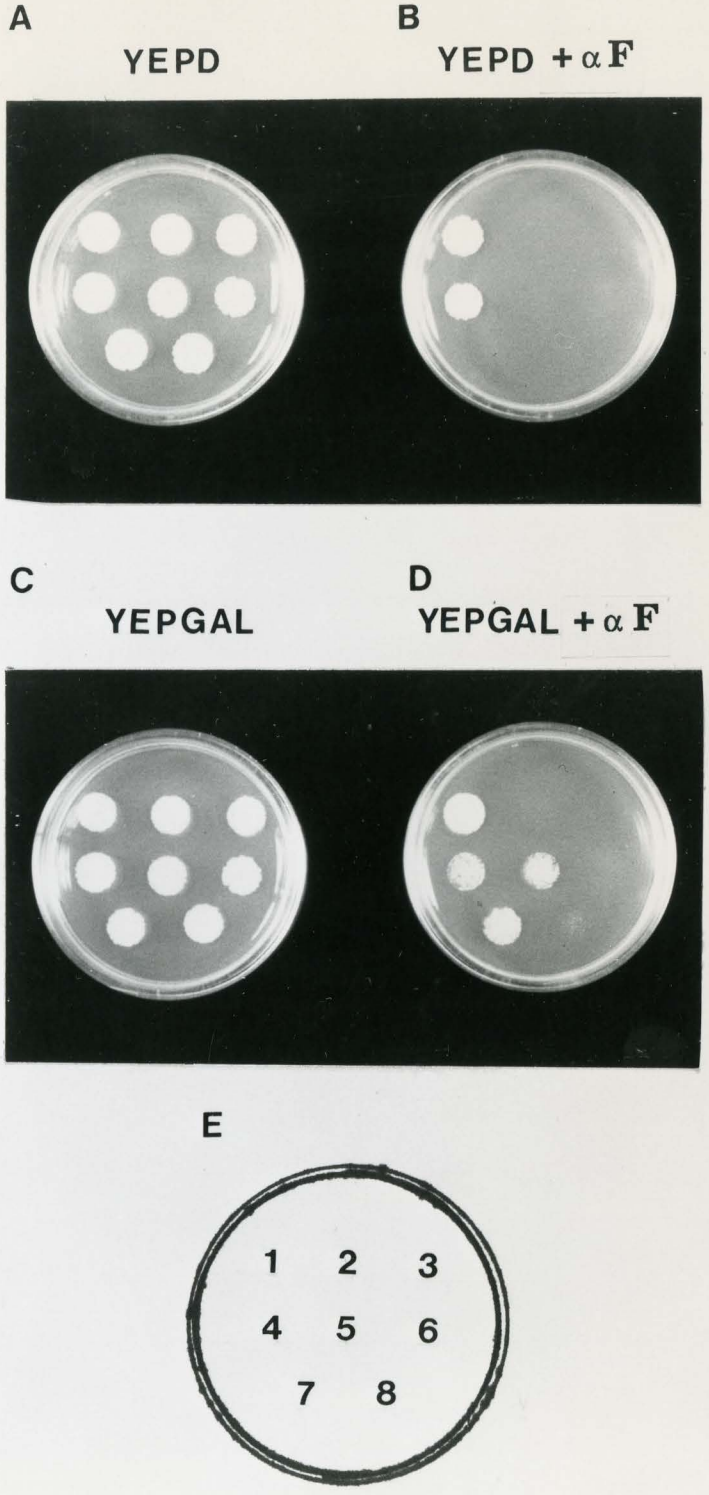

A
26.63
338-3b
1000
B
27.39
28.53
pCGS110
+Gal
CELL NUMBER
0
12
24
36
48
60
72
C
15.98
25.87
3(1)
+Gal
D
17.12
26.63
9(1)
+Gal
CELL VOLUME (μm³)

Figure 15. Induction of α-factor resistance. Cells were first grown in YNB (-ura) media then plated out on to various types of YEP plates containing glucose +/- $5x10^{-6}$ M α-factor (in panels **A** and **B**) or galactose +/- $5x10^{-6}$ M α-factor (in panels **C** and **D**). Approximately 1000 cells were spotted onto the plate in 5 µl volumes and allowed to grow for 2-4 days at 30°C. α-factor resistant cells were scored by their ability to form colonies. Cells which contained pCGS110 alone or had lost their plasmid were not α-factor resistant (3, 6 and 8 panel **D**). Cells sensitive to α-factor grown in the presence of glucose remained sensitive (5 and 7 in panel **B**). All cells grew in the presence of galactose or glucose in the absence of α-factor (panels **A** and **C**). In **E** 1. *BF350-12c* (*MATα CLN3*$^+$ *leu2 ura3 his G418*^{R}) 2. *BF338-3b* (*MATa CLN3*$^+$ *ade1 leu2 ura3 his3 G418*^{R}), 3. *BF338-3b* + pCGS110 (*URA*$^+$), 4. *BF338-4b* (*MATa CLN3-1 leu2 his3 lys2*), 5. clone 3(1) (*BF338-3b* + pGal1RD84) originally grown on YNB (-ura), 6. clone 3(1) originally grown on YEP which had lost pGal1RD84, 7. clone 9(1) (*BF338-3b* + pGal1BK84T_4) originally grown on YNB (-ura), 8. clone 9(1) originally grown on YEP which had lost pGal1BK84T_4.


A
YEPD
B
YEPD + αF
C
YEPGAL
D
YEPGAL + αF
E
1 2 3
4 5 6
7 8

presence of α-factor. *BF338-3b* cells, cells that had lost their pGal1 constructs, or contained the vector alone with no insert failed to grow on the same plates (Figure 15, #3, 6 and 8). All transformants grew in the absence of α-factor (Figure 15, Panel A). *BF350-12c MAT*α (*MAT*α are not sensitive to α-factor) cells as predicted grew in the presence of α-factor on all carbon sources. Cells containing the pGal1 constructs (*CLN3*$^+$ or *CLN3-1*) grown in the presence of galactose and glucose were not α-factor resistant (data not shown).

These experiments indicate that both the pGal1RD84 and pGal1BK84T$_4$ constructs expressed the *CLN3*$^+$ or *CLN3-1* gene on the appropriate carbon source and confer to the cells both small cell size and α-factor resistance. Recently, this has been confirmed using antibodies that detect the CLN3$^+$ or CLN3-1 protein. Much more protein (CLN3$^+$ or CLN3-1) is expressed by strains containing the pGal1 contructs (M. Tyers, personal communication).

DISCUSSION

Studies using the yeasts *S. cerevisiae* and *S. pombe* have defined two key control points during the cell cycle, one in G1 called Start and the other at the G2/M boundary (reviewed by Nurse, 1985). Several genes and gene product interactions directly involved with Start and the G2/M boundary have been found. These genes and their product interactions control the entry of the cell into a new round of the cell cycle and control the entry to and exit from mitosis.

5.1 Search for *S. cerevisiae* Homologs of *S. pombe cdc25*$^+$ and *wee1*$^+$

Direct genetic and molecular evidence suggests eukaryotic cells share general mechanisms of control at Start (reviewed by Pringle and Hartwell, 1981; Nurse and Bissett, 1981) and at the G2/M boundary (Piggott *et al.*, 1982; Nurse and Thuriaux, 1980). Much of the evidence for the existence of general cell cycle control mechanisms comes from complementation experiments and conserved structural similarities in proteins. Gene sequences show that cell cycle control proteins are highly conserved from yeast to man. Surprisingly often, genes from one organism are able to function in distantly related organisms. Since there exists increasing evidence of general control mechanisms at Start and at the G2/M boundary, it seemed likely that *S. cerevisiae* might have homologs to the *cdc25*$^+$ and *wee1*$^+$ genes of *S. pombe* (Russell and Nurse, 1986; Russell and Nurse, 1987a). Investigation of potential homologous regulatory genes is important in comparing cdc2/Cdc28 cell cycle control between the two yeasts. The projects original purpose was

to isolate a *cdc25*$^+$ or a *wee1*$^+$ homolog in *S. cerevisiae*, using DNA cross-hybridization to *cdc25*$^+$ or *wee1*$^+$ of *S. pombe* as the method of identification.

Synthetic oligonucleotides or DNA fragments covering the entire *wee1*$^+$ gene and DNA fragments covering the entire *cdc25*$^+$ gene were used as probes to search *S. cerevisiae* banks for their respective homologs. Colony hybridizations and Southern analysis were carried out on *S. cerevisiae* total DNA (strain *LL20*) for both genes. Under low stringency conditions DNA clones that hybridized to the *cdc25*$^+$ and *wee1*$^+$ probes were isolated. A potential *cdc25*$^+$ *S. cerevisiae* clone (*GTcdc25*$^+$) was isolated through its DNA cross-hybridization to the *S. pombe cdc25*$^+$ probe. After cloning and sequencing, *GTcdc25*$^+$ was found not to be a *cdc25*$^+$ homolog but was unfortunately found by chance DNA cross-hybridization to a small region of DNA. A potential *wee1*$^+$ homolog was isolated through some DNA cross-hybridization to the *wee1*$^+$ synthetic oligonucleotide probes, but further analysis suggested that it too was probably picked up by chance DNA cross-hybridization.

An explanation for not finding a *cdc25*$^+$ or *wee1*$^+$ homolog in *S. cerevisiae* could be that it is possible that these do not exist. Different proteins may be required at the different points of control to regulate Cdc28 or cdc2. An alternative method to look for a *cdc25*$^+$ or a *wee1*$^+$ homolog in *S. cerevisiae* would be to take the genetic approach and use complementation as the criterion. *S. cerevisiae* genomic DNA that could complement conditional lethals of *S. pombe* (e.g. *cdc25* temperature-sensitive lethals) at the restrictive temperature would be looked for. The reciprocal experiments could also be done. However, this would depend on heterologous expression and also on conservation of the details of protein-protein interactions.

5.2 *cdc25* $^+$ and Perhaps *wee1* $^+$ Homologs Do Exist in *S. cerevisiae*

An *S. pombe cdc25*$^+$ homolog has been found in *S. cerevisiae*. The homolog,

MIH1 was cloned by its ability to complement the *cdc25-22* mutant in *S. pombe* (Russell *et al.*, 1989). Sequence data from *cdc25*$^+$ and *MIH1* showed some similarity at the protein level (Russell *et al.*, 1989). However, there is very little similarity between the DNA of the *S. pombe cdc25*$^+$ and *MIH1* of *S. cerevisiae*. This explains the failure of DNA cross-hybridization to find the homolog.

There is some evidence suggesting a *wee1*$^+$ homolog in *S. cerevisiae*. Expression of *S. pombe wee1*$^+$ in *S. cerevisiae mih*$^-$ cells prevents the initiation of mitosis (Russell *et al.*, 1989). This closely resembles the failure of mitosis in *cdc25* temperature-sensitive mutants at the restrictive temperature in *S. pombe* cells that have wild-type *wee1*$^+$ (Russell and Nurse, 1987b). The lethal mitotic block in *S. cerevisiae mih*$^-$ cells by *S. pombe wee1*$^+$ can be overcome if *MIH1* is reintroduced. This argues that *S. cerevisiae* may have a *wee1*$^+$ gene of its own.

5.3 Characterization of *CLN3*$^+$

Attention was turned towards working with *CLN3*$^+$, then known as *WHI1*$^+$ because of the "wee" phenotype of the mutant. The gene was cloned in the lab. Together with Rob Nash, I have sequenced both stands of *CLN3*$^+$ and *CLN3-1* (the mutant gene). The most significant finding is that CLN3$^+$ is homologous to cyclins (Nash *et al.*, 1988) (see Table 2.). However, CLN3$^+$ is only distantly related to the mitotic cyclins. It is possible that two sets of cyclins may exist that interact with cdc2/Cdc28. One set may function at the G2/M boundary and the other at the G1 control point at Start. Common to the two sets of cyclins may be their interaction with their respective p34^{cdc2} homologs. G2/M cyclins have already been identified that interact with their p34^{cdc2} homologs (reviewed by Murray and Kirshner, 1989). CLN3$^+$ is the first G1 cyclin to be identified (Nash *et al.*, 1988). Hadwiger *et al.* (1989b) have found two other G1 cyclin homologs, *CLN1* and *CLN2*. The

Table 2. Homologous Cell Cycle Components in Eukaryotes II

		*S.c.**	*S.p.***	Humans	*Xenopus*	Clams/ Urchins	Starfish	*Drosophila*
Cyclin	clb1 clb2 clb3 clb4	CLN1 CLN2 CLN3^{+} (WHI1^{+})	cdc13	p62	B1,B2, A-type	A,B/B	-	A,B
cdc2/CDC28		CDC28	cdc2	p34^{cdc2}	MPF-p32^{cdc2}	MPF-p32^{cdc2}	p34^{cdc2}-H1K	-
cdc2/CDC28 Substrates		p40	cdc13	p62 (cyclin)	p45 (cyclin) (cyclin)	p62 (B1,B2)	- (cyclin)	-
Associated Components		CKS1 (=p13)	suc1 (=p13)	p13	(p13?)	-	p13	-
		-	nim1	-	-	-	-	-
		MIH1 (=cdc25)	cdc25	(cdc25?)	-	-	-	stg
		(WEE1?)	wee1	-	-	-	-	-

* *S. cerevisiae*
** *S. pombe*
- not done

evidence that CLN3$^+$ acts in G1 is that *CLN3-1* mutants allow passage of Start at a reduced critical cell size, and that cells lacking all three cyclin genes *CLN1, 2* and *3* permanently arrest in G1 (Richardson *et al.*, 1989). It will be interesting to investigate the role of CLN3$^+$ cyclin in the G1 cell cycle control.

The similarity of CLN3$^+$ to other cyclins implies that CLN3$^+$ probably helps regulate the cell cycle. Since *CLN3-1* mutants allow passage of Start at a reduced cell size, CLN3$^+$ may play a role in the G1/S transition (Nash *et al.*, 1988). It seems plausible that CLN3$^+$ may interact with Cdc28 in G1. This is like the mitotic cyclins interacting with their p34^{cdc2} homologs at the G2/M boundary, for e.g. cdc13 interacting with cdc2 in the *S. pombe* cell cycle. There may exist other cyclins in *S. cerevisiae*, in particular, G2 cyclins which are analogous in function to cdc13 of *S. pombe*.

CLN3$^+$ potentially encodes a 64 kDa protein of 580 amino acids. *CLN3-1* sequence differs from *CLN3*$^+$ at only one place, nucleotide 1210. This cytosine to thymine transition introduces a stop codon. CLN3-1 is a truncated form of CLN3$^+$. Sequence analysis and computer analysis have yielded some interesting facts leading to interpretations of the phenotypes of *CLN3-1* cells. Extra copies of *CLN3*$^+$ resulted in slightly smaller size cells entering a new round of the cell cycle. A similar phenotype was observed with *CLN3-1*. The point mutation in *CLN3-1* causes a truncation of the wild-type protein and causes the protein to be hyperactive. CLN3$^+$ may also give clues to the reason for the rapid proteolytic destruction of cyclins in that it contains PEST sequences (rich in proline, glutamate, serine, threonine and/or aspartate) (Rogers *et al.*, 1986) in its carboxy-terminus. These protein sequences are found in a number of proteins which have very short half-lives. These PEST sequences may be signals for proteolytic destruction. The mutant form, CLN3-1, has had these potential PEST sequences removed by the point mutation and may be more resistant to proteolytic destruction. Western analysis has shown that the mutant protein is more stable than the wild-type protein (M. Tyers,

personal communication). This probably accounts for the hyperactivity.

CLN3-1 cells are resistant to arrest induced by mating pheromones (Nash *et al.*, 1988; Cross, 1988). When haploid cells encounter mating pheromones from cells of the opposite mating type they arrest in G1 prior to Start and prepare for conjugation (reviewed by Thorner, 1981). If mating does not occur, the cells must recover from the blockage and continue the cell cycle. *MATa CLN3-1* cells are resistant to the mating factor pheromone (α-factor) arrest and continue to grow and divide in its presence. CLN3-1 apprears to be an activator of Start which can overcome the inhibitory effects of α-factor.

CLN3-1 mutants are able to suppress mutants in the *SCG1/GPA1* gene which constitutively activate the signal pathway in response to pheromone (F. Cross, personal communication). This suggests some type of cell cycle control involved with *CLN3-1*. CLN3$^+$ can be considered an activator of Start by its ability to initiate Start too early (over comes the critical size requirement) and by its ability to resist arrest due to mating pheromones.

CLN3$^+$ is not an essential gene -deletion results in larger cell size, but cells are still viable. Cells containing deletions of any two of *CLN1, 2* or *3* have morphological and growth abnormalities, but are still alive (Hadwiger *et al.*, 1989b; Richardson *et al.*, 1989). However the triple deletion is inviable (Richardson *et al.*, 1989). Unlike *CLN3-1* mutants which are resistant to α-factor (Cross, 1988; Nash *et al.*, 1988; Hadwiger *et al.*, 1989b), *CLN2-1* mutants are only slightly resistant to the pheromone . The important observation that CLN3$^+$ is homologous to cyclins raises the possibility of finding an interaction between CLN3$^+$ and Cdc28, analogous to the cdc2-cdc13 complex in *S. pombe*.

5.4 Future Studies on *CLN3*$^+$

CLN3$^+$ must be characterized at the protein level. Antibodies to CLN3$^+$ are

required and once a good set of antibodies have been prepared, CLN3$^+$ and CLN3-1 characterization can take place. The effects of the truncated CLN3$^+$ (CLN3-1) may be due to an increase in stability of the protein, because of the elimination of the PEST sequences in CLN3$^+$.

The periodical rise and fall of the cyclins correlates with the onset and completion of mitosis (Evans *et al.*, 1983). Does CLN3$^+$ follow a similar pattern but at Start? In order to test this, protein from cells taken from various stages of the cell cycle can be used in western analysis. The levels of protein can be quantitated with the antibodies. The level of protein found in a particular stage of the cell cycle can be investigated.

CLN3$^+$ appears to be important in some aspect of cell size control. Since cell size is monitored at Start in *S. cerevisiae*, it is likely that this is where CLN3$^+$ functions. Also, the G1 arrest phenotype of the *CLN1, CLN2* and *CLN3* triple deletion observed by Richardson *et al.* (1989) and the resistance to α-factor observed in *CLN3-1* strains (Cross, 1988; Nash *et al.*, 1988) strongly suggests a G1 function for CLN3 and not a role in mitosis.

The most important characterization of CLN3$^+$ or CLN3-1 involves their potential interaction with Cdc28. cdc13 the mitotic cyclin of *S. pombe* appears in a complex with cdc2 prior to and at mitosis (Draetta *et al.*, 1988; Booher *et al.*, 1989). cdc13 is associated with maximal activity of the cdc2 kinase and has been shown to play a role in the substrate specificity of the cdc2 kinase. cdc2 alone phosphorylates casein and not Histone H1, but will phosphorylate Histone H1 in the presence of cdc13 (Draetta *et al.*, 1988; Brizuela *et al.*, 1989; Booher *et al.*, 1989). If CLN3$^+$ worked with Cdc28, similar to the cdc2-cdc13 complex of *S. pombe*, it seems that the time where CLN3$^+$ would be the most abundant would occur at the control point Start in G1, where the major Cdc28 function is found. CLN3$^+$ may help to regulate the substrate specificity of Cdc28.

There is some evidence that the sea urchin (Pines and Hunt, 1987), clam A cyclin (Swenson *et al.*, 1986) and cdc13 (Booher *et al.*, 1989) are modified by phosphorylation. In the sea urchin and clam, the cyclins are modified just prior to mitosis as indicated by a decrease in the migration rate in one dimensional gels. This change may be due to phosphorylation, since *in vitro*, clam A cyclin when mixed with sea urchin extracts migrates more slowly (reviewed by Minshull *et al.* 1989). Immunoprecipitates containing the cdc2 kinase complex (cdc2, suc1 and cdc13) from *S. pombe* were shown to phosphorylate the cdc13 product *in vitro* (Booher *et al.*, 1989). CLN3$^+$ is also post-translationally modified by phosphorylation (M. Tyers, personal communication). It would be interesting to investigate the importance of this phosphorylation in regard to the Cdc28 protein kinase with some aspect of controlling the cell cycle.

5.5 Control of the Expression of *CLN3*$^+$/*CLN3-1* using the *GAL1* Promoter

Control of the expression of the *CLN3*$^+$ and *CLN3-1* genes would be useful for a variety of experiments. Over-expression of the genes, lack of expression and the temporal expression of the genes at various times during the cell cycle are experiments of interest. Therefore, both the *CLN3*$^+$ and *CLN3-1* genes were placed downstream of the *GAL1* promoter in the high copy number plasmid pCGS110 creating pGAL1RD84 and pGAL1BKT$_4$ respectively. Expression of *CLN3*$^+$ or *CLN3-1* can be controlled depending on the carbon source presented to the cells. Cells grown in the presence of glucose will turn off the *GAL1* promoter and expression of *CLN3*$^+$ or *CLN3-1* is lost. Conversely, if cells are grown in the presence of galactose in the absence of glucose, the *GAL1* promoter is used and *CLN3*$^+$ or *CLN3-1* is expressed.

Over-expression of either *CLN3*$^+$ or *CLN3-1* resulted in cells that entered the cell cycle at a reduced cell size. These cells were also α-factor resistant. Loss of the

plasmid subsequently yielded cells that were wild-type in size and no longer α-factor resistant. An important observation was that over-expression by a strong promoter on a high copy number allowed *CLN3*$^+$ cells to grow in the presence of α-factor. Nash *et al.* (1988) and Cross (personal communication) using integrated tandem repeats of *CLN3*$^+$ did not obtain any cells that were α-factor resistant. This was attributed to the instability of the wild-type protein. However, this study suggests the when expression of *CLN3*$^+$ is very high, cells can grow in the presence of α-factor, possibly due to large amounts of CLN3$^+$ saturating the proteolytic machinery.

5.6 Interactions with CLN3$^+$ other than Cdc28

What other functions does CLN3$^+$ have? Recent evidence has suggested that CLN3$^+$ may interact with two other proteins which share homology to Cdc28. These two proteins are KSS1 and FUS3 of *S. cerevisiae*. *KSS1* encodes a non-essential protein kinase homologous to the cdc2 and Cdc28 kinases (Courchesne *et al.*, 1989). Cells when exposed to mating pheromones of the opposite type arrest in G1 and prepare for conjugation. Recovery from this G1 block must occur if the cell cycle is to proceed in the absence of cells of the opposite mating-type and this involves the *KSS1* gene product. *sst2* mutations arrest in the presence of very low doses of mating pheromones and remain in the G1 phase. These mutations are thus considered to be supersensitive to pheromone (Chan *et al.*, 1982). Over-expression of *KSS1* in a *sst2* strain (super sensitive to α-factor) suppresses the growth arrest of these cells and in wild-type cells conferred a reduction in sensitivity to pheromone. However this adaptation from α-factor is not possible without a functional *CLN3*$^+$ product. Courchesne *et al.* (1989) have speculated that KSS1 may directly regulate CLN3$^+$ and through the levels of cyclin control the arrest/progression through certain stages of the cell cycle.

FUS3, another Cdc28 homolog, is required for arrest in G1 and for mating (Elion *et al.*, 1990). Mutations were isolated that affected either the G1 arrest point or conjugation. The G1 arrest mutation could be suppressed by a *CLN3* null mutation. These two genes, *KSS1* and *FUS3* interact genetically with *CLN3*$^+$ may involve G1 to S control. This suggests that other protein kinases may interact with cyclins to control different places in the cell cycle. It would be of interest to investigate further the relationship between CLN3$^+$ and KSS1 or FUS3 in hopes of discovering some control mechanism in the G1 to S transition.

5.7 Conclusions

In conclusion, I was unable to isolate either a *cdc25*$^+$ or a *wee1*$^+$ homolog of *S. pombe* in *S. cerevisiae* using DNA cross-hybridization as the method. The approach taken assumed that if homologous genes did exist in *S. cerevisiae* their DNA sequences may be conserved. *S. cerevisiae* does have a *S. pombe cdc25*$^+$ homolog *MIH1*, and potentially a *wee1*$^+$ homolog as well (Russell *et al.*, 1989). Sequence analysis between *MIH1* and *cdc25*$^+$ indicate that there is very little sequence similarity between the two genes at the DNA level. This is the reason for not identifying a *cdc25*$^+$ homolog in *S. cerevisiae* using DNA cross-hybridization. Attention was therefore turned to sequencing and characterizing *CLN3*$^+$ and *CLN3-1*. CLN3$^+$ is the first G1 cyclin to be identified (Nash *et al.*, 1988). Since cyclins are important in the control of mitosis, it seems likely that cyclins (G1 cyclins) play an equally important role at the G1/S control point. CLN3-1 is a hyperactive form of CLN3$^+$ (truncated form of CLN3$^+$) which allows cells to enter a new round of the cell cycle at a reduced cell size and also confers α-factor resistance (Nash *et al.*, 1988). Control of the expression of both *CLN3*$^+$ and *CLN3-1* was made possible by the use of the *GAL1* promoter of *S. cerevisiae*. Both genes were placed downstream of the inducible promoter. *CLN3*$^+$

and *CLN3-1* cells were both α-factor resistant and small upon induction of the *GAL1* promoter. Future studies of *CLN3*$^{+}$ have been discussed.

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