ANALYSIS OF CELL SIZE CONTROL IN THE YEAST *SACCHAROMYCES CEREVISIAE*: ISOLATION AND CHARACTERIZATION OF *WHI3*, A DOSE-DEPENDENT INHIBITOR OF START

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ABSTRACT

Progression through the yeast cell cycle is controlled in the G1 phase at a point called Start. One prerequisite for the completion of Start is growth to a critical cell size. Once critical cell size is obtained and other conditions have been satisfied, cells complete Start and become committed to a full cell cycle. One approach to identifying genes which regulate the cell cycle is to find mutants which confer a small cell size because they proceed through Start prematurely. For example, the *CLN3-1* mutant has precisely these properties and encodes a stable hyperactive G1 cyclin variant that alters the timing of Start through premature activation of the Cdc28 protein kina e.

A tagged Ty1 transposon mutagenesis procedure coupled with size enrichment was used to isolate a novel cell size mutant called *whi3*. The *whi3* mutation is partially dominant and causes cells to commit to cell division at a reduced cell size. *whi3* mutants are still capable of modulating cell size depending on the nutritional conditions. The size effects caused by the *whi3* mutation are multiplicative with those of *CLN3-1*. In addition, the *whi3* mutant is more α-factor resistant than an isogenic wild-type strain and has a slightly reduced mating efficiency. The *whi3 CLN3-1* double mutant is nearly sterile and is largely defective for mating factor induced transcription.

The *WHI3* gene was cloned and was able to complement the size defect of the mutant as well as increase the cell size of a wild-type strain. Extra copies of the gene carried on single copy vectors or integrated into the yeast genome increase cell size in a dose-dependent manner. Thus, *WHI3* may

encode a cellular metric used to measure cell size or mass. Sequence analysis indicates that this 71 kDa protein contains a domain common to a family of RNA binding proteins, the RNP motif. The deduced sequence of the protein is rich in serine residues and contains a small region rich in glutamine residues. Deletion of the *WHI3* gene results in a small cell size phenotype, increased α-factor resistance and reduced mating efficiency, phenotypes shared with the Ty induced mutation. When *WHI3* is placed under the control of the *GAL1* promoter and induced, cells arrest in the G1 phase of the cell cycle. These results suggest that *WHI3* encodes a dose-dependent inhibitor of Start by affecting the critical cell size requirement. It is hoped that these studies will increase our understanding of Start through the identification of molecules which regulate this transition.

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ATP	adenosine triphosphate
BAP	bacterial alkaline phosphatase
β-gal	β-galactosidase
bp	base pair
BSA	bovine serum albumin
can	canavanine
CCB	cell cycle box
CDC	cell division cycle
CHEF	contour clamped homogeneous electric field gel electrophoresis
cen	centromere
Ci	Curie
DAPI	4,6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
DEPC	diethylpyrocarbonate
FITC	fluoroscein isothiocyanate
5-FOA	5-fluoroorotic acid
G1	gap 1
kb	kilobase
kDa	kilodalton
median	estimate of the median
MAP kinase	microtubule associated protein kinase
MTOC	microtubule organizing center

neo	neomycin phosphotransferase
PVP	polyvinylpyrrolidone
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
RNA	ribonucleic acid
U	units
V	volts

Introduction

1.1 Overview of the Yeast Cell Cycle and Life Cycle

The budding yeast *Saccharomyces cerevisiae* is a powerful system for the study of a variety of biological problems. There are numerous reasons for using yeast including: its short generation time, genetic tractability, small genome size, ease of transformation and ability to accept targeted gene replacements. In addition, the life cycle and cell cycle of yeast have been characterized extensively.

The budding yeast *Saccharomyces cerevisiae* can exist in both a haploid and a diploid state. In the presence of adequate nutrients, haploid cells propagate vegetatively by growing to a critical cell size and then committing to DNA replication, nuclear division and cytokinesis. Cell cycle position can be monitored morphologically as a bud is formed concurrent with the initiation of DNA replication and enlarges with cell cycle progression. In the absence of adequate nutrients haploid cells arrest in the G1 phase of the cell cycle and enter stationary phase. Haploid cells exist as one of two mating types *MAT***a** or *MAT*α. The mating process is mediated by mating pheromones which activate a signal transduction pathway leading to cell cycle arrest and cellular differentiation. This is followed by cellular and nuclear fusion to produce a stable diploid. In the diploid state cells are capable of vegetative reproduction when nutritional requirements are satisfied. When starved for nitrogen, diploid cells arrest in the G1 phase of the cell cycle and enter the developmental

pathway leading to sporulation. The sporulation process includes two meiotic divisions converting the cellular DNA content from 2N to 1N followed by encapsulation of the meiotic products into four spores which constitute a tetrad.

1.2 Cell Cycle Control and CDC Mutants

The yeast cell cycle consists of four distinct phases: G1 (gap phase), S (DNA replication), G2 (gap phase) and M (mitosis). During the cell cycle the cellular complement of DNA is replicated and segregated with high fidelity into two cells. Our present understanding of the cell cycle is based on the isolation and characterization of a large number of temperature sensitive mutants (Hartwell *et al.*, 1970). Among this collection of mutants are a subset of temperature sensitive mutants that arrest at specific stages of the cell cycle with a uniform cellular and nuclear morphology, referred to as the terminal arrest phenotype (Hartwell *et al.* 1970). These mutants are known as cell division cycle or cdc mutants as the function of these gene products is required for progression through specific stages of the cell cycle.

These mutants have been subdivided into two groups depending on the number of cell cycles completed at the restrictive temperature. Members of the first group complete several cycles at the non-permissive temperature before they display the terminal arrest phenotype (Hartwell *et al.*, 1973). Mutants in the second group exhibit a first cycle arrest. Cells located before the execution point arrest in the first cycle and cells located after this point arrest in the subsequent cycle (Hartwell *et al.*, 1973).

Reciprocal-shift experiments with *cdc* mutants and chemical

inhibitors and the analysis of double mutants allowed Hartwell and colleagues to order a series of dependent pathways containing interdependent steps (reviewed by Hartwell, 1974; Hartwell *et al.*, 1974; Hartwell, 1976; Pringle and Hartwell, 1981). These include the processes of DNA replication, spindle pole body duplication and bud emergence. Mutants defective in DNA synthesis are still able to undergo both bud emergence and spindle pole body duplication when arrested and mutants in either of the latter pathways are still capable of synthesizing DNA (Pringle and Hartwell, 1981). Thus, in addition to dependent pathways the available evidence suggests the existence of several independent pathways.

1.3 Start

The *cdc28* mutant was isolated in the original collection of cell division cycle mutants. This mutant is unique as it is required for the initiation of several independent pathways. This observation coupled with additional observations suggest the existence of a unique control point termed Start. Start is defined as the control point located in the G1 phase of the cell cycle where nutrient status, growth, and events of the previous cycle are monitored. It is at this point that cells acquire a molecular commitment to cell division (Hartwell *et al.*, 1974). It also defines the point at which mating pheromone arrests cell division in haploids and the point at which diploid cells commit to meiosis and sporulation (Hartwell *et al.*, 1974).

Start mutants are now classified in two groups, called Class I and Class II (Reed, 1980). Class-I mutants arrest at or before the mating factor sensitive step (Start B), continue to grow and are able to conjugate at the block

(eg. *cdc28*). Three additional class-I mutants, *cdc36*, *cdc37*, and *cdc39* were identified in a screen designed to select for conditional mutants that could conjugate at the block (Reed, 1980). The *cdc36*, *cdc39* and an additional class-I mutant *gpa1* (*cdc70*) arrest because of mutational activation of the pheromone response pathway. The Cdc36 and Cdc39 proteins appear to function as negative regulators of the pheromone response pathway while Gpa1 is homologous to the α subunit of mammalian G proteins (Neiman *et al.*, 1990; Barros-Lopes *et al.*, 1990; Jahng *et al.*, 1988). Class-II Start mutants also arrest at or before the mating factor sensitive step (Start B) but are similar to nutritionally starved cells as they are unable to grow and cannot conjugate at the block (eg. *cdc25*, *cdc33* and *cdc35*). The gene products of some of the class-II mutants have been identified and some encode components of the cAMP pathway in yeast. The *CDC35* gene encodes adenylate cyclase while the *CDC25* gene encodes a GTP/GDP exchange factor for the Ras proteins and in general terms is an upstream activator of the RAS-cAMP pathway (Matsumoto *et al.*, 1982; Boutelet *et al.*, 1985; Camonis *et al.*, 1986). Others such as *cdc19* and *cdc33* encode products required for metabolism and protein synthesis respectively (Pringle and Hartwell, 1981; Brenner *et al.*, 1988).

Since the isolation of the original Start mutants other screens have been employed to identify additional Start mutants. These include: *cdc60*, *cdc62*, *cdc63* (*prt1*), *cdc64*, *cdc65* (*dna33*), *cdc67* and *cdc68* (Bedard *et al.*, 1981; Prendergast *et al.*, 1990). *cdc62*, *cdc63* and *cdc68* are class-I Start mutants; *cdc60* and *cdc64* are class-II Start mutants, while *cdc65* and *cdc67* cannot be easily classified as they arrest as large unbudded cells, retain an intermediate level of biosynthetic activity but cannot conjugate to form zygotes at the restrictive temperature (Bedard *et al.*, 1981; Prendergast *et al.*, 1990).

The product of the *CDC63* gene is required for translational initiation and the *CDC68* gene encodes a transcription factor (Hanic-Joyce, *et al.*, 1987; Rowley *et al.*, 1991). *Cdc68* mutants arrest at Start as a consequence of a reduction in cyclin gene expression (Rowley *et al.*, 1991). *cdc63* mutants may arrest at Start because of a deficiency in the translation of the G1 cyclins, essential activators of Start (see Section 1.8) (Cross *et al.*, 1989). In addition to these mutants, temperature sensitive alleles of the *SIT4* protein phosphatase display a class-I start defect and *RPC53*, a subunit of RNA polymerase III displays a large unbudded arrest characteristic of Start mutants (Sutton *et al.*, 1991; Mann *et al.*, 1992).

1.4 Coordination of Growth and Division and the Critical Cell Size Requirement

Many of the *cdc* mutants described above are defective in discontinuous or stage-specific processes. Dividing cells also perform a series of continuous processes. These include RNA synthesis, protein synthesis and the production of energy (Pringle and Hartwell, 1981). The coordination of continuous and discontinuous processes is critical to the survival of the organism. For example, a tight coupling must exist between the processes of growth and division so that successive rounds of DNA synthesis do not exceed the processes of growth and result in the production of abnormally small cells.

Size homeostasis is used to coordinate the processes of growth and division (Johnston *et al.*, 1977a). When progress through the cell division cycle is blocked by incubating *cdc* mutants at the restrictive temperature, most mutants continue to accumulate mass. However, when growth processes are

blocked by nutritional starvation cells complete initiated cell cycles and arrest in the following G1 phase as small unbudded cells. The daughter cells produced under these conditions are abnormally small and are unable to enter into a new cell cycle until nutrients are restored (Johnston *et al.*, 1977a). These observations indicate that growth rather than the completion of a specific event in the cell cycle is rate-limiting for progression (Johnston *et al.*, 1977a).

When heterogeneously sized stationary phase cells are reinoculated into fresh media there is a striking correlation between the size of the cells and the time required for reentry into the cell cycle (Johnston *et al.*, 1977b). This suggests that the completion of an event coincident with or prior to the *cdc28* sensitive step (Start) is dependent on the attainment of a critical cell size (Johnston *et al.*, 1977a). However, once Start is completed cells become committed to completing the full cell cycle and can accomplish this in the absence of further growth (Johnston *et al.*, 1977a; 1977b).

Additional evidence supports the idea that growth to a critical cell size is required for passage through Start. Yeast cells bud asymmetrically and mother cells are larger than daughter cells. Consistent with this proposal, daughter cells have a longer unbudded interval (G1) than their larger mothers, while both cell types spend a similar length of time in the budded interval of the cell cycle (Hartwell and Unger, 1977; Carter and Jagadish, 1978; Lord and Wheals, 1981; Brewer *et al.*, 1984). Cells starved for nitrogen arrest at Start as small unbudded cells. When nutritional limitation is relieved by placing cells into fresh medium of varied carbon source, the small cells bud after an interval of time that is inversely related to volume (Johnston *et al.*, 1979). Small unbudded cells obtained by elutriation bud after they reach a volume that is characteristic of the strain and the growth condition (Tyers *et al.*, 1993). The

critical cell size requirement is also nutrient modulated. Cells shifted from poor to rich media have an increased size requirement which is satisfied prior to Start in the unbudded interval of the cell cycle (Johnston *et al.*, 1979; Jagadish and Carter, 1977; Lorincz and Carter, 1979; 1983).

1.5 Molecular Models of Size Control

Numerous molecular models have been proposed to explain how critical cell size is monitored and tethered to Start. These models propose that the accumulation of specific molecules directly influences the ability of cells to transit Start. The accumulation of ribosomal RNA has been proposed to play such a role because ethionine, a methionine analog, causes G1 arrest by affecting rRNA levels (Singer *et al.*, 1978). Treating cells with chelating agents causes similar effects on rRNA accumulation and results in G1 arrest (Johnston and Singer, 1978). However, it has been argued that since these inhibitors also inhibit mRNA synthesis, the G1 arrest may result from a decrease in the synthesis of proteins made from newly synthesized mRNAs at Start (Popolo *et al.*, 1982). In addition, when cells are treated with low doses of cycloheximide, the rate of protein synthesis is reduced while rRNA synthesis is unaffected yet the completion of Start is delayed (Popolo *et al.*, 1982).

Protein synthesis may play an important role by affecting the accumulation of a critical signalling molecule. To investigate the requirement for a threshold protein synthetic capacity various steps in the sulfate assimilation pathway have been perturbed (Unger and Hartwell, 1976). Single perturbations such as methionine starvation or the use of a strain with a temperature sensitive methionyl-tRNA synthetase mutation reduce this

capacity and result in G1 arrest (Unger and Hartwell, 1976). Combined perturbations result in non-specific arrest as cells are no longer able to complete cycles which have been initiated (Unger and Hartwell, 1976). Therefore, a threshold protein synthetic capacity is specifically required for passage through Start.

Similar conclusions concerning the requirement for a critical protein content or a critical rate of protein synthesis for the completion of Start have been reached by a number of groups. In these studies limiting concentrations of cycloheximide alone or in combination with other block-release techniques have demonstrated a protein synthetic requirement for the completion of Start (Hartwell and Unger, 1977; Shilo *et al.*, 1979; Popolo *et al.*, 1982; Moore, 1988). Cells treated with α-factor can be divided into two subpopulations, fast and slow budding cells (Moore, 1988). Cycloheximide treatment can be used to convert fast budding cells into cells with a slow rate of bud emergence (Moore, 1988). This suggests that there is a requirement for a critical rate of protein synthesis (Moore, 1988). Even though fast budding cells have achieved this critical rate of protein synthesis they still require additional protein synthesis (Moore, 1988). This suggests that the synthesis of a specific and labile protein is essential for the completion of Start and α-factor treatment inactivates this protein or prevents its synthesis (Moore, 1988). The requirement for protein synthesis 4-13 minutes prior to Start, as defined by the α-factor sensitive step, has been termed the last synthetic prestart step. A labile protein with an estimated half life of about 6 minutes may be required for passage through Start and the period of time required for its synthesis probably defines the last synthetic prestart step (Hartwell and Unger, 1977; Shilo *et al.*, 1979; Ko and Moore, 1990).

1.6 Cell Size Mutants

To identify specific signalling molecules that might be important for the timing and activation of Start, investigators identified mutants which were defective in these controls. *whi1-1* was originally isolated in a screen designed to identify small cell size mutants altered in the timing of Start (Carter and Sudbery, 1980). The screen involved the size separation of mutagenized cells. Fractions containing small cells were reinoculated into fresh media until they had reached a median cell volume of 25 μm^3 at which time α-factor was added. After a six hour incubation cells were separated using zonal centrifugation to enrich for small cell size mutants (Carter and Sudbery, 1980). Wild-type cells were expected to arrest in the first cycle as they were α-factor sensitive until reaching a median cell volume of 38 μm^3. However, small cell size mutants would escape first cycle arrest as they had achieved the critical cell size required to complete Start. These cells were expected to arrest in the second cell cycle (Carter and Sudbery, 1980). It was further reasoned that schmoos formed by these mutants would be smaller and therefore separated more efficiently by centrifugation. The semi-dominant small cell size mutant *whi1-1* was isolated with this selection scheme (Carter and Sudbery, 1980). A second allele, *DAF1-1*, was isolated in a screen designed to identify dominant α-factor resistant mutants. It was reasoned that dominant mutants might encode targets of the division arrest signal generated by pheromone or might act to alter cell cycle regulation so that the arrest would be bypassed (Cross, 1988). The *whi1-1* and *DAF1-1* mutations have been renamed *CLN3-1* and *CLN3-2*.

The properties of the *CLN3-1* and *CLN3-2* mutants are consistent with those expected of an activator of Start. The mutants have a cell volume

that is about 60% that of wild-type cells (Carter and Sudbery, 1980; Sudbery *et al.*, 1980; Cross, 1988). The small volume of the mutant is correlated with a corresponding reduction in both protein and RNA content (Carter and Sudbery, 1980; Cross, 1988). In addition, mutants have a shorter G1 phase than wild-type cells (Cross, 1988; Nash *et al.*, 1988). These properties suggest that the Cln3 protein performs an integral role in the coordination of growth and division by influencing the cell size and hence the timing of the commitment event (Cross, 1988; Nash *et al.*, 1988). Extra copies of the wild-type gene reduce cell size in a dose-dependent manner and extra copies of the mutant allele almost completely eliminate the G1 phase (Cross, 1988; Nash *et al.*, 1988). Deleting *CLN3* increases both cell size and the length of the G1 phase (Cross, 1988; Nash *et al.*, 1988). The cell size and length of G1 are proportional to the dose of *CLN3* indicating that *CLN3* may encode a cellular metric used to measure mass or volume (Nash *et al.*, 1988). The properties of the mutant gene are consistent with a hyperactive but similar function as the wild-type gene. In addition, both the *CLN3-1* and *CLN3-2* mutants are α-factor resistant yet both display a normal mating factor transcriptional response (Cross, 1988; Nash *et al.*, 1988). This suggests that Cln3 performs an important role in recovery and that the mutant has a hyperactive recovery response (Nash *et al.*, 1988).

The small cell size mutant *whi2* was identified as a result of a spontaneous mutation. Exponentially growing *whi2* mutants have a critical cell size similar to wild-type cells. As *whi2* mutants enter late log phase and approach stationary phase cell size decreases (Sudbery *et al.*, 1980). In addition to the small cell size phenotype, *whi2* mutants do not arrest in G1 upon entry into stationary phase but arrest randomly in the cell cycle (Sudbery *et al.*, 1980). These mutants arrest with a phase dark appearance

characteristic of actively dividing cells and with a final cell density that is higher than that of wild-type cells (Sudbery *et al.*, 1980). Furthermore, *whi2* mutants do not adopt normal stationary phase characteristics. They do not become heat shock resistant, do not accumulate glycogen and do not become resistant to cell wall degrading enzymes (Saul *et al.*, 1985). Additional evidence supports the proposal that the Whi2 protein may function as a negative regulator of catabolite repression (Mountain and Sudbery, 1990).

The *RCS1* gene has also been implicated in the control of critical cell size (Gil *et al.*, 1991). Strains disrupted for *RCS1* are twice the size of control strains and initiate buds at a cell volume which is on average twice that of wild-type strains (Gil *et al.*, 1991). Construction of the appropriate diploids suggests that the cell size phenotype of the disruption is dominant. Transformation of a haploid with centromeric plasmids containing the disrupted or wild-type alleles confirms this result (Gil *et al.*, 1991). Multiple copies of the wild-type *RCS1* gene cause a 16% decrease in mean cell volume (Gil *et al.*, 1991). The *RCS1* gene product may therefore function as a positive cell cycle regulator by acting at the G1/S phase transition (Gil *et al.*, 1991).

Components of the RAS/cAMP pathway have also been implicated in size control. The gene products of two of the class-I Start mutants, *cdc25* (GTP/GDP exchange factor for RAS proteins) and *cdc35* (adenylate cyclase) are known to function as components of the RAS-cAMP pathway (reviewed in Pringle and Hartwell, 1981; Broach, 1991). These mutants arrest with low cAMP levels and phenotypically resemble nutritionally starved cells (Camonis *et al.*, 1986; Pringle and Hartwell, 1981; Broach, 1991). Mutations which activate the pathway like $RAS2^{Val\text{-}19}$ and *bcy1-1* do not arrest properly under conditions of nutrient starvation and have elevated cAMP levels (Matsumoto *et al.*, 1983,

Toda *et al.*, 1987a). At the permissive temperature *cdc25-1* mutants have a longer G1 phase and a smaller critical cell size requirement. (Baroni *et al.*, 1989). The *cdc25-1* size effect is more evident in rich media. The activated $RAS2^{Val-19}$ allele suppresses the *cdc25-1* induced increase in the length of the G1 phase and also increases the critical cell size requirement in both rich and poor media (Baroni *et al.*, 1989).

To test the effects of cAMP on the critical cell size and the timing of Start a cAMP-permeable strain (*rca1*) has been used (Baroni *et al.*, 1992). Raising the intracellular level of cAMP increases the critical cell size requirement and delays the G1/S transition for small starved cells (Baroni *et al.*, 1992). There is less of an effect on large fast growing cells (Baroni *et al.*, 1992). A reciprocal shift positions this inhibitory effect coincident with or just prior to Start I (Start B) (Baroni *et al.*, 1992). It is argued that these results are not at odds with defects in the RAS/cAMP pathway which lower cAMP levels (Baroni *et al.*, 1992). In this case the growth lesion affects protein synthesis and blocks cells at Start II (Start A) (Baroni *et al.*, 1992).

Accumulating evidence suggests that the effects of the RAS/cAMP pathway on the critical cell size requirement may involve Cdc28 and the G1 cyclins. When glucose is added to cultures of yeast cells there is a reduction in the levels of the G1 cyclin proteins (G. Tokiwa, personal communication). This effect may be mediated through cAMP as glucose addition is known to cause a spike in intracellular cAMP levels. The critical cell size requirement also increases when cells are shifted to rich carbon sources. This nutritional modulation of critical cell size may therefore be mediated through the RAS/cAMP pathway. Furthermore, when a strain with a galactose-inducible TPK1 gene (TPK1 encodes one of the three cAMP-dependent protein kinases) is

induced with galactose there is a reduction in the levels of the G1 cyclins and the kinase activity associated with the Cdc28/G1 cyclin complexes (G. Tokiwa, personal communication). When *cdc25* mutants are shifted to the non-permissive temperature there is a decrease in the intracellular levels of cAMP (Camonis *et al.*, 1986). When *cdc25* mutants are incubated at the restrictive temperature the levels of the *CLN1* and *CLN2* G1 cyclin transcripts remain elevated (Fernandez-Sarabia *et al.*, 1992). Lowering the cAMP levels should lower the critical cell size and may do so by increasing the levels of the G1 cyclins directly or may do so through the positive feedback loop (see Section 1.9). To summarize, these results suggest that nutritional modulation of the critical cell size requirement may be mediated through the RAS-cAMP pathway.

The isolation of cell size mutants has proven to be a useful means of identifying components which regulate the commitment event. The study of mutants such as *CLN3-1* has increased our understanding of the molecular requirements needed for Start. The properties of this mutant and the characterization of the wild-type gene have provided useful information regarding the regulation of Start. These studies identified Cln3 as a G1 cyclin which functions as a dose-dependent activator of Start. The stability and hyperactive properties of the mutant protein have also facilitated studies that identified this G1 cyclin as an activator of the Cdc28 protein kinase. Such studies may not have been possible without the *CLN3-1* mutant because of the low abundance, instability and weak kinase activity associated with the wild-type Cln3 protein (see Section 1.10). Therefore, the isolation and characterization of cell size mutants is a proven and tested approach for identifying and analyzing the regulatory molecules which control the G1/S phase transition.

1.7 The Cdc28 Protein Kinase Performs a Dual Role during the Cell Cycle

The importance of Cdc28 in the yeast cell cycle was first recognized with the observation that temperature sensitive alleles arrested at a point coincident with the α-factor arrest point or Start (Hartwell, 1970; Hartwell, 1973). The gene was cloned and sequence analysis indicated homology with protein kinases of the *cdc2* family (Lorincz and Reed, 1984; Hindley and Phear, 1984). Antibodies which recognized the Cdc28 protein were produced and an *in vitro* kinase activity was demonstrated (Reed *et al.*, 1985). The phenotype of a novel allele of *CDC28* suggested that the action of this protein kinase might be required at two points in the yeast cell cycle. The *cdc28-1N* allele was unique in that cells arrested in G2 and were defective in nuclear migration (Piggott *et al.*, 1982). Alleles of *CDC28* with a Start defect were discovered to have a cryptic nuclear division defect. This defect was uncovered when cells were shifted to the restrictive temperature just after the completion of Start using an α-factor block-release (Piggott *et al.*, 1982). This division defect might normally be masked due to its temporal proximity to the Start defect (Piggott *et al.*, 1982).

Mitotic cyclins have been identified in other eukaryotes as components of maturation promotion factor, an activity that induces mitosis. The mitotic cyclins oscillate in abundance and through association with the Cdc2 protein kinase promote the entry into mitosis (reviewed by Murray and Kirschner, 1989; Nurse, 1990). The conservation in this control suggested that yeast should also contain mitotic cyclins. High copy number suppressors of the *cdc28-1N* allele and the polymerase chain reaction were used to identify four yeast B-type cyclins (CLBs) (Surana *et al.*, 1991). The terminal phenotypes

of CLB deletions, the arrest of the *cdc28-1N* allele and the results of conditional CLB expression studies support a role for Clb3 and Clb4 in the promotion of spindle formation and a role for Clb1 and Clb2 in the promotion of spindle elongation (Fitch *et al.*, 1992; Richardson *et al.*, 1992). An association between the Clb1 protein and Cdc28 has been described and this complex has an associated kinase activity suggesting that these cyclins may associate with and activate Cdc28 (Ghiara *et al.*, 1991).

1.8 The Identification of G1 Cyclins

The isolation of dosage-dependent suppressors of a temperature sensitive allele of *CDC28* and studies of the *CLN3-1* and *CLN3-2* mutants have been instrumental in furthering our understand of the role of the Cdc28 protein kinase at Start. These studies have resulted in the identification of three genetically redundant G1 cyclins that function as specific regulators of the Cdc28 protein kinase and hence the G1/S phase transition. In addition, dominant mutations in two of these cyclins advance cells through the cell cycle (shorten G1) by reducing the critical cell size. This indicates that the G1 cyclins coordinate the processes of growth and division by controlling the cell size at which Start is executed.

The properties of the *CLN3-1* (*WHI1-1/DAF1-1*) mutant and gene dosage studies have demonstrated that the Cln3 functions as an activator of Start and as a cellular metric controlling the timing of Start. As previously mentioned, the *CLN3-1* mutation makes cells small, α-factor resistant and reduces the length of time that cells spend in the G1 phase of the cell cycle (Sudbery and Carter, 1980; Cross, 1988; Nash *et al.*, 1988). Extra copies of the

hyperactive *CLN3-1* allele almost eliminates the G1 phase indicating that Cln3 affects the timing of Start (Cross, 1988; Nash *et al.*, 1988). Increasing the copy number of *CLN3* reduces cell volume and deletion increases cell volume suggesting that the gene product acts as a dose-dependent activator of Start (Cross, 1988; Nash *et al.*, 1988). When *CLN3-1* cells are grown in low doses of cycloheximide or in poor carbon sources the proportion of cells in the G1 phase of the cell cycle increases (Cross, 1989; Nash *et al.*, 1988). *CLN3-1* therefore relaxes but does not eliminate the G1 cell size control (Nash *et al.*, 1988; Cross, 1989).

Although *CLN3-1* strains are α-factor resistant they display a transient cell cycle arrest and induce the α-factor transcriptional response (Cross, 1988; Nash *et al.*, 1988). The *CLN3-1* mutant suppresses the division arrest of an *SCG1* disruption, which arrests because of constitutive activation of the mating pathway (Cross, 1988). The *cdc28-13* mutation partially suppresses the α-factor resistance of *CLN3-1* (Cross, 1989). These results suggest that the Cln3 gene product functions in the α-factor recovery process. The hyperactive mutant may be more efficient but still requires an active Cdc28 protein (Cross, 1988; Nash *et al.*, 1988).

The sequence of Cln3 displays weak but significant homology with the mitotic cyclins (Nash *et al.*, 1988). The degree of homology, the effects of the mutation and dosage studies suggest that Cln3 may represent the first identified member of a novel class of cyclins that act to promote the G1/S phase transition (Nash *et al.*, 1988). A second feature of interest is a PEST rich region in the C-terminal third of the wild-type protein (Nash *et al.*, 1988). PEST (proline, glutamate, serine, threonine and aspartate) rich regions exist in a number of short lived proteins and may target these proteins for degradation

(Rogers *et al.*, 1986). The activating mutations in *CLN3-1* and *CLN3-2* are stop codons that prematurely terminate the protein resulting in the loss of the PEST regions (Cross, 1988; Nash *et al.*, 1988). This would explain the hyperactive phenotype of the mutant as the mutation might increase the stability of the protein (Nash *et al.*, 1988).

A search for dosage dependent suppressors of the temperature sensitive defect of the *cdc28-4* allele identified two additional G1 cyclins called *CLN1* and *CLN2* (Reed *et al.*, 1989; Hadwiger *et al.*, 1989b). These cyclins are 75% identical with each other but only 20-25% identical with *CLN3* (Hadwiger *et al.*, 1989b). The phenotype of a dominant truncated allele suggests that Cln2 is a rate-limiting activator of Start (Hadwiger *et al.*, 1989b). Strains containing the *CLN2-1* allele have a small cell size, a short G1 phase and are more α-factor resistant than wild-type strains (Hadwiger *et al.*, 1989b). In addition, while wild-type cells arrest in G1 when limited for nitrogen or when entering stationary phase, *CLN2-1* mutants arrest heterogeneously (Hadwiger *et al.*, 1989b). This suggests that Cln2 is rate-limiting for commitment to cell division. The mutant has properties consistent with an inability to respond to environmental conditions that normally arrest cells in G1.

Disruption of either *CLN1* or *CLN2* has no discernible phenotype. Disruption of both *CLN1* and *CLN2* results in slow growth, a G1 delay and an increase in cell size (Hadwiger *et al.*, 1989b). The elimination of the three *CLN* genes is a lethal event and indicates that the three G1 cyclins are genetically redundant (Richardson *et al.*, 1989). This is expected as the elimination of rate-limiting activators of Start should cause a G1 arrest. Strains deleted for *CLN1*, *CLN2* and *CLN3* but kept alive on galactose with a plasmid borne copy of *CLN1* or *CLN3* under the transcriptional control of the *GAL1* promoter have

been used to determine the specificity of the essential function (Richardson *et al.*, 1989; Cross, 1990). When these strains are switched from galactose to glucose, transcription of the plasmid borne cyclin is repressed. Cells display a first cycle G1 arrest with growth processes unaffected (Richardson *et al.*, 1989; Cross, 1990). This indicates that the essential function of the G1 cyclins is required for passage through the G1 phase of the cell cycle. When the *CLN3-2* coding sequences are placed under *GAL* control in the triple deletion strain multiple cell cycles are completed before cells arrest in G1 (Cross, 1990). This supports the idea that the C-terminal third of *CLN3* confers instability to the protein (Cross, 1990).

Three additional cyclin homologs have recently been identified and may function in the G1 to S phase transition. The *HCS26* gene is a high copy suppressor of an allele of *SWI4*, a transcription factor needed for the cell cycle specific oscillation of G1 cyclin transcripts (see Section 1.9) (Ogas *et al.*, 1991). *ORFD*, a previously identified open-reading frame is homologous to *HCS26* (Frolich *et al.*, 1991; T. Hunt, personal communication). The *CLB5* gene is a suppressor of the lethality of a *cln1 cln2 cln3* strain (Epstein and Cross, 1992). The *CLB5* transcript peaks in G1 coincident with the peak of *CLN2* transcription (Epstein and Cross, 1992). It is also required in conjunction with *CLN3* for viability on glycerol in strains which are deleted for *CLN1* and *CLN2* (Epstein and Cross, 1992).

1.9 A Positive Feedback Loop Controls G1 Cyclin Accumulation

A considerable amount of evidence supports the proposal that a positive feedback loop exists to amplify a small amount of Cln associated

kinase activity and ensure a rapid commitment into the cell cycle. The original proposal is based on observations regarding the transcriptional regulation of the G1 cyclins (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). *CLN1* and *CLN2* mRNA levels are cell cycle regulated and peak in G1 (Wittenberg *et al.*, 1990). Both *CLN1* and *CLN2* mRNA levels decrease in response to α-factor treatment (Wittenberg *et al.*, 1990). In contrast, the *CLN3* mRNA is not cell cycle regulated and is actually induced two to three fold by α-factor treatment (Nash *et al.*, 1988; Wittenberg *et al.*, 1990). When *CLN3-1* or *CLN3-2* mutant strains are treated with α-factor, *CLN1* and *CLN2* transcription is no longer turned off (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). A feedback loop may therefore positively regulate *CLN1* and *CLN2* expression at Start (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). When a strain surviving on *GAL-CLN3* alone is shifted to glucose cells arrest in G1 and there is a drop in the mRNA levels of a non-functional *cln2* reporter construct (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). When galactose is added back to Cln deficient G1 arrested cells, *CLN3* is induced and an increase in the level of a *cln2* reporter transcript is observed (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). The Cln dependent positive feedback loop requires a functional Cdc28 protein kinase and is α-factor sensitive (Cross and Tinkelenberg, 1991). Any functional G1 cyclin in combination with Cdc28 can activate the expression of *CLN1* and *CLN2* (Cross and Tinkelenberg, 1991). This explains the strong transcriptional periodicity associated with *CLN1* and *CLN2* expression and provides a mechanism for rapid signal amplification and transit through Start (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991).

Two transcription factors, encoded by the *SWI4* and *SWI6* genes, are

active components of this feedback loop. The cell cycle dependent increase in *CLN1* and *CLN2* mRNAs requires the Swi4 and Swi6 transcription factors (Nasmyth and Dirick, 1991). In contrast, the expression of *CLN3* is independent of Swi4 and Swi6 (Nasmyth and Dirick, 1991). Strains deleted for *SWI6* and conditional for Swi4 function (*swi4-29*^{ts} allele) arrest at the restrictive temperature in the G1 phase as large unbudded cells with a single nucleus (Nasmyth and Dirick, 1991). When *CLN2* is expressed from the constitutive *S. pombe ADH* promoter the conditional lethality is suppressed (Nasmyth and Dirick, 1991). The lethality therefore results from a lack of CLN expression. Genetic interactions suggest that *CLN1* is dependent on both Swi4 and Swi6 while *CLN2* is dependent on Swi4 (Nasmyth and Dirick, 1991). In addition, genetic interactions suggest that Cln3 function is dependent on Swi4 and Swi6 (Nasmyth and Dirick, 1991). When *swi4* mutants are grown under non-permissive conditions the transcription of all of these genes is reduced (Ogas *et al.*, 1991). In addition, *CLN1*, *CLN2* and *HCS26* are able to suppress this conditional *swi4* growth defect (Ogas *et al.*, 1991). All of these genes contain upstream sequences resembling the cell cycle box (CCB) sequence (CACGAAAA), an element identified upsteam of the HO endonuclease gene to which the Swi4 protein binds (Andrews and Herskowitz, 1989). This combined evidence implicates the Swi4 and Swi6 transcription factors in the positive feedback loop.

Swi4 and Swi6 are both components of the cell cycle box factor and bind *in vitro* to *CLN2* and *HCS26* promoter fragments containing the CCB sequence (Nasmyth and Dirick, 1991; Ogas *et al.*, 1991). Swi4 alone is capable of binding these promoter sequences via its N-terminus and binds to Swi6 via its C-terminus (Primig *et al.*, 1992). When Swi6 binds to Swi4 it may expose

the DNA binding domain of Swi4 or increase its affinity for CCB sequences (Sidorova and Breeden, 1993). In *swi6* mutants the periodicity of G1 cyclin transcription is greatly diminished implicating Swi6 as the target of cell cycle specific regulation (Dirick *et al.*, 1992). This may occur through the phosphorylation of Swi6, possibly by Cdc28, converting Swi6 from a repressor to an activator at Start (Dirick *et al.*, 1992).

The *SIT4* protein phosphatase has also been implicated in the accumulation of *CLN1*, *CLN2*, and *HCS26* mRNAs in late G1 phase. The *sit4-102*^{ts} allele arrests at the restrictive temperature with low levels of *CLN1*, *CLN2* and *HCS26* mRNAs. Genetic interactions suggest that *SIT4* may be acting through *SWI4* to effect the accumulation of the G1 cyclins, *CLN1* and *CLN2* and *HCS26* (Fernandez-Sarabia, 1992). The specific target of the *SIT4* phosphatase in the feedback loop has yet to be clearly established.

1.10 The Cdc28/G1 Cyclin Complexes

Early biochemical studies suggested that the active form of the Cdc28 protein kinase existed in a 160 kDa complex (Wittenberg and Reed, 1988). The activity of Cdc28 was found to coincide with the appearance of this complex and led to the proposal that the activity of the kinase was cell cycle regulated by complex assembly and disassembly (Wittenberg and Reed, 1988). The 160 kDa complex was detected using gel filtration chromatography and was considered to be the active form of the CDC28 kinase as these fractions were capable of phosphorylating an endogenous substrate of 40 kDa, and histone H1, an exogenous substrate (Wittenberg and Reed, 1988). This complex was absent in stationary phase cells and in α-factor arrested cells

(Wittenberg and Reed, 1988).

The development of reagents that specifically recognize the G1 cyclins has facilitated studies aimed at understanding the role of the association between Cdc28 and the G1 cyclins in promoting passage through Start. The Clns have been tagged with three tandem copies of the hemagglutinin HA1 epitope and used to replace the chromosomal copies (Tyers *et al.*, 1991; Tyers *et al.*, 1992; Tyers *et al.*, 1993). The tagged proteins can be detected with the 12CA5 anti-epitope monoclonal antibody. Tagged versions of Cln3 and Cln3-1 have been expressed under the control of the *GAL1* promoter to overcome problems associated with low abundance. Promoter shut off experiments have been used to compare the half-lives of the Cln3 and Cln3-1 proteins (Tyers *et al.*, 1992). Cln3 is very unstable with a half-life of about 10 minutes and Cln3-1 is very stable with a half-life of about 2 hours (Tyers *et al.*, 1992). The Cln2 protein is also unstable with a half-life of 15 minute or less (Wittenberg *et al.*, 1990). This is in keeping with the idea that the C-terminal PEST rich region is required for the rapid turnover of these proteins. The continuous instability of the Cln3 protein would provide a mechanism for tethering growth with division as this would make Cln3 levels responsive to the metabolic state of the cell (Tyers *et al.*, 1992).

A Cln3 associated kinase activity phosphorylates the exogenous substrate histone H1 and two endogenous substrates, Cln3 and p45 (Tyers *et al.*, 1992). The kinase activity associated with Cln3 is Cdc28 dependent (Tyers *et al.*, 1991; Tyers *et al.*, 1992). The kinase activity of the Cln3-Cdc28 complex is regulated by phosphorylation and proteolysis (Tyers *et al.*, 1991; Tyers *et al.*, 1992). The kinase activity of the complex requires phosphorylation as alkaline or potato acid phosphatase treatment eliminates this activity and inclusion of

phosphatase inhibitors prevents this inactivation (Tyers *et al.*, 1991; Tyers *et al.*, 1992). The kinase activity is elevated in a *cdc34*$^{-}$ background as is the abundance of the phosphorylated form of Cln3 (Tyers *et al.*, 1992). *CDC34* encodes a ubiquitin conjugating enzyme that may target proteins for degradation. The Cdc34 protein may not be working through the PEST rich tail of Cln3 as a similar elevation of kinase activity is found associated with *Cln3-1* mutants in a *cdc34* background (Tyers *et al.*, 1992). It is possible that the active form of Cln3 is the phosphorylated form and this form is specifically targeted for degradation by Cdc34 (Tyers *et al.*, 1992). There may therefore be two independent pathways for Cln3 degradation, one working through the PEST rich tail of Cln3 causing constitutive instability and a second which specifically degrades the active phosphorylated form of Cln3 and requires Cdc34.

A comparative study of the three G1 cyclins has revealed striking differences between these regulatory molecules. As mentioned, sequence comparisons, differences in mRNA expression patterns and the feedback loop independent mode of *CLN3* expression suggests that *CLN3* and the other G1 cyclins are different. Biochemical studies indicate that the Cln3 protein is less abundant than Cln1 or Cln2 and has a much weaker associated kinase activity (Tyers *et al.*, 1991; Tyers *et al.*, 1992). Cln3 kinase activity does not oscillate through the cell cycle and is unaffected by α-factor addition (Tyers *et al.*, 1992). In contrast, both Cln1 and Cln2 histone-H1 kinase activities oscillate quite dramatically through the cell cycle with the peak of activity in the G1 phase (Tyers *et al.*, 1993). In the presence of α-factor both protein levels and associated kinase activities decrease (Tyers *et al.*, 1993).

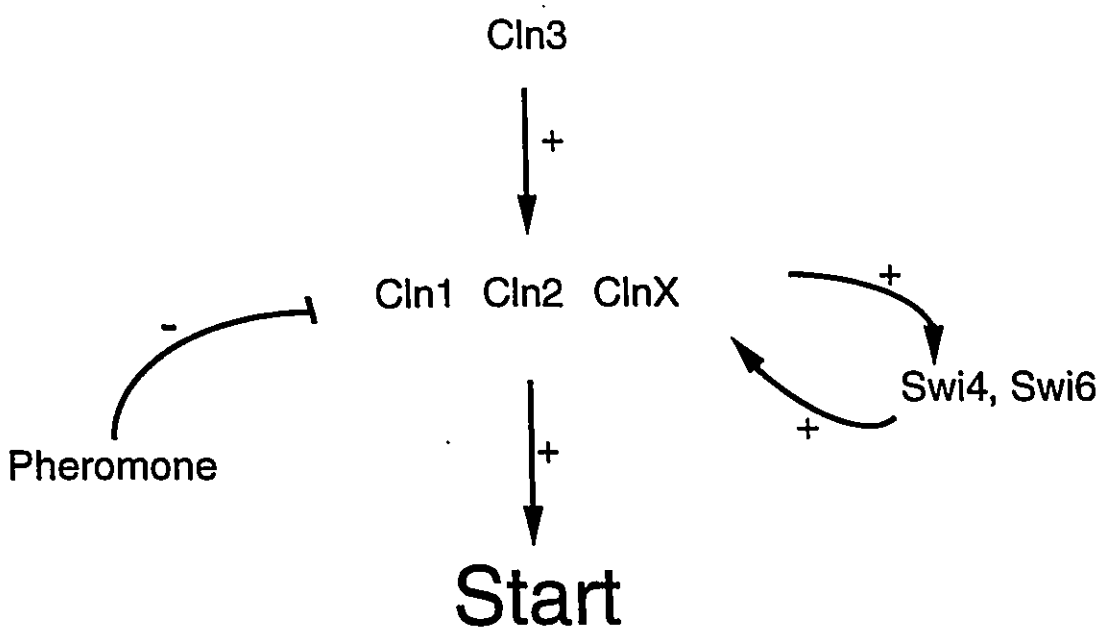
Present evidence favors a position for Cln3 as an upstream activator

of Cln1, Cln2 and the other G1 cyclins (Figure 1.1). The α-factor resistance of *CLN3-1* strains depends on the presence of both *CLN1* and *CLN2* (Tyers *et al.*, 1992). The Cln2 associated kinase activity recovers more rapidly after α-factor treatment in the presence of Cln3-1 (Tyers *et al.*, 1993). A brief induction of *GAL1-CLN3* results in a rapid increase in the expression of *CLN1*, *CLN2*, *HCS26*, *ORFD*, *CLB5* and *SWI4* (Tyers *et al.*, 1993). When a *cln1 cln2 GAL1-CLN3* strain is shifted to raffinose there is a large decrease in the expression of *HCS26*, *ORFD* and *CLB5* mRNAs and a brief pulse of galactose results in a rapid induction of *HCS26*, *ORFD*, *CLB5* and *SWI4* mRNA levels (Tyers *et al.*, 1993). These results and other findings support a model in which Cln3 acts upstream of the other G1 cyclins and provides a low constitutive "pilot light" kinase activity (Figure 1.1) (Tyers *et al.*, 1993). This would activate the feedback loop and lead to an increase in the expression of the downstream cyclins until a critical level of kinase activity results in the completion of Start.

1.11 The Mating Pheromone Response Pathway

The binding of mating pheromone to cell surface receptors activates a signal transduction pathway and leads to cellular differentiation and cell cycle arrest. The differentiation process includes the transcriptional induction of genes required for cellular and nuclear fusion (reviewed by Marsh *et al.*, 1991). The α-factor receptor (Ste2) is coupled to a heterotrimeric G protein. When α-factor binds to the receptor, the stimulated receptor causes the α subunit (Gpa1/Scg1) to switch from the GDP bound to the GTP bound state and dissociate from the Gβγ (Ste4, Ste18) subunits. The free Gβγ subunits activate a protein kinase cascade (Figure 1.2) (reviewed by Marsh *et al.*, 1991).

Figure 1.1. Model for activation of Start. In this model Cln3 activates the downstream cyclins Cln1, Cln2 and ClnX, where ClnX refers to Hsc26, OrfD, Clb5 and perhaps others, which in turn activate Start. Cln3 functions as a pilot light for the other cyclins by activating transcription through Swi4 and Swi6 and possibly also by phosphorylating downstream Cln kinase complexes. This would activate the downstream cyclins and the positive feedback loop. In this model the downstream cyclins may all be inactivated by α-factor and dependent on Swi4 and Swi6 for transcription. Taken directly from Tyers *et al.* (1993).


Cln3
+
Cln1 Cln2 ClnX
+
Swi4, Swi6
+
-
Pheromone
+
Start

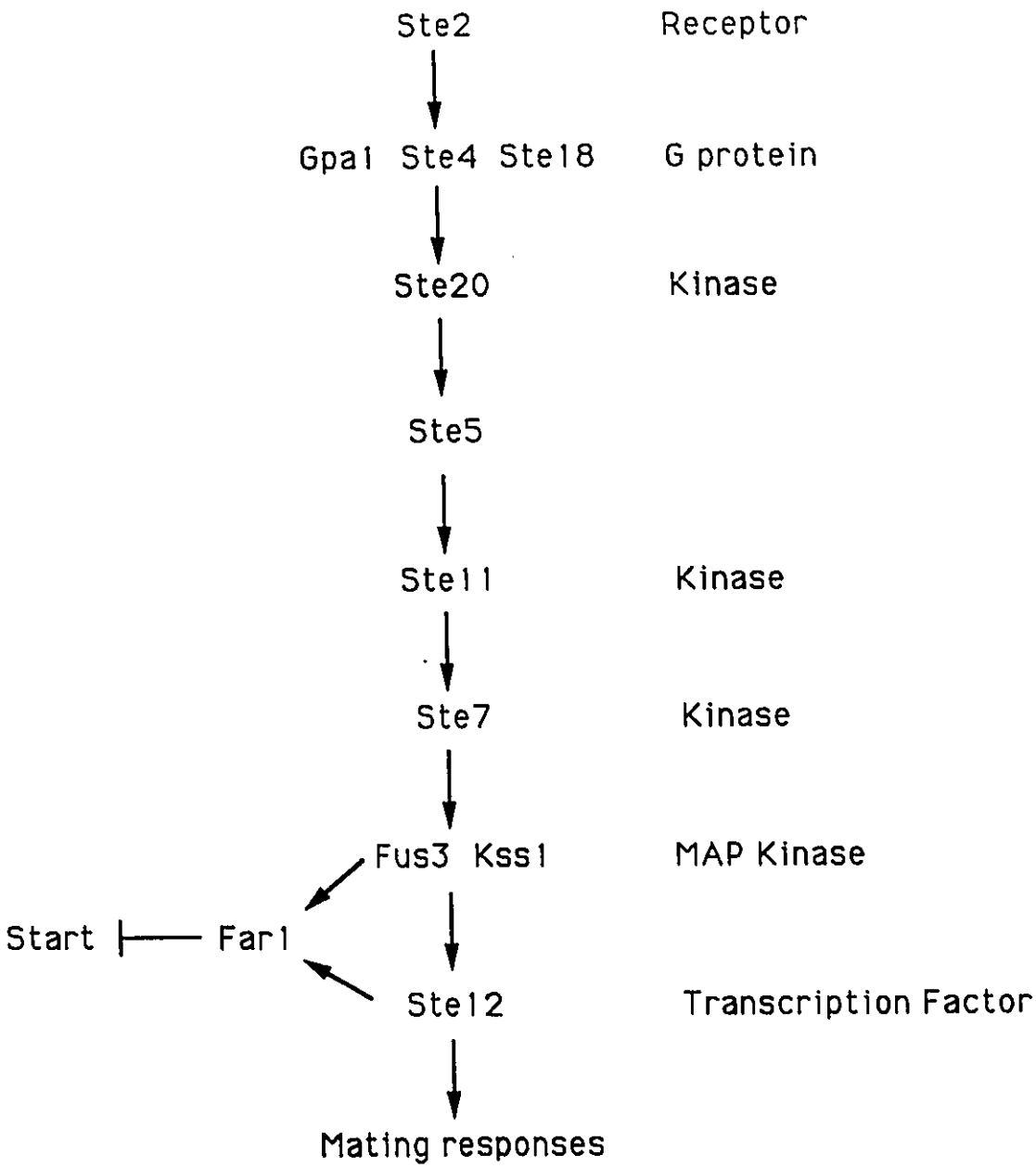
At the bottom of the cascade are the Fus3 and Kss1 MAP kinase homologs. These kinases are functionally redundant for the transcriptional response while Fus3, but not Kss1, is also required for α-factor induced cell cycle arrest (Elion *et al.*, 1991). The final step in the signal transduction pathway involves the binding of the Ste12 transcription factor to the pheromone response element. This factor induces the expression of genes required for: cellular fusion, nuclear fusion, pheromone biosynthesis, the signal transduction pathway and cell cycle arrest (reviewed by Marsh *et al.*, 1991). The ability of Ste12 to activate transcription is increased by phosphorylation and this activation may occur through the action of the Fus3/Kss1 and Ste7 kinases (Figure 1.2) (Song *et al.*, 1991).

In addition to the effects of the signal transduction pathway on the transcriptional response, α-factor treatment also results in cell cycle arrest (Pringle and Hartwell, 1981; Marsh *et al.*, 1991). Recent evidence supports the idea that α-factor induced G1 arrest may result from the inactivation of the G1 cyclins thus preventing the activation of Cdc28. For example, the addition of α-factor results in a large decrease in the levels of *CLN1* and *CLN2* mRNAs, proteins and associated kinase activities (Wittenberg *et al.*, 1990; Tyers *et al.*, 1992). The *CLN3* gene is actually induced 2-3 fold and there are no significant changes in the level of the protein or its associated kinase activity (Nash *et al.*, 1988; Tyers *et al.*, 1992). If Cln3 functions as an upstream activator of the other G1 cyclins its associated kinase activity may be important to initiate recovery but may not be sufficient to trigger Start.

Two negative cell cycle regulators perform an important role in pheromone induced cell cycle arrest. In addition to the redundant role of Fus3 in the α-factor induced transcriptional response, this MAP kinase homolog also

performs an essential function in promoting cell cycle arrest (Elion *et al.*, 1990; Elion *et al.*, 1991). The *fus3-1* and *fus3-2* mutants, originally identified because of a cellular fusion defect, are also defective in α-factor induced G1 arrest (Elion *et al.*, 1990). A *cln3* deletion suppresses the α-factor resistance and G1 arrest defect of *fus3* mutants and suggests that Fus3 may inactivate Cln3 (Elion *et al.*, 1990). A second mutant, *far1*, is defective in α-factor induced G1 arrest but maintains an intact transcriptional response (Figure 1.2) (Chang and Herskowitz, 1990). The *cln2* null allele reverses the *far1* arrest phenotype and implicates Far1 as a specific inhibitor of Cln2 (Chang and Herskowitz, 1990). In the presence of α-factor, Far1 becomes a substrate of the Fus3 protein kinase (Peter *et al.*, 1993). This phosphorylation increases the affinity of Far1 for Cln2 (Peter *et al.*, 1993). An association between Far1 and the Cln1/Cdc28 and Cln3/Cdc28 complexes also occurs upon the addition of α-factor and is dependent on Fus3 phosphorylation (Tyers and Futcher, submitted). The kinetics of the association between Far1 and the Cln3/Cdc28 complex are slower than for the Cln1 and Cln2 complexes and does not have a major effect on the Cln3 associated kinase activity (Tyers and Futcher, submitted). This interaction may therefore be important for recovery rather than for arrest (Tyers and Futcher, submitted). After association with the Cln/Cdc28 complexes, Far1 becomes a substrate for the Cdc28 kinase (Peter *et al.*, 1993; Tyers and Futcher, submitted). The role of this phosphorylation is unknown. Finally, it has been proposed that the association between Far1 and the Cln/Cdc28 complexes may inhibit the kinase activity of Cdc28, prevent the association between kinase and substrate or may induce cyclin degradation (Peter *et al.*, 1993; Tyers and Futcher, submitted).

Figure 1.2. Model for the mating pheromone signal transduction pathway. When pheromone binds to the receptor the Gα subunit dissociates from Gβγ and the Gβγ subunits activate a protein kinase cascade. A protein kinase C homolog (Ste20) may transmit the signal from Gβγ to the Ste5 phosphoprotein (Leberer *et al.*, 1992). The signal is then transmitted to Ste11 which hyperphosphorylates Ste7 (Stevenson *et al.*, 1992; Cairns *et al.*, 1992). Ste7 may directly phosphorylate the Fus3/Kss1 MAP kinases (Gartner *et al.*, 1992; Stevenson *et al.*, 1992). The MAP kinases might then activate the Ste12 transcription factor by phosphorylation. Upon activation, Ste12 induces the expression of a number of genes including Fus3 and Far1 both of which are required for cell cycle arrest (Elion *et al.*, 1990; Chang and Herskowitz, 1990).


Ste2
Receptor
Gpa1 Ste4 Ste18
G protein
Ste20
Kinase
Ste5
Ste11
Kinase
Ste7
Kinase
Fus3 Kss1
MAP Kinase
Start
Far1
Ste12
Transcription Factor
Mating responses

1.12 Scope of This Work

The isolation of cell division cycle mutants has provided us with a basic understanding of how the cell cycle is regulated. The study of a subset of these, the Start mutants, has allowed investigators to gain insight into the components of the Start machinery and the processes that are important for entry into a new cell cycle. To ensure that successive rounds of division do not exceed cellular growth these processes are coordinated in the G1 phase of the cell cycle. This is achieved by requiring that cells attain a critical cell size before completing Start. Cells must therefore monitor growth processes and may do so with cellular metrics or measuring devices. The isolation of small cell size mutants has proven to be a useful method of identifying molecules that regulate Start. For example, *CLN3-1* is a small cell size mutant that completes Start prematurely. The phenotype results from a relaxed G1 size control. The properties of the mutant and dosage studies suggest that the *CLN3* gene encodes an activator of Start and may be a cellular metric. The stability of the mutant protein and its hyperactive properties have also proven to be useful in studies aimed at understanding the function of this protein as an activator of the Cdc28 protein kinase. Such studies would not have been possible without this mutant because of the low abundance, instability and weak kinase activity associated with the wild-type Cln3 protein. The isolation and characterization of additional cell size mutants is a tested means for identifying regulatory molecules that control the G1/S phase transition.

A screen designed to enrich for small cells was used in combination with tagged Ty1 mutagenesis to genetically and physically tag mutations in size control genes. A small cell size mutant called *whi3* was identified with this

screen and the Ty tag allowed us to clone the wild-type gene. The *WHI3* gene was sequenced in the hopes that the primary sequence would provide us with insight into protein function. The phenotype of the mutant and the results of gene-dosage studies suggested that *WHI3* encoded a dose-dependent inhibitor of Start. This fulfilled one of the goals of the work, the isolation of a size control gene which affects the cellular requirements for Start.

In the second part of this thesis various aspects of Whi3 function were analyzed. *WHI3* was overexpressed to determine whether its inhibitory effects were specific to the G1 phase of the cell cycle. In addition, the size phenotype of *whi3 CLN3-1* double mutants were analyzed to determine whether Whi3 and Cln3 were affecting the same or different processes. This also included the analysis of mating responses in single and double mutants since both mutants were more α-factor resistant than wild-type strains. The *WHI3* transcript was also studied to gain insight into its regulation. The *whi3* mutant was crossed to the *cdc* Start mutants to check for genetic interactions. The phenotypes of the *whi3* mutant were considered with respect to the cell size requirements needed for passage through Start.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Critical reagents were obtained from the following sources:

Geneticin (G418 sulfate)	Gibco laboratories
5-Fluoroorotic acid	PCR incorporated, Gainesville, Fl

All other chemicals, reagents and media were of standard laboratory grade and were purchased from the Sigma Chemical Co., Biorad, Aldrich or Baxter.

2.1.2 Radioisotopes

Adenosine 5'-[γ^{32}P]triphosphate (3000 Ci/mmol) and deoxyadenosine 5'-[α^{32}P]triphosphate (3000 Ci/mmol) were obtained from Amersham. Deoxyadenosine 5'-[α-thio][^{35}S] triphosphate (1200 Ci/mmol) was obtained from New England Nuclear.

2.1.3 Enzymes

Restriction enzymes were purchased from Boehringer Mannheim,

Bethesda Research Labs, New England Biolabs or Pharmacia. Klenow was obtained from either Bethesda Research Labs or Boehringer Mannheim. T4 DNA ligase was purchased from New England Biolabs or Bethesda Research Labs. T4 polynucleotide kinase was obtained from Boehringer Mannheim. Bacterial alkaline phosphatase was purchased from IBI. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim. Zymolyase was obtained from Seikagaku Kogyo Co. Ltd., Japan. Glusulase was obtained from Dupont. Agarase was purchased from Sigma. Proteinase K was obtained from Boehringer Mannheim. Sequenase sequencing kits were purchased from U.S. Biochemical.

2.1.4 Cloning and Miscellaneous Vectors

The yeast shuttle vectors used throughout this study were obtained from P. Hieter (Sikorski and Hieter, 1989). The yeast integrating vector, pRS306, contains the yeast selectable marker *URA3*. The yeast centromeric vector, pRS315, contains a CEN/ARS cassette to provide mitotic stability and autonomous replication and the *LEU2* selectable marker. The yeast high copy number vector, pRS425, contains the yeast 2μm circle and the *LEU2* selectable marker. The single strand producing vectors pUC118 and pUC119 were obtained from Dr. J. Viera (Viera and Messing, 1987). The Ty1 mutagenesis vector used in this study, pJEF1105, was obtained from J. Boeke (Boeke *et al.*, 1988). The lambda clone #5025 was obtained from L. Riles. The high copy number yeast genomic library was obtained from S. Cameron and contained *Hind*III partially digested yeast genomic DNA inserted into YEp213 (Toda *et al.*, 1987b). The plasmids pBF30 and pWJ310 contain the *CLN3-1* mutant gene

and the *CLN3::URA3* deletion respectively and have been described previously (Nash *et al.*, 1988). The plasmids and sources of various other fragments used are listed in Table 2.1.

2.1.5 Oligonucleotides

The oligonucleotides listed in Table 2.2 were used as sequencing primers and as probes in hybridizations. They were synthesized by the Cold Spring Harbor Laboratory Oligonucleotide Facility or by Operon Technologies Ltd, Alameda, CA. Strand polarity is indicated by (-) and (+) and indicates complementarity to the non-coding (mRNA) strand and coding (template) strand, respectively. The oligonucleotide WHI306 differs from the wild-type sequence as it is missing a C at position 1221. The oligonucleotide WHI310 differs from the wild-type sequence as it is missing a G at position 1169. The endpoints of the oligos 3'JUNC and 5'JUNC were not listed in the table as they span the junction of the integrated Ty element and the *WHI3* gene at the position of Ty1 insertion. Oligonucleotides were received in a lyophilized state and had been deprotected and desalted after synthesis. They were resuspended in water and quantified by measuring UV absorbance at 260 nm. The concentration was then determined assuming that single-stranded oligonucleotides at a concentration of 33 μg/ml have an absorbance of 1.0 A.U.

2.1.6 Media

All media were commonly prepared in 2 liter flasks and autoclaved for 25-35 minutes at a temperature of 121°C and a pressure of 20 lbs/in^2.

Table 2.1 List of additional plasmids

Plasmid	gene	source
pAJ50	*neo*	R. Rodriguez
TT115	*RAS2*	R. Ballester
pART5	*LEU2*	L. Molz
pMR438	*GAL1*	M. Rose
pYEE74	*FUS3*	E. Elion
pSB202	*FUS1*	C. Devlin
pASP6	*ACT1*	K. Arndt
pJD14	*ADH1*	E. Young
pA1a	*CLN2*	T. Connolly

Table 2.2 List of oligonucleotides

Oligo	strand	sequence	ends	
WHI301	(-)	5'-CTCAAATCTGGCAATGATA-3'	480	462
WHI302	(+)	5'-GGAGCTACAGCCTAAAATT-3'	299	317
WHI303	(+)	5'-GCCAGATTTGAGTTATT-3	469	485
WHI304	(+)	5'-GAGGAAGCGCAAGTGGG-3'	155	171
WHI305	(+)	5'-GTTATCTTCTCAAGGCC-3'	849	865
WHI306	(+)	5'-GGCACTCTCTGCAGCTC-3'	1209	1226
WHI307	(-)	5'-GATGAAATACTATAAGA-3'	-636	-652
WHI308	(+)	5'-CACCTCCTGCCAACCCT-3'	1574	1590
WHI309	(-)	5'-GAGGTAGTAGAGTTATTC-3'	1505	1488
WHI310	(-)	5'-GGAGCTGCGTTTGCATT-3'	1176	1159
WHI311	(-)	5'-GATTACATTGTTGTGGT-3'	823	807
WHI312	(-)	5'-CCAAGGGCAAGTGTGTT-3'	239	223
WHI313	(+)	5'-TATTCCGTCCACATATT-3'	-286	-270
WHI314	(-)	5'-TTGGGATCAGGAACTGA-3'	-948	-964
WHI315	(-)	5'-CCGTGACTATGACCATT-3'	1745	1729
WHI316	(-)	5'-GACCAACATTAGAATTG-3'	1972	1956
WHI317	(+)	5'-GGCAGTGGTAACCCAAA-3'	1912	1928
WHI318	(+)	5'-ACTATTACATTACAACG-3'	2199	2215
WHI319	(-)	5'-GAGATAAAGTATTATCG-3'	2173	2157
WHI320	(-)	5'-CGGTATAGGAGTTCGCA-3'	2321	2305
WHI321	(-)	5'-GAGCGATATTCGATAGT-3'	2435	2419
WHI322	(+)	5'-CTCACTATCGAATATCG-3'	2416	2432
3'JUNC	(-)	5'-TGTTGTTATTGAGAAATG-3'		
5'JUNC	(-)	5'-ATTCCAACAGTTATCGTG-3'		

Solutions which could not be autoclaved were filter sterilized with Nalgene (0.2 μm pore size) filter units. Plates were prepared by cooling media to 50°C and pouring about 25 ml of medium per plate in a laminar flow hood. Plates were allowed to air dry for 1-2 days at room temperature and were then put in bags and stored at 4°C.

i. Yeast Media

Listed below are the common media used throughout the course of this work. Details of the media described and additional information can be found in Rose *et al.* (1990). YEPD was the standard medum used for strain propagation and contains: 10 g of Difco bacto-yeast extract, 20 g of Difco bacto-peptone and 900 mls of deionized water. D-glucose (20g) was dissolved in 100 ml of water, autoclaved separately and added to the other components after autoclaving. When required, sugars such as raffinose or galactose (20 g/l) were substituted for glucose. When preparing plates, 20 g/l of Difco-BiTek agar was added. When required geneticin (G418 sulfate) was added to a final concentration of 0.5 mg/ml.

When defined medium was required for selective purposes YNB minimal medium was used and contains: 6.7 g of Difco bacto-yeast nitrogen base, 0.45 g of sodium phosphate dibasic and deionized water to 900 ml. Glucose (20g) was dissolved in 100 ml water, autoclaved separately and added to the other autoclaved components. Sugars such as raffinose and galactose were substituted as required. When solid medium was desired 20 g/l of Difco BiTek Agar was added. Amino acids and bases were added as required to the final concentrations listed in Table 2.3. If necessary, amino acids were spread

onto the surface of plates from liquid stocks. When required, L-canavanine was added to a final concentration of 30 µg/ml. When selecting for complementation of a specific auxotrophic requirement drop-out medium was used. Drop-out medium was supplemented with all of the common amino acids except for the one being tested or selected. This medium was often made as a powdered stock containing all components except for glucose and agar.

When converting *URA3* prototrophs to *ura3* auxotrophs, defined plates containing the compound 5-fluoroorotic acid (5-FOA) were used. 5-FOA is converted to the toxic compound 5-fluorouracil by the *URA3* gene. This compound is therefore toxic to *URA3*$^+$ strains but not to *ura3*$^-$ strains. To make these selective plates, 5-FOA was added to a final concentration of 1 mg/ml in solid form to an autoclaved precooled solution containing: yeast nitrogen base, sodium phosphate, the amino acids listed in Table 2.3 and 600 ml of water. After completely dissolving the 5-FOA, 50 ml of a sterile 40% D-glucose solution was added. Finally, an autoclaved partially cooled solution containing agar and 350 ml of water was added and mixed gently but completely.

Diploid strains were propagated overnight on GNA, a rich media, before transfering them to sporulation plates. GNA plates contain: 10 g of Difco bacto-yeast extract, 30g of Nutrient broth, 50 g of D-glucose, 20 g of Difco BiTek agar and water to one litre. Sporulation plates contain: 10 g of Potassium Acetate, 1 g of D-glucose, 1.25 g of Difco bacto-yeast extract and water to one litre.

Table 2.3 Concentrations of Amino Acids and Bases

Constituent	Final concentration (µg/ml)
L-alanine	50
L-arginine-HCl	50
L-asparagine	50
L-aspartic acid	50
L-cysteine	50
L-glutamic acid	50
L-glutamine	50
L-glycine	50
L-histidine-HCl	50
L-isoleucine	50
L-leucine	50
L-lysine-HCl	50
L-methionine	50
L-phenylalanine	50
L-proline	50
L-serine	50
L-threonine	50
L-tryptophan	80
L-tyrosine	50
L-valine	50
adenine sulphate	32
inositol	50
uracil	50
p-aminobenzoic acid	5

ii. Bacterial Media

The standard bacterial media used throughout the course of this work are described below. Recipes for additional bacterial media and details of the media described below can be found in Sambrook *et al.* (1989). The standard media used for the propagation of *E.coli* was Luria broth (LB) which contains: 10 g of Difco bacto-tryptone, 5 g of Difco bacto-yeast, 10 g of sodium chloride and water to 1 litre. Solid medium was prepared by adding 20 g/l of Difco BiTek agar prior to autoclaving. When used for the growth of bacteria infected with bacteriophage, medium was supplemented with 10 mM magnesium chloride and 0.2% (w/v) maltose. When selecting for antibiotic resistant strains of bacteria, filter sterilized antibiotics were added after the medium was autoclaved. Ampicillin (Sigma) was added to a final concentration of 100-125 µg/ml. Kanamycin (Sigma) was added to a final concentration of 50-100 µg/ml.

The defined growth medium M9 was used for selective purposes and was made up as a 20X stock solution. The stock solution contains: 120 g of sodium phosphate dibasic, 60 g of potassium phosphate monobasic, 20 g of ammonium chloride, 10 g of sodium chloride and water to 1 litre. This solution was autoclaved and stored refrigerated after adding 2 ml of chloroform to maintain sterility. To make M9 plates, 50 ml of M9 media was added to 400 ml of water. After autoclaving, 15 ml of a 20% glucose solution, 1 ml of a 1% vitamin B1 solution (thiamine), and 1 ml of a 1M magnesium sulphate solution were added. In a separate flask, 16 g of agar and 550 ml of water were combined and autoclaved. After autoclaving 0.1 ml of 1M calcium chloride was added. Finally, these two solutions were combined, mixed and poured. When

required 5 g casamino acids were added. Ampicillin was added to a final concentration of 100 µg/ml. To make M10 plates, 0.6 ml of 0.1M ferric chloride and 1 ml of 1M manganese chloride to the autoclaved 20X M10 stock solution.

SOB was used for the outgrowth of transformed strains and contains 20 g of Difco bacto-tryptone, 5 g of Difco bacto-yeast extract, 584 mg of sodium chloride, 186 mg of potassium chloride and water was added to 1 litre. To make SOC, SOB was supplemented with 10 mM magnesium sulphate, 10 mM magnesium chloride and 20 mM glucose.

Two additional growth media were used for the growth of phage infected bacteria. 2X YT contains: 16 g of Difco bacto-tryptone, 10 g of Difco bacto-yeast extract, 10 g of sodium chloride and water to 1 litre. The pH of the medium was adjusted to 7.5. NZCYM contains : 10 g of NZ amine, 5 g of sodium chloride, 5 g of Bacto yeast-extract, 1 g of casamino acids, 2 g of magnesium sulphate and water to 1 litre. The final pH of the medium was adjusted to 7.5. When plates were required 20 g/l agar was added. For the growth and propagation of strains infected with bacteriophage media was supplemented with 10 mM magnesium chloride and 0.2% (w/v) maltose.

2.1.7 Strains

i. Yeast Strains

The strains and relevant genotypes of the yeast strains are listed in Table 2.4. When appropriate, the source of the strain or the study which describes the strain is included.

Table 2.4 List of Yeast Strains

Strain	Genotype	Source
BF328-2c	*MAT*α *his3 CLN3-1*	a
BF328-4a	*MAT***a** *his3*	a
BF328-4b	*MAT*α *his3*	a
BF328-5a	*MAT***a** *his3 CLN3-1*	a
BF328-6b	*MAT*α *his3 CLN3-1*	a
BWG1-7a	*MAT***a** *ade1 ura3-52 leu2 his4*	b
MDMy256	*MAT***a** *his7 tyr1 met8 cdc28-13*	c
RN100-1a	*MAT***a** *his4 leu2 ura3*	d
RN100-1d	*MAT*α *his3 leu2 whi3 G418*^{R}	d
RN100-3c	*MAT*α *his3 his4 leu2 ura3 whi3 G418*^{R}	d
RN100-3d	*MAT*α*his3* or *his4 leu2*	d
RN101-1d	*MAT*α *his3* or *his4 tyr1 cdc39-1 whi3 G418*^{R}	d
RN101-2b	*MAT***a** *his3* and/or *his4 cdc39-1*	d
RN210-3a	*MAT*α *his4 ade1-100 ura3-52 WHI3::LEU2*	d
RN210-3b	*MAT***a** *his4 his3? WHI3::LEU2*	d
RN210-3c	*MAT*α *his4 his3? ade1-100 ura3-52 leu2*	d
RN210-3d	*MAT***a***his4 his3? leu2*	d
RN210-13d	*MAT***a** *his3 his4 ade1-100 ura3-52 WHI3::LEU2*	d
RN230-3a	*MAT***a** *his3* or *his4 leu2 ura3 whi3 G418*^{R}	d
RN230-3b	*MAT*α *his3* or *his4 leu2 2X WHI3* at *URA3*	d
RN230-3c	*MAT*α *his3* or *his4 leu2*	d
RN230-3d	*MAT***a** *his3* or *his4 leu2 whi3 G418*^{R} *2X WHI3* at *URA3*	d
SR661-2	*MAT***a** *trp1 ura1 tyr1 gal1 cdc36-16*	e
SR665-1	*MAT*α *met2 tyr1 cyh2 gal1 cdc39-1*	e

SR672-1	*MAT***a** *ura1 cyh2 cdc37-1*	e
T25-6	*MAT*α *leu2 ura3 trp1 can1 cdc25-1*	f
T504b-1a	*MAT***a**	d
T504b-1b	*MAT***a** *his3 his4 whi3 G418*^{R}	d
T504b-1c	*MAT*α *leu2 whi3 G418*^{R}	d
T504b-1d	*MAT*α *his3 his4 leu2*	d
T504b-3d	*MAT*α *his4 leu2 whi3 G418*^{R}	d
T504b-5c	*MAT***a** *his3* and/or *his4 leu2 whi3 G418*^{R}	d
T504b-6a	*MAT*α *his4 leu2 whi3 G418*^{R}	d
T654b-4b	*MAT***a** *his3 leu2 CLN3-1 whi3 G418*^{R}	d
T654b-4c	*MAT*α *leu2 CLN3-1*	d
T654b-4d	*MAT***a** *his3 whi3 G418*^{R}	d
YPH80	*MAT***a**/α *ura3-52/ura3-52 lys2-801/lys2-801 ade2-101/ade2-101 trp1-Δ1/trp1-Δ1*	g
YPH49	*MAT*α ura3-52 lys2-801 ade2-101 his7 *trp1-Δ1*	g
YPH149	*MAT*α *ura3-52 lys2-801 ade2-101 his7* (CFVII(*RAD2*.p.YPH149) [CFVII(*RAD2*.d. YPH146.*TRP1*)] ρ$^-$	g
699α	*MAT*α *ade2-1 trp1-1 leu2-3 leu2-112 his3-11 his3-15 ura3 can1-100 GAL pst*$^+$	h

a Nash *et al.*, 1988
b Boeke *et al.*, 1988
c M. Mendenhall
d this study
e yeast genetic stock center
f M. Wigler
g Gerring *et al.*, 1991
h K. Nasmyth

ii. Bacterial Strains

The bacterial strains used in this study and their sources are listed in Table 2.5. JA226 was most commonly used as this strain had a short doubling time and transformed very efficiently by electroporation. This strain was also *leu*⁻ so that it could be used for selecting LEU⁺ transformants. DH5α and DH10B were used for propagating recombinogenic plasmids and for β-gal blue-white screens. X82 was used for the selection of *URA3*⁺ constructs. MV1193 was used for the production of single stranded DNA (for sequencing) after infecting with the helper phage M13K07.

2.2 Yeast and Bacterial Methods

2.2.1 Strain Culture Techniques

Standard sterile techniques were used in the plating and culturing of both bacterial and yeast strains. For most purposes yeast strains were grown on solid media at 30°C in an air incubator. Bacterial cultures were grown at 37°C. Small liquid cultures in test tubes were grown in an air incubator equipped with a roller drum while larger liquid cultures were grown in orbital shakers or in shaking water baths. Temperature sensitive strains of yeast were propagated at room temperature and shifted to the restrictive temperature (37-38°C) when required.

Yeast and bacterial suspensions were plated using a spreader that was sterilized by dipping in 95% ethanol and flaming. When required serial dilutions of yeast were made in sterile water. Electroporated yeast were diluted

in 1M sorbitol. Bacteria were diluted in water or Luria broth. Liquid cultures were inoculated with either a flame sterilized platinum loop or disposable plastic loops. Sterile wooden toothpicks were used for streaking bacterial and yeast strains onto solid media. Yeast and bacterial colonies were often transferred by replica plating from the master plate onto a piece of sterile velvet using a replica plating block. This was then used used as a template to transfer colonies to other plates. Bacteria and yeast strains were stored at 4°C for short periods of time and were stored permanently in 20% glycerol at -70°C.

For galactose induction studies involving the yeast *GAL1* promoter, strains were first grown to mid-log phase in the presence of 2% raffinose. Induction was achieved by adding galactose to a final concentration of 2% (v/v). The *GAL1* promoter could be rapidly repressed by adding glucose to a final concentration of 2% (v/v).

2.2.2 Genetic Analysis of Yeast

To construct diploids, haploid strains of opposite mating type were grown overnight on YEPD plates (unless plasmid maintenance was essential). These freshly growing cells were mixed in equal quantities on a fresh plate. Plates were incubated overnight and then replica plated to select for the desired diploid by complementation of auxotrophic requirements. When auxotrophic markers were unavailable, mixed haploid cells were incubated for 2-4 hours to allow for the formation of zygotes. A portion of the mating mix was then streaked onto a fresh plate. Zygotes were identified by microscopy. A Singer micromanipulator was used to move these zygotes to a defined position on the plate. The micromanipulator was equipped with a glass needle containing a

Table 2.5 List of Bacterial Strains

JA226 [*c600, hsdR (rk-), hsdM (mk+), recBC, lop11, thi, leuB6 str*^{R}] source, J. Carbon.

DH5α [*F-, endA1, hsdR17(rk-mk+), supE44, thi-1, l, recA, gyrA96, relA1, Δ(argF-laczya), U169, F80 lacZDM15*] source, Bethesda Research Labs.

DH10B [*F-, mcrA (mrr-hsdRMS-mcrBC), F80dlacZDM15, DlacX74, deoR, recA1, araD139 D(ara, leu)7697, galU, galK, rpsL, endA1, nupG*] source, Bethesda Research Labs

X82 [*trpC60 (trp1), pyrF (ura3), hisG, lacZ, rpsL8*] source, B. Cipriano

MV1193 [*Δ(lac-proAB),thi, supE D(sr1-recA) 306::tn10(tet*^{R}*;F: traD36 proAB lac*^{q}*ZDm15)*] source Dr. J. Viera

small length of fibre optic and was positioned so that the needle of the dissection apparatus was directly under the 10X objective of a Zeiss binocular microscope (Eichinger and Boeke, 1990).

Diploids were grown overnight on rich media (GNA plates) and streaked onto sporulation plates. They were then incubated at room temperature for 3-4 days. This procedure was used for many crosses but a modified procedure was used for small cell size mutants. It has previously been demonstrated that the initiation of sporulation is subject to size control (Haber and Halvorson, 1972; Calvert and Dawes, 1984). Diploids homozygous for the *CLN3-1* mutation sporulate poorly but brief treatment with hydroxyurea, which inhibits cell cycle progression but not mass accumulation, enabled these mutants to sporulate (Calvert and Dawes, 1984). Similar problems were encountered when attempting to sporulate diploids heterozygous for *whi3* and *CLN3-1*. To overcome these problems, diploids were grown overnight to a density of about 2 X 10^7 cells/ml at which time hydroxyurea was added to a final concentration of 6 mg/ml. Cells were incubated in hydroxyurea for 3 hours. Cells were pelleted, washed in 3% potassium acetate and resuspended in 1.5 volumes of 3% potassium acetate. Resuspended cells were placed in petri dishes and incubated for 3-4 days at room temperature on an orbital shaker.

Prior to the dissection of sporulated cultures enzymatic treatment was required to digest the ascus wall that holds the four spores of the tetrad in place. Sporulated cultures were incubated in a 5% solution of glusulase at 37°C for 30 minutes. This was superseded by a method that used enzymatic treatment with zymolyase. The sporulated culture was suspended in 1 M sorbitol and incubated in the presence of zymolyase (37.5 µg/ml final

concentration) at 37°C for 25 minutes. Tubes were placed on ice and about 5 µl of the suspension was gently spread onto the surface of one end of a YEPD plate. Spores from 8-10 complete tetrads were placed at marked positions on the plate by micromanipulation. Plates were incubated for 3-4 days and spore clones were picked, streaked and replica plated to test for auxotrophic requirements.

When required, *URA3*$^+$ prototrophs were converted to *ura3* auxotrophs by UV irradiation followed by 5-FOA selection. 5-FOA is a selective agent that is converted to the toxic compound 5-fluorouracil by the product of the *URA3* gene. This compound can therefore be used as a negative selection to isolate 5-FOA resistant, *ura3*$^-$ strains. Approximately $1x10^8$ cells were spread onto 5-FOA plates and mutagenized by brief treatment with UV light. Plates were wrapped in tinfoil to inhibit light induced repair processes and incubated for 3-4 days at 30°C. 5-FOA resistant colonies were mated with ura$^-$ tester strains to determine which were *ura3*$^-$. Cell size and doubling times were checked before using strains for other purposes.

2.2.3 Measurement of Cell Volume and Cell Number

Yeast cell volumes were measured using the Coulter Channelyzer as previously described (Nash *et al.*, 1988). Cells were grown to mid-log phase, placed on ice and then sonicated with a Virsonic 300 (Virtis Co. Ltd.) at a setting of 3 for about 10 sec. Sonicated cultures were diluted in Isoton buffer and cell volumes were measured using the Coulter Counter Model ZM equipped with a 70 µm aperture and a Coulter Channelyzer Model 256. The Coulter Channelyzer was calibrated with 5.11 µm diameter plastic beads. An estimate

of the median cell volume was determined and volume profiles were printed.

When calculating doubling times and plotting growth curves yeast cell density was measured with the Coulter Counter Model ZM. When estimating cell concentrations for plating and for routine dilutions an Improved Neubauer haemocytometer slide (0.1 mm deep) was used. Bacterial cell density was measured using a Beckman spectrophotometer at a wavelength of 550-600 nm.

2.2.4 Propidium Iodide Staining and FACS Analysis

Propidium iodide staining was performed as described (Nash *et al.*, 1988) with one additional modification. After incubating the cells in RNase A, proteinase K was added to a final concentration of 1 mg/ml and incubated for 1 hour at 50°C. After staining with propidium iodide cells were filtered through a 36 µm mesh and analyzed with a Coulter Model Epics-C Flow Cytometer. Peaks were quantified as described (Nash *et al.*, 1988).

2.2.5 Tubulin and DAPI Staining of Yeast Cells

Yeast cells were fixed and stained according to the methods described in Rose *et al.* (1990). Rat anti-yeast-α-tubulin antibody (YOL1/34) (Accurate Chemical and Scientific Corp., Westbury, NY) was used at a dilution of 1:50. Goat anti-rat IgG, FITC conjugated (Boehringer Mannheim) was used at a dilution of 1:1000. DAPI (Accurate Chemical and Scientific Corp., Westbury, NY) was prepared in distilled water and stored frozen at -20°C in the dark. It was diluted to 1 µg/ml just prior to use. Stained cells were viewed

with a Nikon optiphot microscope equipped with the appropriate filters and a UV light source. The final magnification was 1000X. Photographs were taken with a Nikon FX35a, 35mm camera.

2.2.6 Transformation of Yeast and Bacteria

Yeast transformations were performed on mid-log phase cells using the lithium acetate method (Ito *et al.*, 1983). This procedure was replaced by a method based on electroporation (Becker and Guarente, 1991). Electroporation was simple to perform and resulted in higher transformation efficiencies. *E.coli* were transformed according to a procedure developed by Hanahan (1983). When large volumes of ligation mix were used reactions were buffered with a tenth volume of 1 M MES (pH 6.3). This method was replaced by a method based on electroporation that consistently gave higher transformation efficiencies. A Biorad Gene Pulser was used for both yeast and bacterial electroporations.

2.2.7 Transposon Mutagenesis and Size Enrichment

The mutagenesis procedure used for the isolation of *whi3* was based on a Ty transposon tagging procedure (Garfinkel *et al.*, 1988; Garfinkel and Strathern, 1991). The plasmid pJEF1105 (pGTyH3neo) was obtained from J. Boeke. This high copy plasmid contains the neomycin phosphotransferase gene inserted into a non-essential region of a class 1 Ty element. This physically tagged Ty element was under the control of the *Gal1* promoter. Transposition of the tagged Ty1 element was induced upon the addition of

galactose and repressed with the addition of glucose. As a consequence of the induction, copies of the tagged element transposed to novel chromosomal loci so that these loci became physically and genetically tagged.

After confirming the integrity of pJEF1105, the yeast strain BWG1-7a was transformed and *URA*$^+$ prototrophs were selected. Transformants were plated on galactose containing selective media at a density of about 1000 cells/plate and incubated at 22°C for a period of 4-5 days. This resulted in the production of colonies of variable sizes which may have occurred because of the deleterious nature of certain transpositions. The Ty mutagenized colonies were suspended in water, scraped from plates and pooled. Pooled cells were stored briefly at 4°C and were used to inoculate overnight cultures for size enrichment.

A method based on unit gravity velocity sedimentation through a preformed gradient of sorbitol was used to enrich for small cell size mutants among the mutagenized population. The method and the device have been described previously (Peterson and Evans, 1967). Cells move through the gradient according to size with larger cells falling faster then smaller cells. A continuous linear gradient of 1.0-1.75% sorbitol was first formed with the use of a gradient maker. Mid-log phase cultures of mutagenized cells were concentrated by centrifugation and sonicated. Approximately 10^9 cells were gently layered onto the top of the preformed gradient. After three hours of differential sedimentation, a concentrated solution of sorbitol (3% w/v) was pumped into the bottom of the tank and 20 ml fractions were collected from the top of the gradient. Fractions were concentrated and sized using the Coulter Channelyzer. Early fractions containing small cells were reinoculated and grown overnight to mid-log phase. The enrichment procedure was repeated

three times. After the final size enrichment, fractions were sized and early fractions were concentrated and plated to obtain single colonies. Colonies were inoculated into liquid media and after overnight growth were sonicated and sized using the Coulter Channelyzer.

2.2.8 Quantitative Mating, Mating Pheromone Resistance and Cycloheximide Resistance Assays

Mating assays were performed as described (Sprague, 1991). The tester strain (699α) was mated with strains whose mating efficiency was being determined at a ratio of 3:1 and a final cell number of $1x10^7$ cells. After thoroughly mixing cells of the two strains, a 25 mm easy pressure syringe filter holder (Gelman Sciences, Ann Arbor, MI) and 25 mm diameter nitrocellulose filter disks (type HA, Millipore, Bedford, MA) with a pore size of 0.45 μm were used to collect the cells. The filters were placed on YEPD plates and incubated at 30°C for 5 hours. The mating mix was then resuspended in 5 ml of sterile water, sonicated, diluted and plated on the appropriate plates. Cells were plated directly or replica plated onto selective plates to determine the number of diploids and the number of diploids plus unmated haploid **a** cells, whose mating efficiency was being determined. Mating efficiency was expressed as the titer of **a**/α cells divided by the titer of **a**/α plus **a** cells (Sprague, 1991). This was used to calculate the relative mating efficiency assuming a mating efficiency of one for the wild-type control.

The sensitivity of strains to mating factor was quantitated by plating 1×10^3 sonicated mid-log phase cells on YEPD plates containing increasing amounts of α-factor (Nash *et al.*, 1988). The ability of strains to form colonies

was determined after 2-3 days of growth at 30°C.

The cycloheximide sensitivity of yeast strains was determined by growing strains to mid-log phase and plating about 2 X 10^3 sonicated cells onto YEPD plates containing increasing amounts of cycloheximide. The ability of cells to form colonies was monitored after 2-3 days growth at 30°C and again after incubating plates for 2 weeks.

2.3 Nucleic Acid Preparation and Analysis

2.3.1 Preparation and Quantitation of DNA and RNA

Yeast genomic DNA was prepared as described by Holm *et al.*, (1986) or as described by Rose *et al.*, (1990). In the first procedure zymolyase was used to enzymatically digest the yeast cell wall (Holm *et al.*, 1986). This procedure was fairly rapid and resulted in good yields of high molecular weight genomic DNA. In the second procedure vortexing with glass beads was used to obtain cell lysis (Rose *et al.*, 1990). This procedure was faster but the quality of the prepared DNA was more variable and the average size of the genomic DNA was smaller.

In both of these procedures centrifugation was not required for the precipitation of nucleic acid if the concentration of genomic DNA was high enough. Two volumes of cold ethanol were layered onto the aqueous layer and genomic DNA was balled out of solution by gently inverting the tube several times. The solution was removed with a pipette tip or by gently inverting the tube. Higher molecular weight DNA was retained while contaminating materials remained suspended and could be removed. DNA was then washed

with 70% ethanol, air dried and resuspended gently in buffer.

The alkaline lysis method was used to prepare plasmid DNA from *E.coli* (Sambrook *et al.*, 1989). Large scale preparations of plasmid DNA were purified by centrifugation through cesium chloride gradients. Gradients were run overnight at 55K rpm in a Sorvall TV-865 rotor or a Beckman VTi-65.2 rotor. Bands were removed, extracted with organics and precipitated according to Sambrook *et al.* (1989). Residual cesium chloride was removed using a sodium chloride exchange reaction (NaCl to 300 mM) followed by precipitation with ethanol.

Bacteriophage lambda DNA was prepared on a small scale after concentrating the phage with DEAE-cellulose (Whatman DE52) columns (Helms *et al.*, 1987). Large-scale lambda DNA was prepared according to Sambrook *et al.*, (1989) with one modification. Solid polyethylene glycol (PEG 6000) was added to a final concentration of 7% w/v and solid NaCl to a final concentration of 0.5 M (Wendy Bickmore, personal communication).

Yeast genomic and plasmid DNA concentrations were determined by the fluorometric method of Morgan *et al.* (1979). The relative fluorescence resulting from the intercalation of ethidium bromide into DNA was measured and compared to that of a standard DNA solution of known concentration. A Sequoia-Turner Model 450 fluorometer was calibrated to a value of 100 using 0.5 μg of a standard DNA solution in pH 11.8 buffer. For genomic samples, readings were taken and compared to the value obtained with the standard. For plasmid DNA, readings were taken before and after heating and cooling the samples. Samples were heated for 2 minutes at 90-100°C and then cooled for 2 minutes in room temperature water. Linear and nicked circular DNA denatures during the heating procedure and reassociates very slowly after

cooling while plasmid DNA snaps back upon itself rapidly and regains its ethidium bromide associated fluorescence. The first reading was used to measure the total DNA concentration while the reading after heating and cooling was used to measure intact plasmid DNA.

Total yeast RNA was isolated by vortexing cells in the presence of hot phenol and SDS (Kohrer and Domdey, 1991). This procedure resulted in high yields of relatively pure RNA free of DNA. The concentration and purity of RNA samples were measured using the fluorometric method of Morgan *et al.*, (1979) using a pH 8.1 buffer. The fluorometer was calibrated by setting a value of 100 using a 5 µl sample of purified calf thymus DNA (100 µg/ml) standard. Sample readings were taken both before and after the addition of RNase A to the assay tube so that the amount of RNA and contaminating DNA could be calculated. Sample concentrations were then determined by comparison to the value obtained for the standard. The accurate quantitation of RNA was important so that samples for Northerns could be equally loaded.

2.3.2 Restriction Digests

Restriction digests were carried out according to the manufacturers specifications. BSA was added to a final concentration of 100 µg/ml in all digests. Digests involving yeast genomic DNA were carried out in the presence of 5 mM spermidine to facilitate complete digestion. The Klenow fragment of DNA polymerase was used to fill in 5' overhangs and to create blunt ends. Calf intestinal alkaline phosphatase or bacterial alkaline phosphatase was used to remove 5' phosphates from DNA. Calf intestinal phosphatase was used according to manufacturers specifications and was then heat inactivated and

phenol/chloroform extracted. Bacterial alkaline phosphatase (BAP) was incubated with digested DNA at 65°C using the recommended buffer or added to completed restriction digests in restriction enzyme buffer. BAP was inactivated by treating with proteinase K at a final concentration of 0.5 µg/µl followed by two phenol/chloroform extractions, a chloroform extraction and ethanol precipitation.

2.3.3 Gel Electrophoresis, Southern and Northern Transfer

Agarose gels contained between 0.5-0.8% agarose depending on the size of fragments to be separated and 1X Tris-Borate EDTA (Sambrook *et al.*, 1989). The running buffer also contained 1X Tris-Borate EDTA. Gels were run in MAX submarine trays (Hoefer) at constant voltage. For analysis of digested plasmid DNA about 0.2 µg was loaded per lane and for genomic DNA 2-2.5 µg was loaded per lane. The 1 kb ladder (BRL) was generally used as a marker. When higher molecular weight markers were required lambda DNA was digested with *Hind*III or high molecular weight DNA markers were obtained from BRL. Electrophoresed DNA was visualized with ethidium bromide and a UV light source. When yeast genomic DNA was electrophoresed for Southern blot analysis, gels were run at low voltage (20-40 V) for the required length of time. When transferring large molecules of genomic DNA gels were depurinated to facilitate efficient transfer. Gels were treated in 0.05 N HCl for 15 minutes or in 0.3 M sodium citrate (pH 3) for 2 hours prior to the denaturation and neutralization steps. Gels were transferred to nitrocellulose or Nytran membranes (Schleicher and Schuell) using the sponge method and 20X SSC (Sambrook *et al.*, 1989). After transfers were complete the positions of wells

were marked on the membrane with a pencil and membranes were washed briefly in 5X SSC. Membranes were blotted dry and baked in a vacuum oven at 80°C for 2-3 hours or UV crosslinked with a UV Stratalinker (Stratagene) according to the manufacturers specifications.

RNA was separated according to size by electrophoresis through denaturing formaldehyde gels. RNA samples (7.5-15 µg) were dried down, resuspended in 4.5 µl of DEPC-treated sterile water and then 17.5 µl of RNA sample buffer was added. Sample buffer contained: 2 µl of 10X running buffer, 10 µl deionized formamide, 3.5 µl formaldehyde, 2 µl formamide dye mix and 320 mg/ml sucrose. RNA samples were heated to 65°C for 10-15 minutes and electrophoresed through 1% agarose-6.6% formaldehyde gels at 60-80 V for 3-5 hours (Sambrook *et al.*, 1989). Gels were washed briefly in water to remove excess formaldehyde and transferred to Nytran by capillary transfer (Sambrook *et al.*, 1989). Membranes were then treated similar to Southern blots.

2.3.4 Isolation and Purification of DNA Fragments

DNA fragments were gel purified from low melt agarose using agarase (Sigma). Gels contained 0.5-1.0% low melt agarose and 0.5 X TBE. Gels were run at 4°C at low voltage to increase resolution. Gels were stained during or after the run with ethidium bromide and bands were visualized on a UV light box. Gel slices containing the band of interest were removed with a scalpel, incubated at 65°C until liquified and then mixed with an equal volume of buffer (100 mM NaCl, 20 mM Tris-HCl (pH 7.5) and 5 mM EDTA). Agarase was added to a final concentration of 50 units/ml and reactions were incubated at 37°C for 4-8 hours. Agarased samples were extracted twice with an equal

volume of phenol/chloroform, once with an equal volume of chloroform and precipitated with two volumes of ethanol. Gel purified fragments were rerun as required and DNA was quantitated by fluorometry or by comparison to the band intensities on gels containing standards of known concentrations.

2.3.5 Labeling and Hybridization of DNA Probes

Oligonucleotides were 5' end labelled with T4 polynucleotide kinase using forward reaction conditions. Reactions were carried out in a 30 µl volume containing 6 pmol of oligonucleotide, 30 µCi of [γ-^{32}P]ATP (3000 Ci/mmol) and 0.5-1U of T4 polynucleotide kinase in the appropriate buffer conditions. Reactions were incubated at 37°C for 45 minutes and then placed on ice.

DNA fragments were labelled with [α-^{32}P]dATP using the random primer labelling method (Feinberg and Vogelstein (1983). In this procedure random hexamers are mixed with and hybridize to the heat denatured DNA fragment to be labelled and become primers for the Klenow fragment of DNA polymerase. Primers were elongated by including dGTP, dCTP, dTTP, radioactive [α-^{32}P]dATP (30-35 µCi, 3000 Ci/mmole) and 5-10U of Klenow in the appropriate buffer conditions. Reactions were carried out at room temperature for 1 hour or greater.

The percentage of incorporated hot nucleotide was calculated by determining the Cerenkov counts emitted from a 1 µl spot of the reaction mix. Two pieces of DE81 paper were spotted and allowed to air dry. One of these was washed for 30 minutes in 0.5 M NaH_2PO_4, followed by a 2 minute wash in water and a 2 minute wash in 70% ethanol. After air drying the washed and

unwashed DE81 papers were placed in a scintillation counter and the number of counts were compared to determine the percentage of hot nucleotide incorporated.

Hybridization of Northern and Southern filters were carried out in sealed plastic bags at varying temperatures depending on the probe used and the stringency required. Two different hybridization solutions were used throughout the the course of this study. Originally, a hybridization solution consisting of 5X Denhardt's solution, 50% deionized formamide, 2.5 mM NaH_2PO_4, 0.1% SDS, 5X SSC and 10 µg/ml of sonicated heat denatured calf thymus DNA was used. Prehybridizations were carried out at 42°C for 1-2 hours followed by the addition of heat denatured radioactive probe and overnight incubation. This method was replaced by the method of Church and Gilbert (1984) as modified by R. Allshire (personal communication). The modified mix contained 7% SDS and 0.25 M Na_2HPO_4, pH 7.2. The 1M phosphate stock was composed of 134 g $Na_2HPO_4 \cdot 7H_2O$ and 4 ml of 85% H_3PO_4 per liter of solution. Prehybridizations and hybridizations were carried out at 65°C. After hybridizations were complete the solution was carefully removed and filters were washed twice with 1X SSC, 0.1% SDS at room temperature and once at 50-65°C depending on the stringency desired. Filters were blotted briefly on 3MM paper, wrapped in Saran wrap while moist and exposed to Kodak XAR film at -70°C with an intensifying screen. When reprobing was required filters were heated to boiling in the presence of 0.1X SSC, 0.1% SDS to remove bound probe followed by a brief wash in 2X SSC.

Oligo quick mix was used for hybridizations involving labeled oligonucleotides. One liter of oligo quick mix contained 0.5 g BSA, 0.5 g PVP, 0.5 g Ficoll, 1 g SDS, 1 g sodium pyrophosphate, 250 ml 20X SSC and 766 ml

water (R. Allshire, personal communication). The hybridization temperature depended on the melting temperature of the oligo and was calculated as follows: Tm = ((A+T) X 2) + ((G+C) X 4). Hybridizations were carried out overnight at 20°C below the calculated Tm of the oligo. Filters were then washed twice for 10-15 minutes in 3X SSC, 0.1% SDS at room temperature and once at a higher temperature which was about 10°C below the Tm of the oligo. Filters were blotted briefly and placed on film as described.

Colony hybridizations were carried out essentially as described for Southern and Northern blots with one exception. The prehybridization solution was changed prior to the addition of probe. Circular nitrocellulose filters were used for this purpose and colony lifts were carried out as described in Sambrook *et al.* (1989).

2.3.6 Plasmid and Bank Constructions

To facilitate the cloning of yeast DNA flanking the position of Ty insertion, the plasmid pRS305neo was constructed and integrated at the tagged Ty locus of the mutant. The plasmid pRS305, a *LEU2* integrating vector, was digested with PvuII and ligated with a 1.6 kb *Pvu*II fragment containing the neomycin phosphotransferase gene (source pAJ50) to create pRS305neo.

The mutant *whi3* locus was cloned by constructing a genomic library from the backcrossed mutant strain T504b-5c. Yeast DNA was prepared and cut to completion with *Nhe*I or *Xba*I, two enzymes which do not cut within the tagged Ty element and liberate a large *whi3* containing fragment as determined by Southern analysis. The centromeric vector, pRS316, was cut with *Spe*I and treated with bacterial alkaline phosphatase. Ligations were set up at an insert

to vector ratio of 4:1 and a final concentration of 20 μg/ml. A small amount of the ligation mix was transformed into the bacterial strain DH5α to determine the frequency of insert containing transformants versus vector only transformants. Most ligation mixes contained between 40-50% insert containing transformants. The majority of the ligation mix was transformed into JA226, a strain which electroporates very efficiently. Ampicillin resistant transformants were selected and pooled. Aliquots were then plated onto kanamycin containing plates and resistant colonies were selected.

For complementation studies, a 5.2 kb *Bgl*II fragment was gel purified from the original bank plasmid #27 and from the lambda clone #5025. These fragments were subcloned into the unique *Bam*HI site of pRS306, a *URA3* integrating vector, to create the plasmids pRN163a and pRN200b. They were also subcloned into pRS315, a *LEU2* centromeric vector, to create the plasmids pRN202a, pRN202b and pRN203b. Finally, these fragments were subcloned into pRS425, a *LEU2* based 2μm high copy number vector, to create the plasmids pRN204b, pRN205a, pRN206a. The plasmids pRN163a, pRN203b and pRN206a contained the 5.2 kb fragment derived from clone #27 while the plasmids pRN163b, pRN202a, pRN202b, pRN204b and pRN205a contained the lambda clone derived fragment. The relative orientation of the 5.2 kb fragment within the vectors was indicated by an a or b.

To delete *WHI3*, a 1.9 kb *Sph*I to *Bst*EII fragment was removed from pRN163b. Restriction sites were blunted with T4 polymerase and phosphatased with bacterial alkaline phosphatase. This fragment was replaced with a 2.1 kb *Hind*III fragment obtained from pART5 and blunted with Klenow. This construction replaced the majority of the open reading frame with the *LEU2* gene.

Isogenic strains which were wildtype or deleted at the *whi3* locus and wildtype, deleted or containing the hyperactive allele of *CLN3* were constructed by gene replacement. In the first step of the constructions, pRN202a was cut with *Xho*I and *Xba*I to liberate the *WHI3* gene. This fragment was used to replace the disrupted copy in strain RN210-13d. Cotransformation with pRS316 was necessary as a direct selection was unavailable. URA3$^+$ transformants were selected and replica plating to SD-leu to identify leu$^-$ transformants. This identified potential candidates in which the deleted *whi3* locus had been replaced by the wild-type gene. In the second step, the *CLN3* locus was replaced with *CLN3::URA3* using pWJ310 cut with *Puu*II or with *CLN3-1* using pBF30 cut with *Puu*II (Nash *et al.*, 1988). These constructs were transformed into the isogenic *whi3* and wild-type strains described above. All were confirmed to be correct by Southern blot analysis and had the expected cell sizes.

The *WHI3* gene was placed under the control of the strongly inducible *GAL1* promoter by first subcloning a 0.75 kb *Eco*RI-*Bam*HI fragment containing the bidirectional *GAL10-GAL1* promoters (pMR438) into pRS316 and pRS426 to create pCGAL1 and pHGAL1. These vectors were digested with *Bam*HI, filled in with Klenow and ligated to the *WHI3* gene on a 2.2 kb blunted *Cla*I fragment gel purified from the lambda clone 5025. The resultant vectors: pCGW2, pCGW5 and pHGW8, contained the bidirectional *GAL10-GAL1* promoter fused to the 5' end of the *WHI3* gene such that the *Bam*HI end (*GAL1*) was attached 40 nucleotides upstream of the *WHI3* start codon.

2.3.7 Sequencing and Sequence Analysis

The sequence flanking the 3' end of the site of Ty integration and the sequence of the 1.3 kb *Eco*RI fragment of the wild-type gene were obtained using single stranded DNA (Vieira and Messing, 1987). Fragments were subcloned into pUC119 and single-stranded DNA was produced using the helper phage M13K07 and the bacterial strain MV1193 (Vieira and Messing, 1987). Single-stranded DNA was recovered according to Vieira and Messing, (1987). The remainder of the wild-type gene and the position of Ty1 insertion in the mutant clones were sequenced with primers (Table 2.2) using double stranded DNA as template. Sequencing reactions were carried out according to manufacturers instructions by the dideoxy method using the reagents supplied in a sequenase kit (US Biochemicals) (Sanger *et al.*, 1977). Samples were run on 6% denaturing acrylamide gels at 55W for 2-8 hours. Gels were dried under vacuum at 80°C for 1-2 hours and placed on Kodak XAR film overnight at room temperature.

The Intelligenetics suite was used for data entry, editing and other sequence manipulations. Current databases were searched locally and at remote sources using the FASTA algorithm (Pearson and Lipman, 1988). Additional sequence analysis and presentation was performed with the Geneworks package designed for the McIntosh.

2.3.8 Mapping

To determine the chromosomal position of the *WHI3* gene, contour-clamped-homogeneous-electric-field (CHEF) electrophoresis was used to

separate yeast chromosomes from the strains YPH49, YPH80, and YPH149. Chromosome-sized yeast DNA molecules were prepared in solid agarose and run on CHEF gels using pulse times of 65 seconds for 27 hours or pulse times of 90 seconds for 14 hours followed by 60 second pulses for 10 hours (Rose *et al*, 1990). DNA in gels was partially depurinated in 200 mls of 0.05 N HCl for 15 minutes prior to denaturation and standard Southern transfer. Chromoblots were hybridized with *WHI3*, chromosome II (*CDC28*) and chromosome XIV (*RAS2*) specific probes. A 1.3 kb *Eco*RI fragment of the *WHI3* gene was sent to L. Riles in M. Olson's lab. This fragment was labelled and used to probe filters that contained an ordered array of overlapping clones covering the majority of the yeast genome (Olson *et al.*, 1986).

RESULTS

3. IDENTIFICATION, CLONING AND SEQUENCING OF *WHI3*

Results presented in this section suggest that the *WHI3* gene encodes a novel regulatory component of the yeast cell cycle. The properties of this mutant are consistent with a defect in the coordination of growth and division. Since the attainment of a critical cell size is a criterion for cellular passage through Start, the phenotypes are suggestive of an alteration in this size requirement and therefore timing of Start. The wild-type gene was cloned and increasing gene dosage resulted in a corresponding increase in cell size. Strains deleted for *WHI3* had a small cell size phenotype. These results are consistent with a dose-dependent inhibitory function for the Whi3 protein.

3.1 Rationale

In the G1 phase of the yeast cell cycle environmental conditions and cell size are monitored by the cell. When appropriate conditions have been met a molecular decision is made to commit to a full round of cell division. Start defines the position in the G1 phase where this molecular decision or commitment event is executed. One approach to identifying genes which regulate the commitment event is to find mutants which confer a small cell size phenotype because they proceed through Start prematurely. The hyperactive G1 cyclin mutant *CLN3-1* is an example of a mutant that fulfills this criterion. To isolate novel cell size mutants a Ty transposon mutagenesis followed by size enrichment was employed. This procedure was chosen over conventional procedures because mutations generated by the transposition of a tagged Ty1 element are physically and genetically tagged. This facilitates genetic studies and provides a way to clone the gene at the site of transposition.

3.2 Isolation and Preliminary Characterization of a Novel Cell Size Mutant

Transposon mutagenesis followed by size enrichment was used to isolate small cell size mutants. A high copy number plasmid, pJEF1105, which contains a Ty1 element under the control of the strongly inducible *GAL1* promoter was obtained from J. Boeke (Boeke *et al.*, 1988). This Ty1 element has the bacterial neomycin phosphotransferase gene inserted into a non-essential region of the transposon. Yeast strains expressing this gene are able to inactivate the drug G418 and therefore become resistant. When strains transformed with this plasmid are grown on galactose, transcription is induced

and the complementary DNA of the tagged Ty element transposes to novel chromosomal locations by a replicative transposition process. The resulting tagged Ty loci can be followed in genetic crosses using the G418 resistance phenotype. Bacterial strains expressing the *neo* gene become kanamycin resistant. This provides a selection for cloning yeast DNA which includes the insertion.

In preliminary experiments the mutagenic effect of induced Ty transposition was quantitated using a fluctuation test. Canavanine is a toxic arginine analog which is transported into cells by the arginine permease. Mutation of the arginine permease gene results in canavanine resistance. The fluctuation test was performed by transformation of the strain BWG1-7a with pJEF1105. Liquid cultures of these transformants were grown to saturation in medium containing glucose where the *GAL1* promoter was repressed or in galactose containing medium where the promoter and transposition were induced. Cultures were grown to saturation at 22°C as transposition rates increase substantially when yeast are grown at lower temperatures (Paquin and Williamson, 1984). Aliquots of these cultures were plated onto canavanine containing medium and the number of resistant colonies were counted. The median number of can^R colonies was determined for uninduced and induced cultures (data not shown). Galactose induction of the Ty plasmid resulted in a six fold increase in the median number of canavanine resistant mutants. These results encouraged us to use this method to mutagenize yeast in attempts to tag genes involved in cell size control.

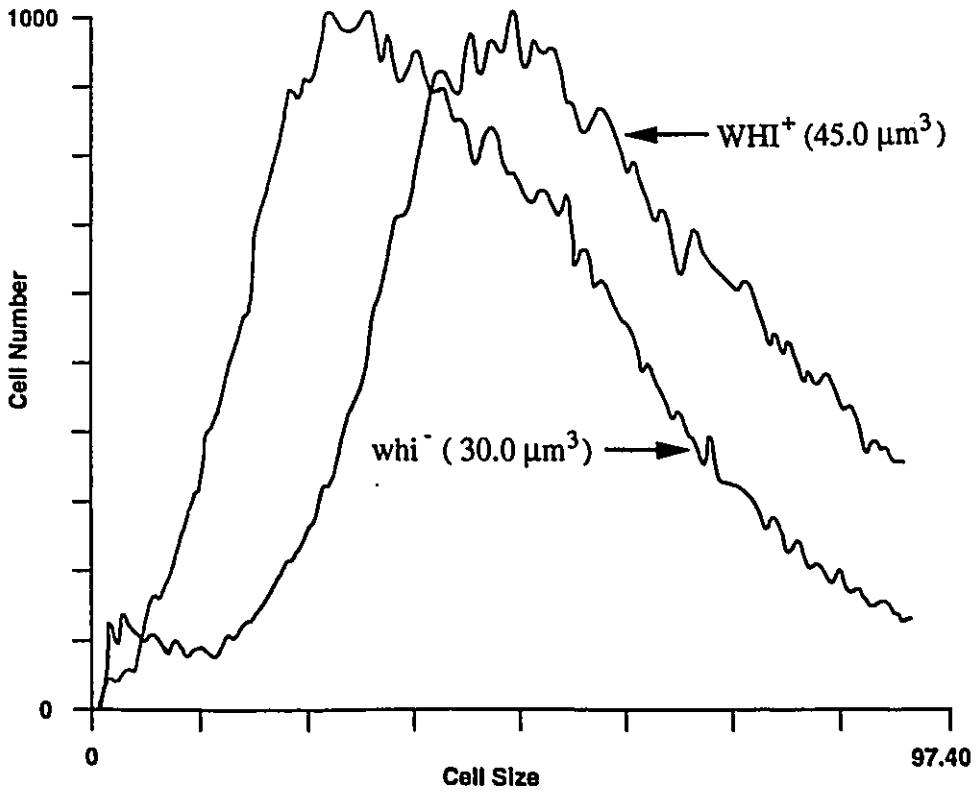
The strain BWG1-7a [pJEF1105] was plated on SC-ura plates containing 2% galactose and incubated for 1 week at 22°C to induce Ty transposition (see Section 2.2.7 for details). To enrich for small cell size

mutants a unit gravity sedimentation device was used (received from Dr R. Barr) (Peterson and Evans, 1967). Log phase cells concentrated by centrifugation were layered onto a preformed gradient of sorbitol and moved through the gradient by gravity according to size. Preliminary reconstruction experiments showed a substantial enrichment for *whi*$^-$ cells over *WHI*$^+$ cells (data not shown). Mutagenized colonies were pooled and grown in culture to mid-log phase. Cells were sonicated, layered on top of a preformed gradient of sorbitol and allowed to settle for 2-3 hours. Fractions enriched for small cell size mutants, as determined by Coulter Channelyzer analysis, were collected, pooled and reinoculated. The size enrichment procedure was repeated three times before plating enriched fractions for single colonies.

Cells from individual colonies were inoculated into liquid media and screened with the Coulter Channelyzer. A number of small cell size mutants were identified with median cell volumes of about 30 μm^3 as compared to the median cell volume of 45 μm^3 for the wild-type parental stain. An example of the size profile obtained for one of these mutants is shown in Figure 3.1. After isolating about 20 such small cell size mutants each was grown non-selectively on YEPD to cure the strains of the Ty plasmid thereby preventing further transposition. Genomic DNA was prepared and Southern analysis was performed to identify novel Ty1 bands common to the mutants but absent in the parental strain. An internal gel purified Ty fragment was used as a probe and numerous novel bands were identified. However, none of mutants had a novel Ty hybridizing band in common (data not shown).

Mutants were backcrossed to a wild-type G418^s strain to segregate away extraneous integrated Ty elements and crossed to a strain containing the *CLN3-1* mutation to test for allelism. All of the original mutants and

Figure 3.1. Small cell size phenotype of a tagged Ty mutant. The wild-type strain BWG1-7a and the tagged Ty mutant S4 were grown overnight in YEPD, placed on ice and then sonicated. Samples were diluted in isoton and cell volume was measured with the Coulter Channelyzer. Cell volume distributions were plotted and median cell volumes determined. Median cell volumes (μm^3) are indicated.


1000
0
Cell Number
0
97.40
Cell Size
WHI+ (45.0 µm3)
whi- (30.0 µm3)

backcrossed strains were crossed to the wild-type parental strains BF328-4a and BF328-4b. The strains BF328-2c and BF328-6b contained the *CLN3-1* mutation and were used to test for allelism. None of the Ty mutants were allelic with *CLN3-1* but the phenotypic effects of combining the two mutations will be discussed in Chapter 4. Backcrossing of some of the mutants (5, 7, S3 and S5) was discontinued due to poor sporulation or strain sterility. This may have occurred as a result of the mutagenesis. Analysis of a few of the mutants (1, 6, S6, S10 and S16) was discontinued because size was not segregating in a 2:2 fashion. This may have been due to a multigenic trait or to strain background differences present in early crosses. Work on another group of size mutants (S2, S4, S8, S11, S13, S14, S17 and S18) was discontinued after completing numerous backcrosses to the wild-type strain and finding that small cell size and G418 resistance segregated independently. A spontaneous mutation, an untagged Ty element or a recombination event may have occurred in these mutants. On average about three crosses to a wild-type strain were required until G418 resistance segregated 2:2. This would suggest that on average each original mutant contained about 8 tagged Ty elements. Some of the original mutants were also intercrossed to determine whether there were multiple linkage groups present. In all of these crosses small cell size segregated in a 4:0 pattern (data not shown). This suggested the existence of a single linkage group but all of the mutants were not intercrossed so it is possible that other linkage groups were present.

The strain T504b was created from the fourth backcross of mutant 9 (T404) with BF328-4a. In this cross a clear 2:2 segregation was observed for both G418 resistance and small cell size and these two phenotypes cosegregated in all tetrads analyzed. This suggested that the small cell

phenotype of this mutant had resulted from the transposition of a tagged Ty element into or near a gene involved in size control. An example of the size segregation observed in tetrads of this cross is shown in Figure 3.2. In this tetrad the spore clones T504b-1b and 1c were G418 resistant and had a reduced cell volume while T504b-1a and 1d were both G418 sensitive and had wild-type cell volumes. The observed small cell size phenotype, the lack of allelism with *CLN3-1* (*whi1-1*) and phenotypic differences compared to those of the *whi2* mutant prompted me to call the new mutation *whi3*. This is in keeping with the original nomenclature used for the *whi1* (*CLN3-1*) and *whi2* small cell size mutants.

To analyze the chromosomal locus of the tagged Ty element in these strains genomic DNA was isolated from spore clones of tetrad T504b-2. DNA was digested with three different restriction enzymes, run on an agarose gel and transferred to nitrocellulose. The Southern blot was probed with a gel purified fragment of the *neo* gene. A hybridizing band was found in two of the four spore clones for all three digests. These two spore clones corresponded to the whi$^-$ segregants (Figure 3.3). Spore clones with wild-type cell size did not contain a hybridizing band. This further supports the idea that the transposition of a tagged Ty element to the locus of a size control gene caused the small cell size phenotype.

To determine whether the *whi3* mutation was recessive or dominant a series of diploid strains was constructed. Homozygous wild-type diploids (*WHI3/WHI3*), heterozygous diploids (*WHI3/whi3*) and homozygous mutant diploids (*whi3/whi3*) were constructed from T504b spore clones and median cell volumes were measured. An example of the Channelyzer plots obtained is shown in Figure 3.4. The average median cell volume of heterozygous diploids

Figure 3.2. The *whi3* phenotype segregates 2:2 and is linked to G418 resistance. Spore clones of the tetrad T504b-1 were grown overnight in YEPD, placed on ice and sonicated. Samples were diluted in isoton and cell volumes were measured with the Coulter Channelyzer. Cell volume distributions were plotted and median cell volumes determined. Average median cell volumes (μm^3) of spore clones are indicated.

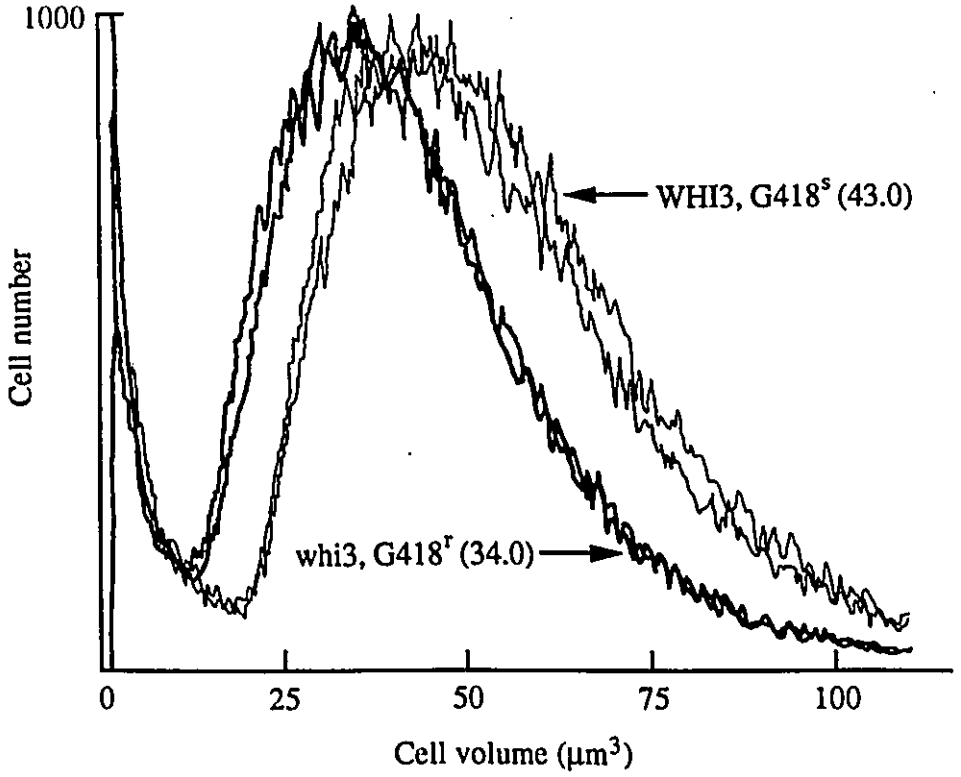

1000
Cell number
WHI3, G418^{s} (43.0)
whi3, G418^{r} (34.0)
0
25
50
75
100
Cell volume (μm^3)

Figure 3.3. The *whi3* tagged Ty1 element segregates 2:2 on Southern blots. Genomic DNA was prepared from spore clones of the tetrad T504b-2. T504b-2a and 2d are *whi3*$^-$ while T504b-2b and 2c are wild-type. DNA from these spore clones was digested with *Eco*RI, *Bam*HI and *Sal*I, run on a 0.8% agarose gel and transferred to nitrocellulose. The resultant Southern blot was probed with a 500 bp *Pvu*II-*Xho*I fragment of the *neo* gene obtained from pAJ50. The sizes of the 1 kb ladder molecular weight markers are indicated.

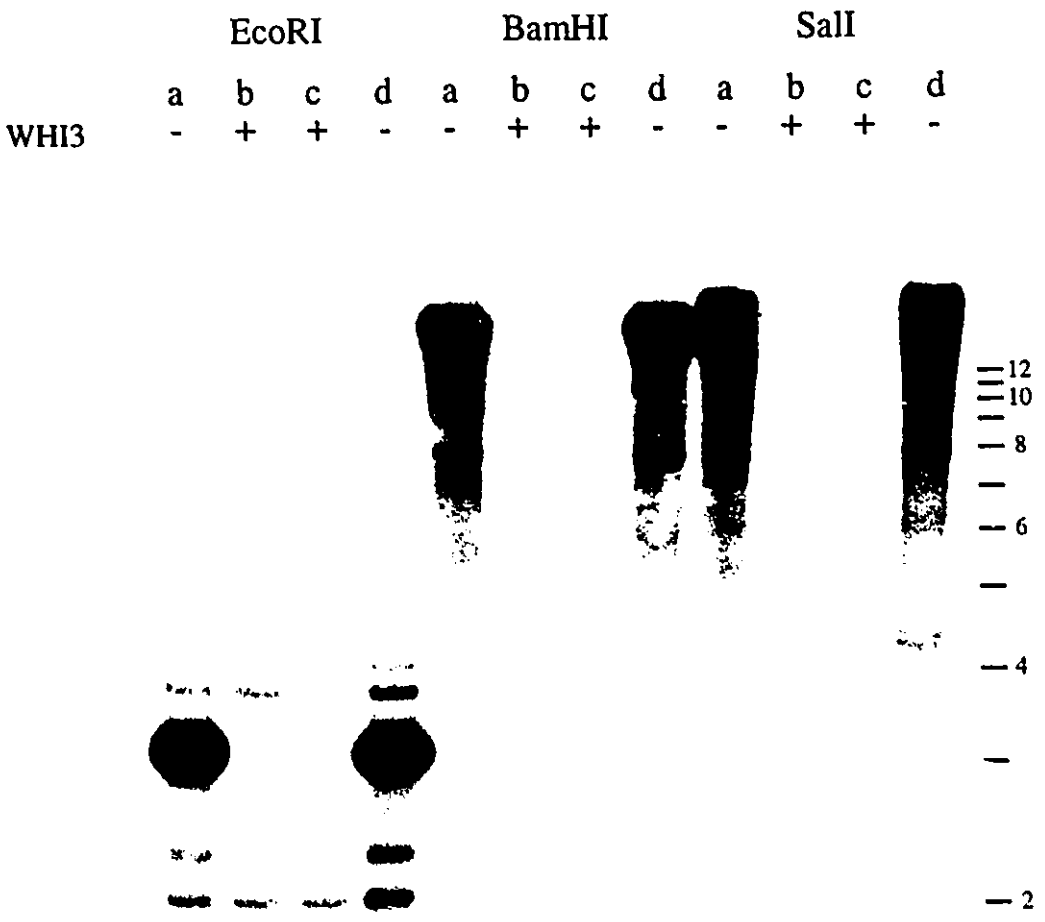
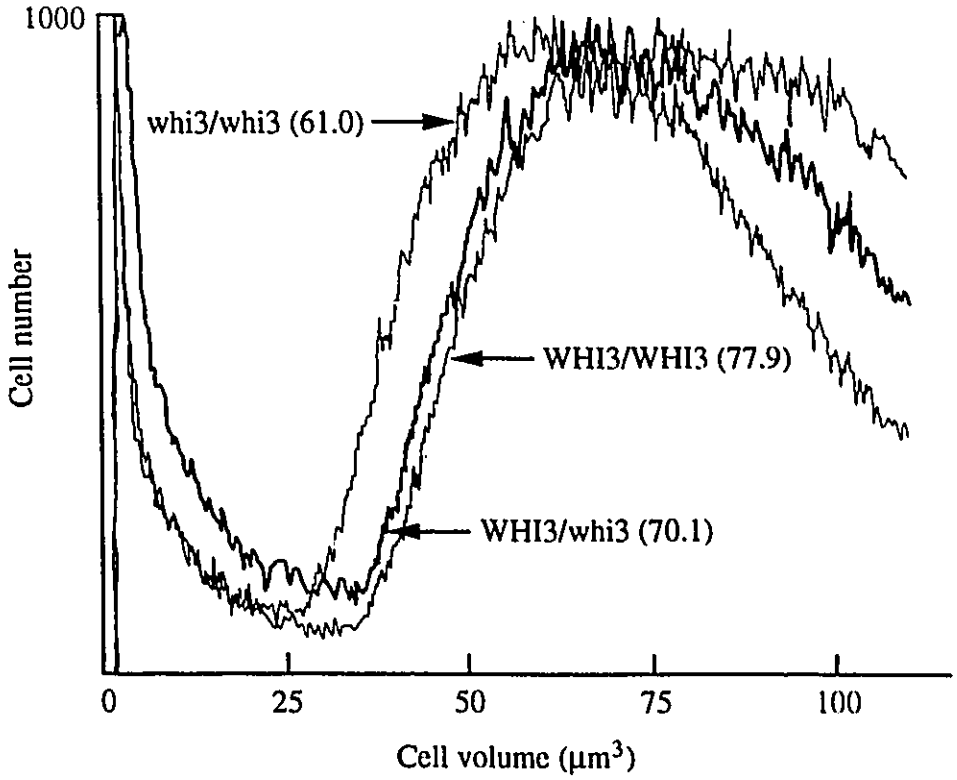

EcoRI
BamHI
SalI
a b c d a b c d a b c d
WHI3 - + + - - + + - - + + -
12
10
8
6
4
2

Figure 3.4. The *whi3* mutation is partially dominant. Homozygous mutant (T504b-2d X T504b-3a), homozygous wild-type (T504b-1c X T504b-4d) and heterozygous diploids (T504b-1b X T504b-1c) were grown overnight in YEPD. After sonication, aliquots were diluted in isoton and cell volumes were measured with the Coulter Channelyzer. Cell volume distributions were plotted and median cell volumes were determined. Median cell volumes (μm^3) are indicated.


1000
Cell number
whi3/whi3 (61.0)
WHI3/WHI3 (77.9)
WHI3/whi3 (70.1)
0
25
50
75
100
Cell volume (μm³)

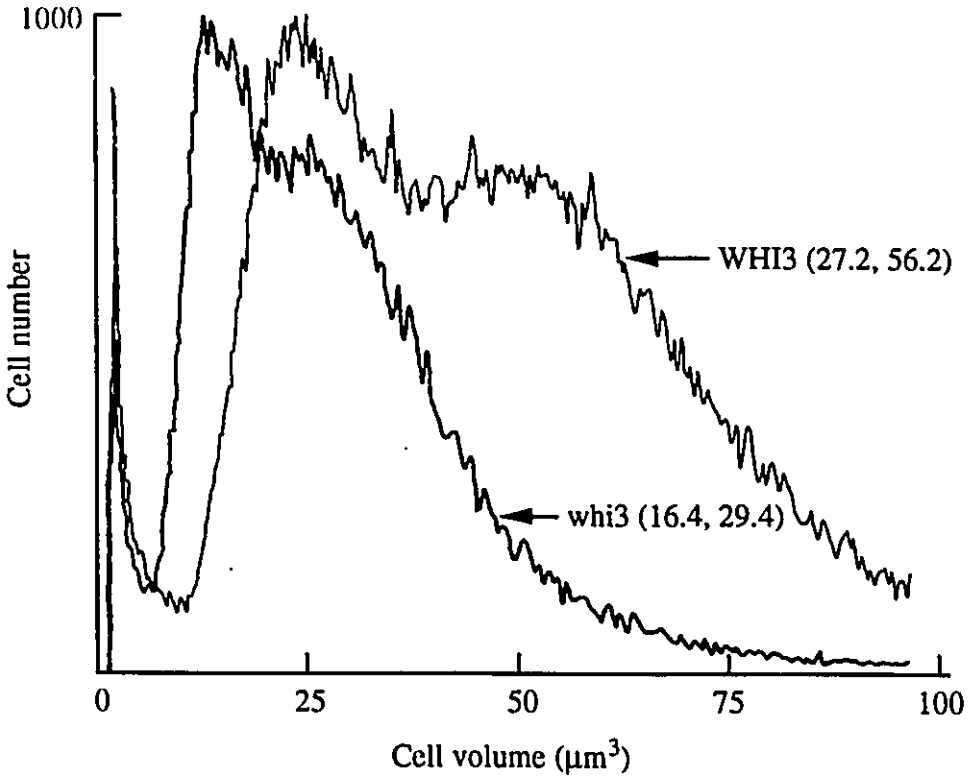
(72 μm^3) was closer to the median cell volume of wild-type diploids (77 μm^3) than to homozygous mutants (61 μm^3). This phenotype can best be termed partially dominant. The single copy of the *WHI3* gene present in the heterozygote appears to be affecting cell size but is not able to fully compensate. This is consistent with a gene product which functions in a dose-dependent manner.

To determine whether the altered cell size requirement in the mutant was specific to carbon source or growth rate, mutant and wild-type strains were grown on glycerol, a poor carbon source. Both *whi3* and *WHI3*$^+$ cells had reduced cell volumes (Figure 3.5) and both had similar slower growth rates (data not shown). *whi3* mutants displayed a biphasic distribution similar to wild-type controls (Figure 3.5). At low growth rates the size difference between mother cells and daughter cells at abscission is increased giving the biphasic distribution. In contrast, *CLN3-1* mutants maintained a monophasic distribution at slower growth rates as a result of mother-daughter division symmetry (Nash *et al.*, 1988; data not shown). *whi3 CLN3-1* double mutants had a smaller cell volume then single mutants when grown in glycerol and had a monophasic distribution similar to *CLN3-1* single mutants (data not shown). Similar results were obtained when ethanol was used (data not shown).

3.3 Cloning of the Mutant Locus

The evidence presented above suggested that a tagged Ty element had transposed into or near to a gene involved in size control. The resulting *whi3* mutation appeared to be partially dominant as the median cell volume of heterozygous diploids was intermediate compared to homozygous mutant and

Figure 3.5. The *whi3* small cell size phenotype is not carbon source or growth rate dependent. The wild-type strain T504b-1a and the isogenic mutant strain T504b-1c were innoculated and grown overnight in YEP glycerol media. Cultures were sonicated, aliquots were diluted in isoton and cell volumes were measured with the Coulter Channelyzer. Cell volumes were plotted and median cell volumes of both peaks were determined. Median cell volumes (μm^3) of each of the biphasic peaks are indicated.


1000
Cell number
WHI3 (27.2, 56.2)
whi3 (16.4, 29.4)
0
25
50
75
100
Cell volume (μm³)

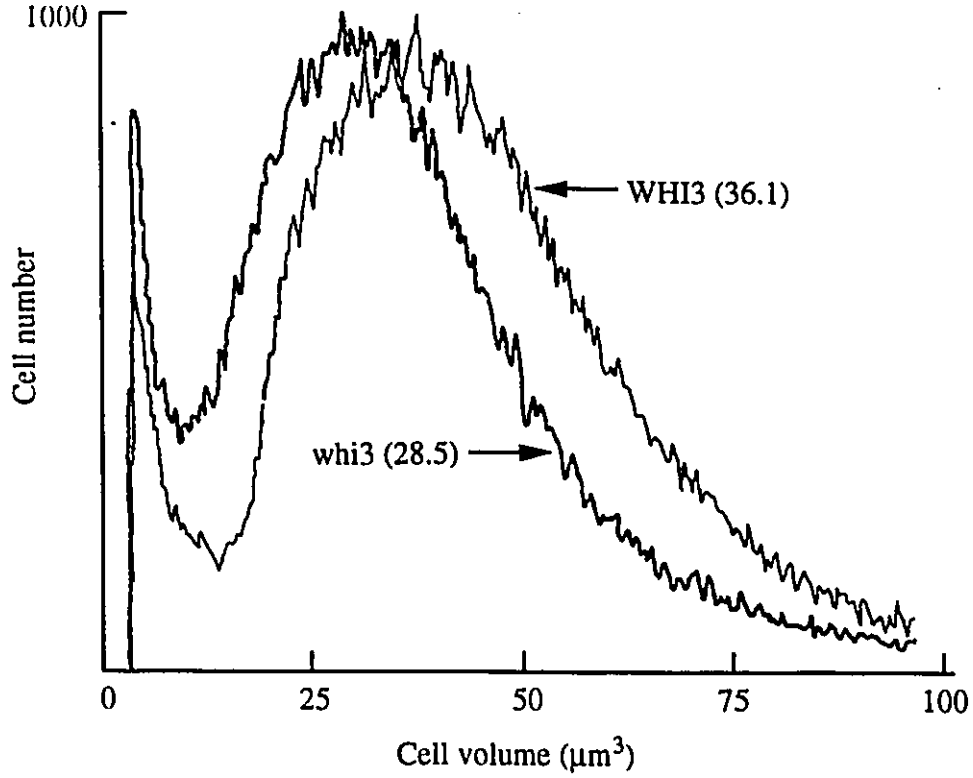
wild-type diploids. In addition, the *whi3* mutant was not defective in carbon source sensing. The critical cell size was modulated by the growth rate. The next goal was to identify and clone the *WHI3* gene so that additional experiments aimed at understanding the role of this gene in cell size control could be started. The first part of the strategy required cloning DNA from the region of the Ty transposon so that flanking DNA could be isolated. This was carried out using the *neo* gene of the tagged Ty element as a selectable marker in bacteria. This would facilitate the cloning of the wild-type gene using a probe obtained from the mutant region. There are then two tests which would show whether the open-reading frame disrupted by the Ty element was in fact the *WHI3* gene. First, the wild-type open reading frame should restore wild-type size. Second, knocking out the open reading frame should cause a *Whi*⁻ phenotype.

To obtain DNA encompassing the mutant locus a genomic library was constructed from a *whi3* strain. The *URA3 cen* vector, pRS316, was digested with *Spe*I and ligated to genomic DNA digested to completion with *Nhe*I or *Xba*I (see Section 2.3.6). These two enzymes do not cut within Ty1 elements. Southern blot analysis of mutant DNA indicated the presence of an 18 kb *Nhe*I band and a 20 kb *Xba*I band containing the *neo* gene (Figure 3.8B, lanes 4, 5 and 8). Ligation mixes were transformed into bacteria and amp resistant colonies were obtained and pooled. The *Nhe*I/*Spe*I bank contained a total of 34,000 insert containing transformants representing about ten yeast genomes. The *Xba*I/*Spe*I bank contained a total of 24,000 insert containing transformants representing about seven yeast genomes. The desired clones were identified using kanamycin resistance as a secondary selection since *whi3* clones should contain the *kan* gene. Many of the amp^R kan^R clones contained

inserts of the expected size when compared to the sizes of bands estimated from yeast genomic Southerns. Southern blot analysis was performed on digested plasmid DNA and specific hybridization was observed with Ty1 specific probes (data not shown). Many of the clones appeared to have rearranged in bacteria as specific bands were missing (data not shown). Two of the clones, #3 from the *XbaI/SpeI* bank and #12 from the *NheI/SpeI* bank were selected for further study.

Fragments containing the Ty element were liberated by digesting with enzymes which cut within the vector polylinker or liberated large insert containing fragments without digesting the tagged Ty1 element. These fragments were used to replace the wild-type locus with mutant DNA by gene replacement. The *WHI3*$^{+}$ strain, RN100-3d, was cotransformed with pRS315 and the fragment containing the Ty1 element. Cotransformation was required so that transformants could be selected independent of the chromosomal replacement event allowing cells time to establish and express the *neo* gene. Leucine prototrophs were selected and replica plated to G418 containing plates to identify resistant colonies. Once G418^{R} transformants were identified, those which had lost the plasmid were chosen. The cell volume of transformants with the replacement was measured with the Coulter Channelyzer. Compared to the *WHI3*$^{+}$ control strain, the median cell volume of stable G418 resistant transformants was reduced and comparable to the volumes of the original *whi3* isolates (Figure 3.6). Similar results were obtained when *whi3* fragments were prepared from either of the mutant bank plasmid clones with all of the enzyme combinations used to liberate the fragment (data not shown). This evidence strongly supports the conclusion that the tagged Ty element was responsible for the small cell size phenotype of the *whi3* mutant. This information was

Figure 3.6. Replacing the *WHI3* locus with DNA from the mutant locus results in a small cell size phenotype. Strain RN100-3d was transformed with a *Bam*HI-*Not*I fragment of the mutant *Xba*I bank clone #3. The control strain and transformant #6 were grown overnight in YEPD, sonicated and cell volume was measured with the Coulter Channelyzer. Cell volume distributions were plotted and median cell volumes determined. Median cell volumes (μm^3) are indicated.


1000
Cell number
WHI3 (36.1)
whi3 (28.5)
0
25
50
75
100
Cell volume (μm³)

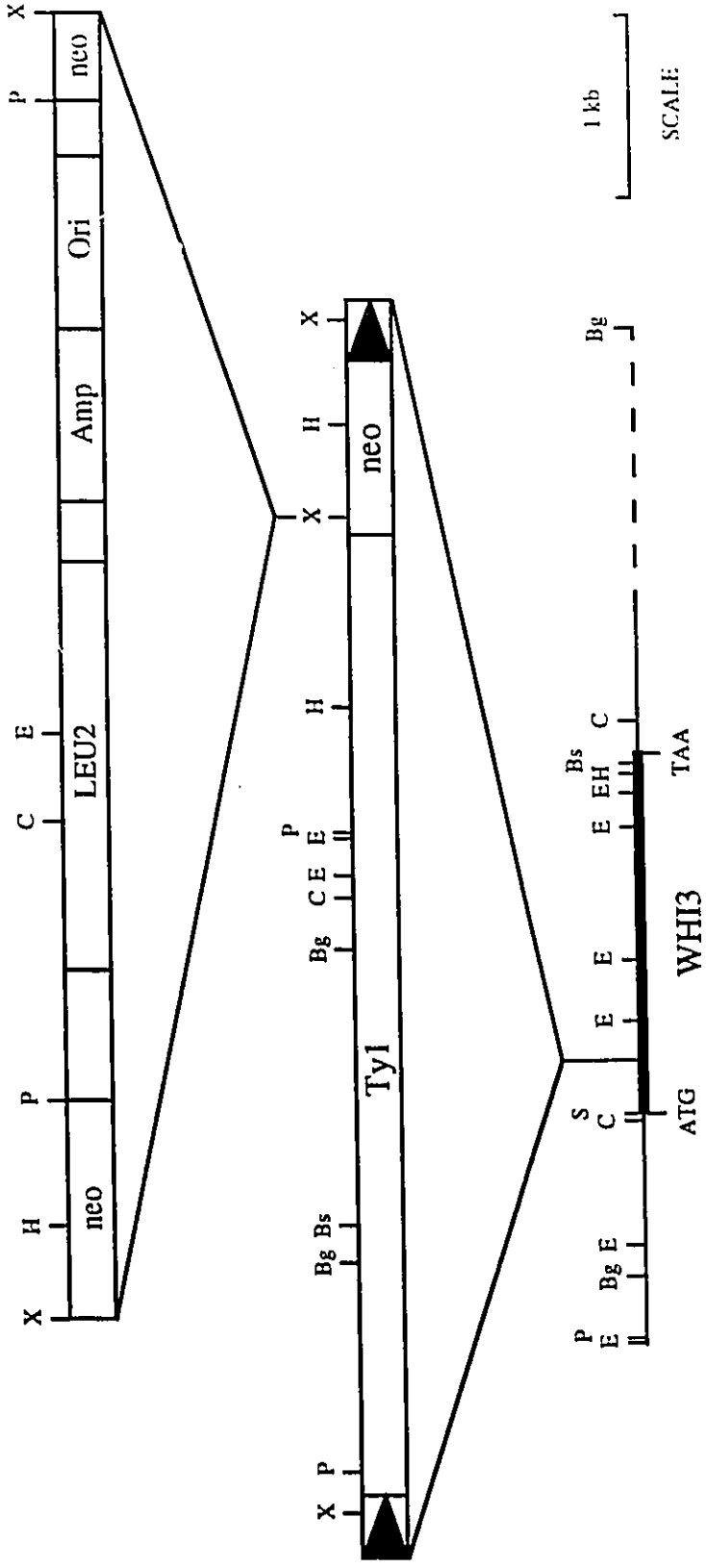
obtained chronologically after yeast DNA flanking the Ty element was cloned (Section 3.4).

3.4 Cloning of *WHI3* and Gene Dosage Studies.

The section above described the cloning of the whole *whi3* region. However, the first *whi3* flanking DNA sequences were obtained using a different strategy. An *E.coli* origin of replication was first integrated at the locus of the tagged Ty element by homologous recombination. This bypassed the requirement for a vector when cloning flanking yeast DNA as linear fragments encompassing this locus would have a bacterial origin of replication and two selectable markers for *E.coli*. The integrating plasmid, pRS305neo (see Section 2.3.6) was linearized within the *neo* gene at the *Xho*I site to direct integration of the origin to the tagged Ty locus. The *whi3* strain T504b-1c was transformed with this fragment and Southern blot analysis was performed on integrants (data not shown). Candidates that contained a tagged Ty element with an internal duplication of the *neo* gene flanking the yeast *LEU2* gene, the amp resistance gene and an *E.coli* origin of replication were identified. The structure of the resultant locus is diagrammed schematically in Figure 3.7.

Yeast genomic DNA flanking the site of insertion was cloned by digesting DNA from T504b-1c A17-*Xho*I with *Eco*RI. *Eco*RI cuts within the *LEU2* gene and beyond the 3' end of the tagged Ty element (Figure 3.7). *Eco*RI digested DNA was then ligated intramolecularly and kanamycin-ampicillin resistant transformants were selected. These clones contained yeast DNA flanking the 3' end of the site of Ty insertion. This flanking DNA was subcloned into pUC119 on a *Hind*III-*Eco*RI fragment and a *Xho*I-*Eco*RI fragment (Figure

Figure 3.7. Restriction map of the wild-type and mutant loci. The *WHI3* open-reading frame is indicated by the solid bar at the bottom and the orientation is as shown. The restriction sites indicated on the solid line (sequenced DNA) were determined by restriction digests and sequencing. The position of the *Bgl*II site located at the end of the dashed line is approximate. The position of insertion of the tagged Ty1 element and linearized pRS305neo insertion are indicated. Selected restriction sites are shown and are as follows: Bg: *Bgl*II, Bs: *Bst*EII, C: *Cla*I, E: *Eco*RI, H: *Hind*III, P: *Pvu*II and X: *Xho*II. Some restriction site information was adapted from Sikorski and Hieter (1989) and Boeke (1989).


X
P
neo
Ori
Amp
LEU2
C
E
P
H
neo
X
1 kb
SCALE
Bg
X
H
neo
X
H
P
C E E
Bg
Ty1
Bg Bs
X P
Bs
EH
C
E
TAA
E
E
WHI3
S
C
ATG
P
E
Bg E

3.7). Single-stranded DNA was prepared and sequenced using the universal primer. This sequence was used to design oligonucleotide probes (WHI301 and WHI302, see Table 2.2) for the purpose of screening a high copy number yeast library for the intact wild-type region. A YEp213 based library containing yeast DNA from a partial *Hind*III digest was chosen for this purpose (Toda *et al.*, 1987b). Approximately 16,000 colonies representing 15-20 yeast genome equivalents were screened with a mixture of the two oligos. A number of potential clones were identified and two of these clones, 7 and 27, were selected for further study after passing a secondary screen designed to identify colonies which hybridized with both oligos individually.

A lambda clone was later received from L. Riles in M. Olson's lab. To obtain the lambda clone a 1.3 kb *Eco*RI fragment was subcloned from the bank clone 27 and gel purified. This fragment was chosen as it hybridized with the oligos WHI301 and WHI302 and specifically hybridized with one genomic band in high-stringency southerns (Figure 3.8). The Olson lab has constructed a lambda library composed of an ordered array of overlapping clones covering the majority of the 14,000 kb yeast genome. This ordered array was probed with the 1.3 kb *Eco*RI fragment and a hybridizing clone (5025) was identified. The lambda phage which contained the hybridizing insert was sent to our lab and used to prepare lambda DNA containing the insert of interest.

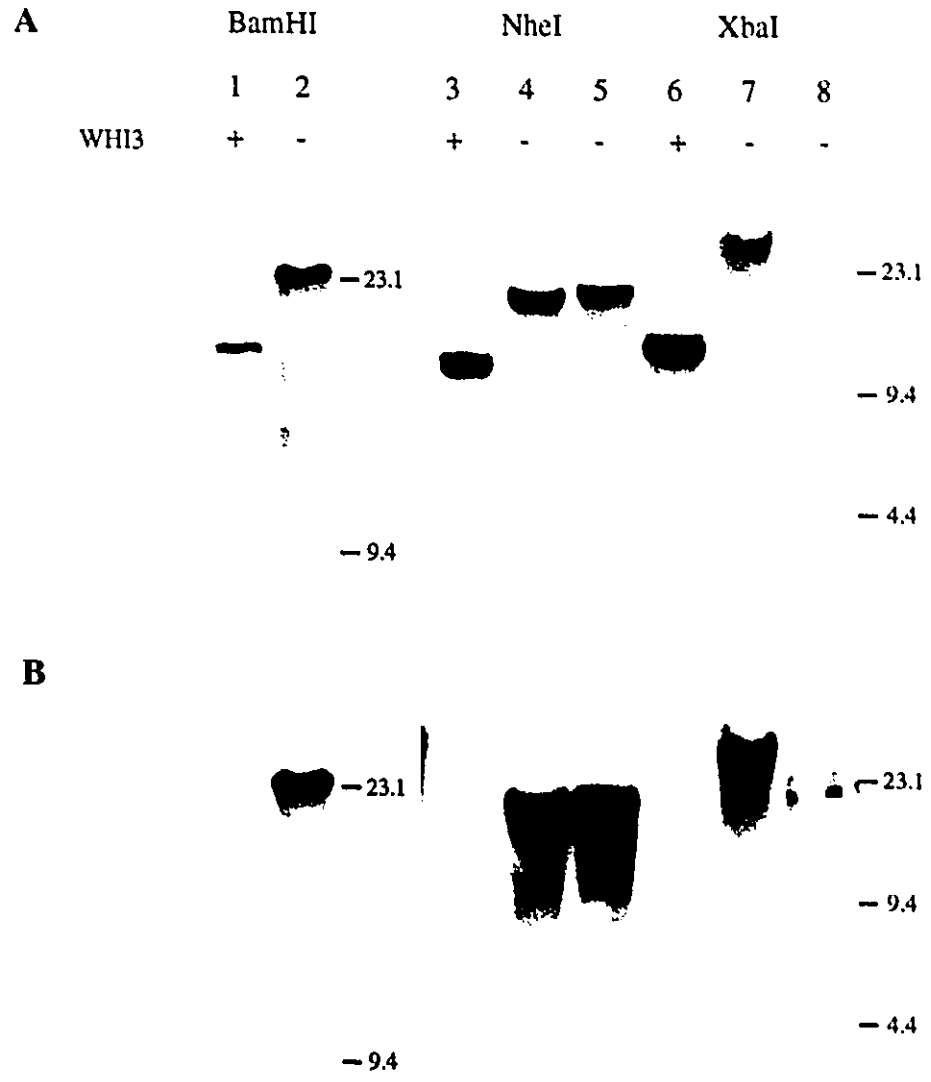
Complementation of the small cell size phenotype of *whi3* was first attempted with the library clones 7 and 27. Clone 7 had an insert of about 13 kb and clone 27 had an insert of about 12.5 kb. Mutant and wild-type strains were transformed with the library clones but no consistent cell size effects were observed (data not shown). Clone 7 was later found to have an incomplete open-reading frame, an artifact of the library construction (see Section 3.7).

The lack of complementing activity associated with clone 27 may relate to the toxic effects associated with expressing *WHI3* at high copy number (see below).

Next, a 5.2 kb *Bgl*II fragment isolated from either the bank clone 27 or the lambda clone 5025 was subcloned into both single and high copy number vectors. This fragment was chosen for a couple of reasons. First, it hybridized with the oligos WHI301 and WHI302 both of which presumably hybridized with the target gene. Second, contained within the 5.2 kb fragment was a 1.3 kb *Eco*RI fragment that hybridized with the same oligos. When the 1.3 kb *Eco*RI fragment was labelled and used as a probe it hybridized with an 11 kb *Nhe*I band and a 13kb *Xba*I band in the wild-type strain T504b-1d and a 16kb *Bam*H1 fragment in RN100-1a (Figure 3.8A, lanes 1, 3 and 6). The size of these hybridizing bands increased by about 7 kb, the size of the tagged Ty1 element, in the *whi3* strains T504b-5c and T504b-1c (Figure 3.8A, lanes 2, 4, 5 and 8). In addition, the band present in lanes containing mutant DNA hybridized with a *neo* specific probe while wild-type DNA did not hybridize with this probe (Figure 3.8B). These results strongly suggested that the *Bgl*II fragment encompassed the genomic position into which the Ty element had transposed in the *whi3* mutant. It was possible that this fragment also contained the full open-reading frame.

The *Bgl*II fragment was gel purified from clone 27 and lambda clone 5025 so that both sources could be examined for complementing activity. These fragments were subcloned into pRS306, pRS315 and pRS425 to create the integrating versions: pRN163a, pRN163b and pRN200b, the centromeric *WHI3* plasmids: pRN202a, pRN202b and pRN203b as well as the high copy number *WHI3* plasmids: pRN204b, pRN205a and pRN206a (see Section 2.3.6).

Figure 3.8. Southern analysis of the *WHI3* locus in mutant and wild-type strains. Genomic DNA was prepared from wild-type and *whi3* mutant strains, digested with *Bam*HI (lanes 1-2), *Nhe*I (lanes 3-5) and *Xba*I (lanes 6-8) and electrophoresed through 0.5% agarose gels. The DNA in lane 7 was undigested. Gels were transferred to nytran and probed with a 1.3 kb *Eco*RI fragment of the *WHI3* gene (panel A) and a 1 kb *Pvu*II-*Hind*III fragment of the *neo* gene (panel B). Lanes 1-8 correspond to the strains RN100-1a, T504b-1c, T504b-1d, T504b-1c, T504b-5c, T504b-1d, T504b-3d and T504b-5c respectively. Relevant genotypes are indicated as are the sizes (kb) of *Hind*III digested lambda DNA.


A
BamHI
NheI
XbaI
1 2 3 4 5 6 7 8
WHI3 + - + - - + - -
23.1
9.4
4.4
B
23.1
9.4
4.4

A great deal of care had to be exercised when propagating these constructs as they were obtained at a very low frequency and when bacteria containing the constructs were propagated they grew slowly and deleted segments of the insert by recombination. Plasmids were checked frequently and complementation analysis was performed with all of the replicating constructs and numerous yeast transformants were analyzed for each construct.

Centromeric plasmids were transformed into both *whi3*$^-$ (T504b-6a #7) and *WHI3*$^+$ (BWG1-7a) strains. While the *cen* control vector caused essentially no change in cell volume, *whi3* and *WHI3*$^+$ strains transformed with single copy vectors containing the 5.2 kb insert from either source increased cell volume by 25%-30% in defined media (Figure 3.9). This provided the first evidence that the 5.2 kb fragment contained complementing activity and suggested that the gene affected cell volume in a dose-dependent manner. The dose dependence associated with increasing copy number is significant as this suggests that the cell is measuring some parameter such as size or mass using the Whi3 protein as its metric.

One and two copies of the complementing fragment were also integrated at the chromosomal *ura3* locus of a *whi3* strain. The integrating plasmid pRN200b, which contained the 5.2 kb *Bgl*II fragment from lambda clone 5025, was digested at a unique *Nco*I site located within the *URA3* gene. The linearized plasmid was used to transform the mutant strain RN100-1d #3, a *ura3*$^-$ derivative of RN100-1d created by UV-mutagenesis and 5-FOA selection. This directed integration of the fragment to the *ura3* locus of the mutant strain. Integrants with one or two tandem copies of the vector were identified by Southern blot analysis (data not shown). Integration of one copy of the fragment increased the median cell volume of the mutant strain by 25%

to about the size of a wild-type strain and integration of two copies increased the median cell volume by a total of 37% (Figure 3.10A). This evidence corroborates the *cen* plasmid results and strengthens the conclusions concerning a complementing activity and dose-dependent activity associated with the 5.2 kb fragment.

The integrant with two copies of the wild-type gene at the *ura3* locus was crossed to a closely related wild-type strain to create RN230. Random segregation of the unlinked *URA3* and *WHI3* loci resulted in spore clones with 0, 1, 2 or 3 copies of the *WHI3*$^+$ gene. The size profiles of spore clones from a tetrad of RN230 with all possible copy number combinations are shown in Figure 3.10B. With each additional copy of the complementing *WHI3* fragment cell volume increased as did the size heterogeneity of the population. These results further extend the observations described above and together strongly suggest that the wild-type *WHI3* gene had been cloned.

To further increase the copy number of the *WHI3* gene, high copy (2μm) vectors with the *Bgl*II fragment were transformed into the mutant strain T504-6a #7 and the wild-type strain BWG1-7a. While vector alone (pRS425) caused no significant increase in cell volume, *whi3* and *WHI3* strains transformed with pRN204b, pRN205a and pRN206a displayed an increase in the median cell volume. An example of one of the the Coulter plots is shown in Figure 3.11. These effects were difficult to interpret because of the size heterogeneity of the profile. The heterogeneity may result from inefficient plasmid segregation and hence unequal partitioning during mitosis. This would lead to plasmid copy number variability and increase the size distribution. In addition, peaks became somewhat biphasic similar to strains grown on poor carbon sources.

Figure 3.9. Single copy *WHI3* plasmids complement the cell size defect of the mutant and increases the cell size of a wild-type strain. Transformants of the mutant strain T504b-6a #7 (panel A) and the wild-type strain BWG1-7a (panel B) containing a *cen* based control vector (pRS315) or a *cen* based *WHI3* plasmid (pRN202a) were innoculated into defined media and grown overnight. Mid-log phase cultures were sonicated and aliquots were diluted in isoton. Cell volume profiles were measured with the Coulter Channelyzer and median cell volumes were determined. Median cell volumes (μm^3) are indicated.

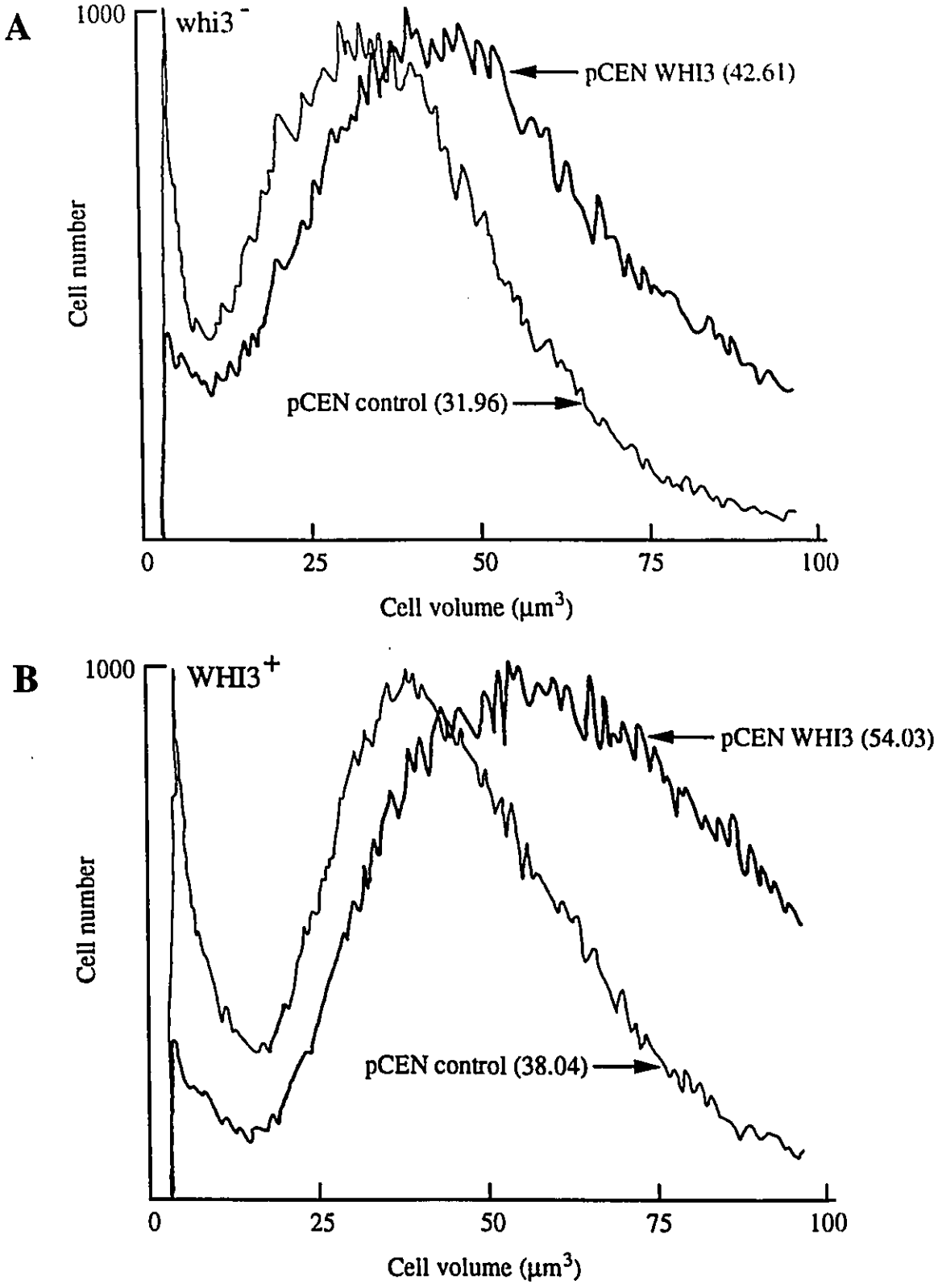

A
1000
whi3⁻
pCEN WHI3 (42.61)
pCEN control (31.96)
Cell number
0
25
50
75
100
Cell volume (μm³)
B
1000
WHI3⁺
pCEN WHI3 (54.03)
pCEN control (38.04)
Cell number
0
25
50
75
100
Cell volume (μm³)

Figure 3.10. Integration of extra copies of the *WHI3* gene increases cell size in a dose-dependent manner. One and two copies of the *WHI3* complementing fragment were integrated at the *URA3* locus of the mutant strain RN100-1d #3 using pRN200b DNA linearized at the *URA3* gene with *Nco*I. Cell volume profiles of the control strain and transformants are shown in panel A. Cell volume profiles of spore clones from the tetrad RN230-3, the result of a cross between the 2x *WHI3* integrant and a wild-type strain are show in panel B. Strains were grown to mid-log phase in YEPD, sonicated and aliquots were diluted in isoton. Cell volume profiles were measured with the Coulter Channelyzer and median cell volumes were determined. Median cell volumes (μm^3) are indicated.

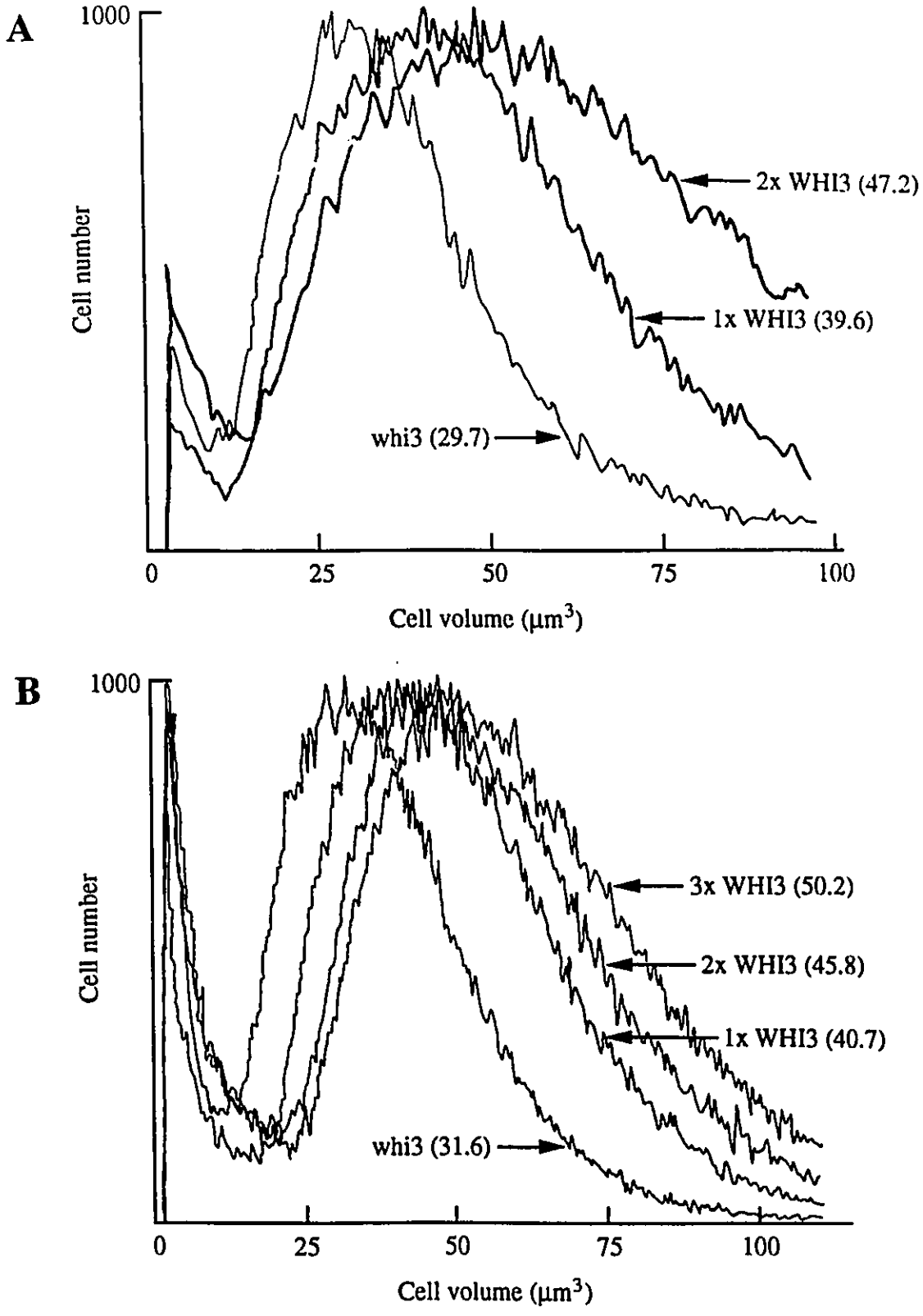
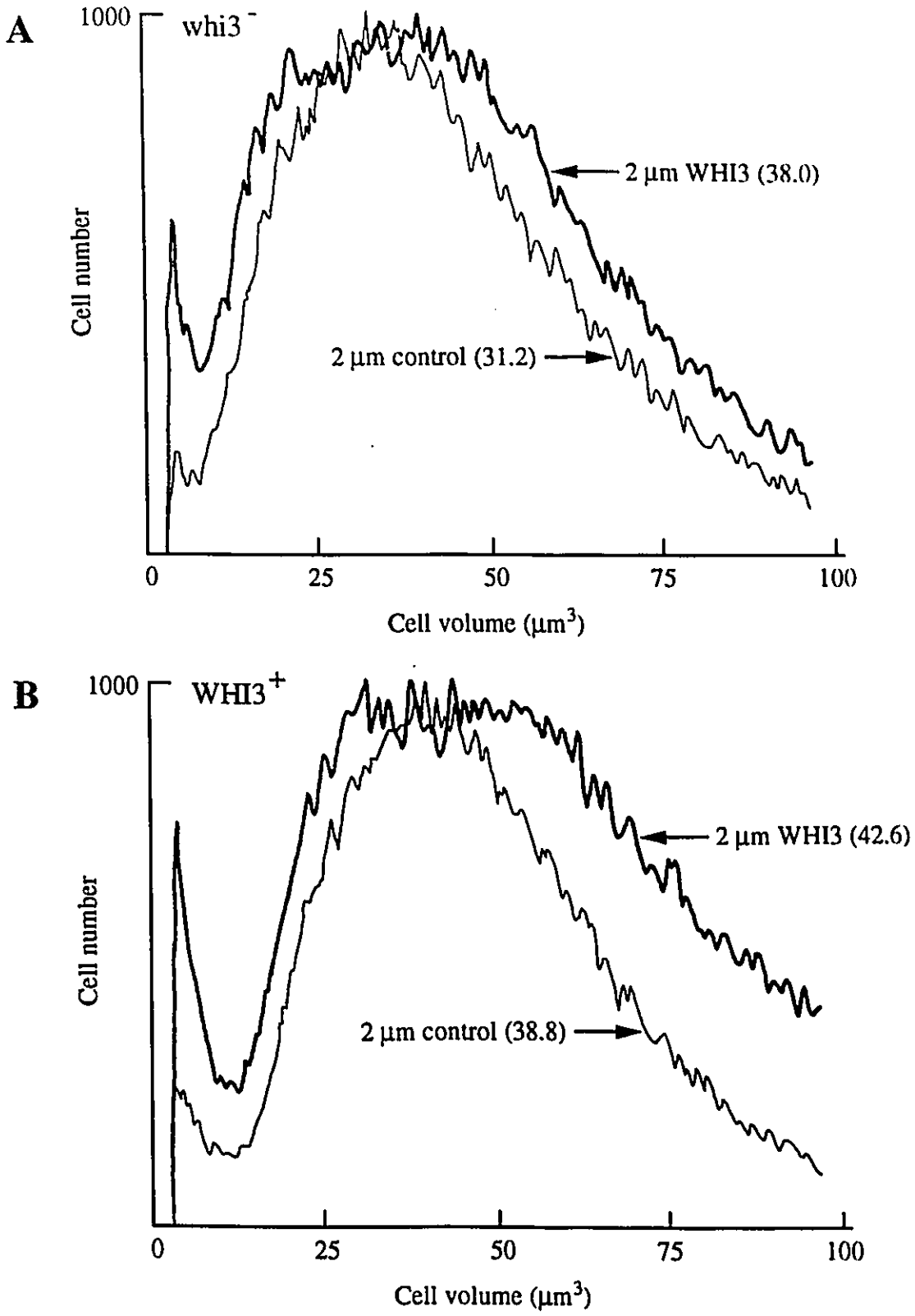

A
1000
Cell number
2x WHI3 (47.2)
1x WHI3 (39.6)
whi3 (29.7)
0
25
50
75
100
Cell volume (μm³)
B
1000
Cell number
3x WHI3 (50.2)
2x WHI3 (45.8)
1x WHI3 (40.7)
whi3 (31.6)
0
25
50
75
100
Cell volume (μm³)

Figure 3.11. High-copy number *WHI3* plasmids affect cell size of wild-type and mutant strains. Transformants of the mutant strain T504b-6a #7 (panel A) and the wild-type strain BWG1-7a (panel B) containing pRS425 (control vector) or pRN205a (panel A) and pRN206a (panel B), two high copy number *WHI3* vectors were grown to mid-log phase in defined media. Cultures were sonicated and cell volumes measured with the Coulter Channelyzer. Median cell volumes are indicated (μm^3).


A
1000
whi3⁻
2 µm WHI3 (38.0)
2 µm control (31.2)
Cell number
0
25
50
75
100
Cell volume (µm³)
B
1000
WHI3⁺
2 µm WHI3 (42.6)
2 µm control (38.8)
Cell number
0
25
50
75
100
Cell volume (µm³)

Wild-type transformants containing high copy *WHI3* plasmids grew very slowly compared to vector alone controls. To assess the toxic or deleterious effects of elevating the copy number of *WHI3* the rate of plasmid loss was measured for mutant and wild-type strains containing the various plasmid constructs. Mutant and wild-type transformants were streaked out for single colonies on non-selective media and plates were incubated for 3 days. After replica plating back to selective media the number of colonies which were unable to grow as a result of plasmid loss was determined. The rate of plasmid loss was similar to vector alone controls for mutant strains containing the *WHI3* gene on a *cen* plasmid and slightly elevated in wild-type strains containing single copy *WHI3*. The rate of loss for 2 μm control vectors was about 10% in mutant and wild-type strains. In comparison, the rate of loss of high copy number *WHI3* plasmids was between 65 and 70% in a *whi3*$^-$ strain (T504b-6a #7) and between 90 and 95% in a wild-type strain (BWG1-7a). Increasing the copy number of the *WHI3* gene has toxic effects on yeast cells and may explain why cell volume did not increase to the expected level in transformants. Cells which contained a higher plasmid copy number due to poor partitioning would grow more slowly and be selected against. As a result cells with a lower plasmid copy number would have a selective advantage and a smaller effect on cell size.

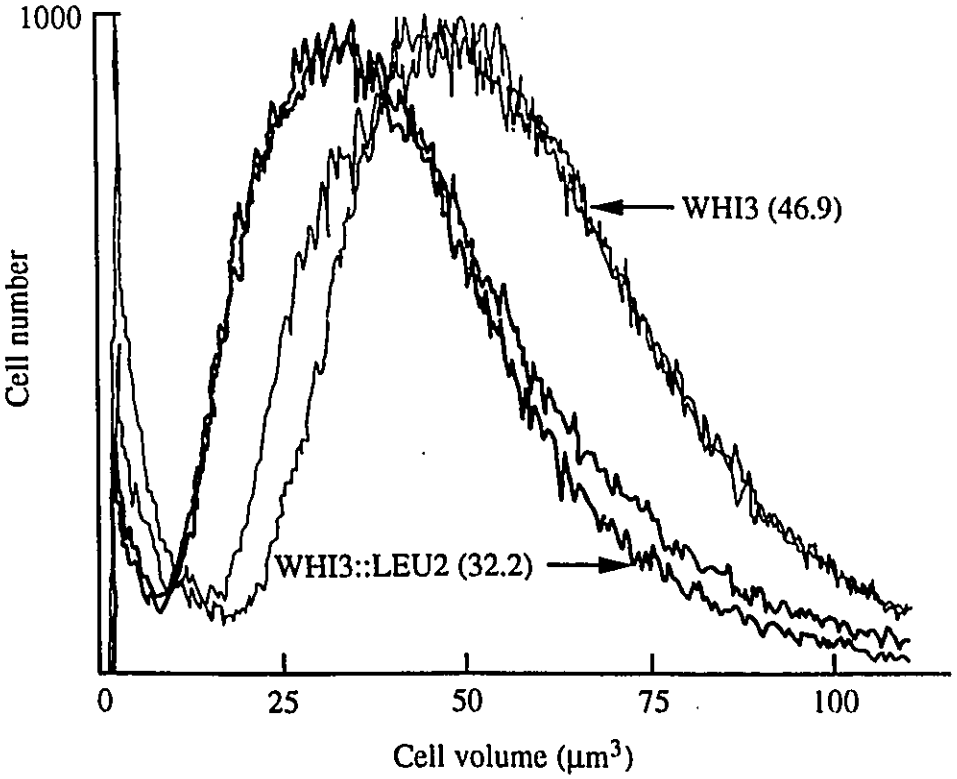
3.5 The *WHI3* Null Allele has a Small Cell Size Phenotype.

To determine the phenotypic consequences resulting from loss of the *WHI3* gene product, the chromosomal locus was deleted. This experiment would confirm whether the Ty element had caused the mutation by inserting

into the *WHI3* open-reading frame. In addition, it would confirm whether the Ty insertion was a loss of function mutation. Before the replacement construct was made, information concerning the size and sequence of the open-reading frame was obtained (see Section 3.7). A 1.9 kb *Sph*I to *Bst*EII fragment (see Figure 3.7) containing the majority of the *WHI3* open-reading frame was replaced with a 2.1 kb *Hind*III fragment containing the yeast *LEU2* gene (see Section 2.3.6). A *Not*I-*Xho*I fragment containing the replaced *WHI3* gene and flanking sequences was introduced into a diploid strain by gene replacement. Southern blot analysis was used to select diploids in which one copy of *WHI3* had been replaced with the *LEU2* gene. Heterozygous diploids were sporulated and tetrads were dissected. A clear 2:2 segregation for small cell size was observed when spore clones from individual tetrads were sized and this phenotype cosegregated with leucine prototrophy (Figure 3.12). Southern blot analysis was used to confirm that cosegregation observed resulted from the proper replacement of the *WHI3* gene with *LEU2* (data not shown). The deletion of *WHI3* therefore resulted in the same small cell size phenotype as the original Ty1 induced *whi3* mutant. This provided additional proof that the Ty element caused the cell size phenotype by inserting into and inactivating a size control gene. When haploid wild-type and mutant strains were transformed with plasmid borne copies of the *whi3* allele there was no effect on cell size. This result is also consistent with a loss of function mutation (data not shown).

To summarize, the evidence obtained suggested that the *WHI3* gene had been cloned. Wild-type clones were isolated using oligonucleotide probes designed using the sequence of the yeast DNA flanking the site of Ty insertion. A subcloned fragment was obtained from one of these clones and independently from a lambda clone and both were able to complement the small cell size

Figure 3.12. Chromosomal replacement of *WHI3* results in a small cell size phenotype. Spore clones of the tetrad RN210-3 were grown to mid-log phase in YEPD. RN210-3a and 3b are *WHI3::LEU2* and RN210-3c and 3d are *WHI3*$^+$. Cultures were sonicated, diluted in isoton and cell volume was measured with the Coulter Channelyzer. Cell volume distributions were plotted and median cell volumes determined. Median cell volumes (μm^3) are indicated.


1000
Cell number
WHI3 (46.9)
WHI3::LEU2 (32.2)
0
25
50
75
100
Cell volume (μm³)

phenotype of the *whi3* mutant. In addition, these inserts also increased the size of wild-type strains. When the *WHI3* open reading frame was replaced with the *LEU2* gene, cells became phenotypically *Whi*$^-$. This supports the idea that the small cell size phenotype of the tagged Ty mutant resulted from the transposition of this element into *WHI3*, knocking out gene function. The results also suggest that the cell may be measuring size or mass using the dose dependent Whi3 protein as a metric.

3.6 Mapping the Chromosomal Location of *WHI3*

The genomic position of the *WHI3* gene was mapped using yeast chromoblots to identify the chromosome. The precise physical map position was then identified by L. Riles with the lambda filter library that contains an ordered array of overlapping genomic inserts. To prepare chromoblots a set of strains (YPH49, YPH80 and YPH149) were obtained from Phil Hieter (John Hopkins School of Medicine, Baltimore). These strains were selected for mapping studies as the majority of the yeast chromosomes in these strains are readily resolved when run on pulse field gels. Yeast chromosomal DNA was prepared in agarose plugs to maintain chromosomal integrity by minimizing shearing forces. Chromosomes were separated by contour-clamped-homogeneous-electric-field (CHEF) electrophoresis using an electrode configuration that generates electric fields in alternating orientations. The gel was transferred to a nylon membrane and hybridized with the 1.3 kb *Eco*RI fragment. Hybridization localized *WHI3* to chromosome XIV or chromosome II but these two chromosomes run very close to one another as they have a similar size. To differentiate between these two chromosomes the *RAS2* gene

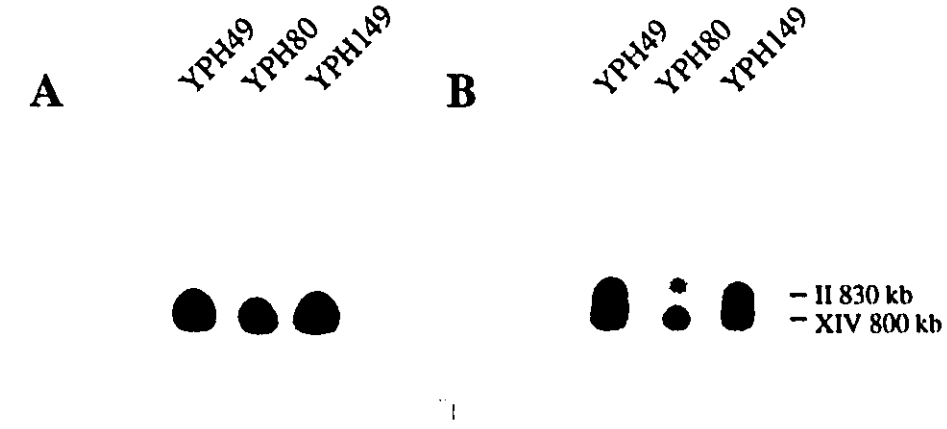
was obtained from M. Wigler's lab (Cold Spring Harbor Labs) as it resides on chromosome XIV and a PCR fragment of the *CDC28* gene was used as a chromosome II specific probe. These fragments were used in combination with the 1.3 kb *Eco*RI fragment to probe chromoblots. When probed with *RAS2* and *WHI3* one band was visualized by autoradiography (Figure 3.13A). When *CDC28* and *WHI3* were used as probes, two bands appeared (Figure 3.13B). The *WHI3* gene therefore resides on chromosome XIV as the mixture of the *RAS2* and *WHI3* probes hybridized only to chromosome XIV while the mixture of the *CDC28* and *WHI3* probes hybridized with sequences from both chromosome II and XIV.

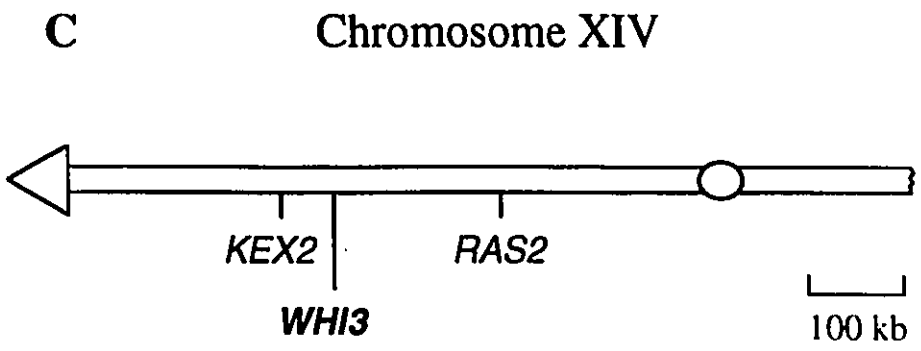
To confirm this result and more precisely position the *WHI3* gene the 1.3 kb *Eco*RI fragment was sent to L. Riles in M. Olson's lab (Washington University Medical Center, St Louis). This fragment was labeled and hybridized with the lambda filter library of Olson *et al.* (1986). Hybridization to clone 5025 placed *WHI3* about 280 kb from the telomere on the long arm of chromosome XIV between *KEX2* and *RAS2* on the linkage map (Figure 3.13C).

3.7 Sequence Analysis of the *WHI3* Gene and the Site of Ty Insertion

To sequence the *WHI3* gene a combined strategy was used. The 1.3 kb *Eco*RI fragment was single and double strand sequenced with the universal and the reverse primer. This fragment and two *Eco*RI-*Sph*I fragments that comprise the 1.3 kb fragment (Figure 3.7) were subcloned into pUC119 and sequenced. The rest of the *WHI3* gene was sequenced on both stands with a series of designed oligonucleotides (Table 2.2). When the two wild-type clones, 7 and 27 were sequenced, a discrepancy was observed starting 125 nucleotides

Figure 3.13. The *WHI3* gene is located on the left arm of chromosome XIV between *KEX2* and *RAS2*. Chromosome sized DNA was prepared and chromosomes were seperated by CHEF electrophoresis. Chromoblots were hybridized with mixed probes. In panel A, the 1.3 kb *Eco*RI fragment, specific for the *WHI3* gene and a 1.2 kb *Hpa*I fragment of the *RAS2* gene were used as probes. In panel B, the 1.3 kb *Eco*RI fragment and an internal PCR fragment of the *CDC28* gene were used as probes. The sizes of chromosomes II and XIV are indicated. A schematic diagram of the left arm of chromosome XIV is shown in panel C. The position of the *WHI3* gene in relation to the telomere and centromere is indicated. The scale of the diagram is as indicated.


A
YPH49
YPH80
YPH149
B
YPH49
YPH80
YPH149
– II 830 kb
– XIV 800 kb


C
Chromosome XIV
KEX2
WHI3
RAS2
100 kb

from the 3' end of the putative *WHI3* ORF. The sequence of the two clones was found to be different after the *Hind*III site. When the clones from the mutant bank were sequenced they were found to be identical to the wild-type clone 27 after the *Hind*III site. This may explain the inability of clone 7 to complement the size defect. During the construction of the partial *Hind*III library two non-contiguous genomic fragments must have ligated into the same vector resulting in an incomplete ORF. A total of 4 kb was sequenced using clone 27 as the template and an open-reading frame of 1983 nucleotides was located using the Intelligenetics sequence analysis package (Figure 3.14). The molecular mass of the deduced protein sequence was calculated to be 71,257 Da and the isoelectric point was calculated to be 8.65 (IgSuite).

Analysis of the 5' end of the ORF suggests that the 5' most AUG is the probable initiator codon. An in-frame stop codon (TAG) is located 54 nucleotides upstream from the predicted start codon and several out of frame stop codons are also present. In addition the next in-frame start codon is 216 nucleotides downstream. The 5' most AUG is embedded in the sequence AGCAUGC which is similar to the eukaryotic translation initiation consensus ACCAUGG especially at the -3 position where a purine has been shown to be required (Kozak, 1986). The region upstream of the *WHI3* open-reading frame is AT rich and this is a common feature of yeast promoter and and intergenic regions (Struhl, 1985). No striking similarities were found when the upstream region was compared with various compiled yeast promoter consensus sequences but a few weak matches to the yeast TATA element were found (Verdier, 1990).

The position of insertion of the tagged Ty element was determined by sequencing the *whi3* mutant library clones (3 and 12) with the oligos WHI301

and WHI304. The *whi3* mutation resulted from the transposition of the tagged Ty1 element between positions +289 and +290 in the open reading frame (Figure 3.14 and 3.15). Integration of the Ty element was accompanied by a duplication of the five base pair target sequence ATAAC located between position +285 to +289 (Figure 3.15). This 5 bp duplication is typical of a Ty element.

The deduced amino acid sequence of WHI3 contains several interesting features. The translated sequence was compared to the contents of the available databases using the FASTA homology search program (Pearson and Lipman, 1988) and to the contents of a database compiled by Mark Goebl (University of Indiana School of Medicine). Both searches revealed homology between the C-terminus of Whi3 and a motif found within a large group of RNA binding proteins (Figure 3.16). This conserved motif has been variously termed the RNA recognition motif (RRM), the ribonucleoprotein (RNP) motif or the consensus type RNA binding domain (CS-RBD) (Banziudis *et al.*, 1989; Kenan *et al.*, 1991). The RNA binding domain is 80-90 amino acids in length and is defined by a conserved octamer called RNP-1 and a conserved hexamer called RNP-2 (Banziudis *et al.*, 1989; Kenan *et al.*, 1991). The Whi3 sequence had a moderate degree of homology with the RNP-1 octamer consensus (amino acids 582-589) and stronger homology with the RNP-2 hexamer consensus (amino acids 540-546) (Figure 3.16). In addition, alignment of this region of *WHI3* with several members of the RRM containing family of proteins indicated that additional conserved residues were located between the two RNP motifs and beyond RNP-1 (Figure 3.16).

Figure 3.14. Sequence of the *WHI3* gene. The nucleotide sequence and the primary amino acid sequence of the open-reading frame are shown. The position of the Ty1 insertion is indicated. The glutamine rich region is underlined and the conserved RNP1 and RNP2 motifs of the RRM are boxed. Selected restriction sites are indicated. Nucleotide position is shown on the left and amino acid position on the right.

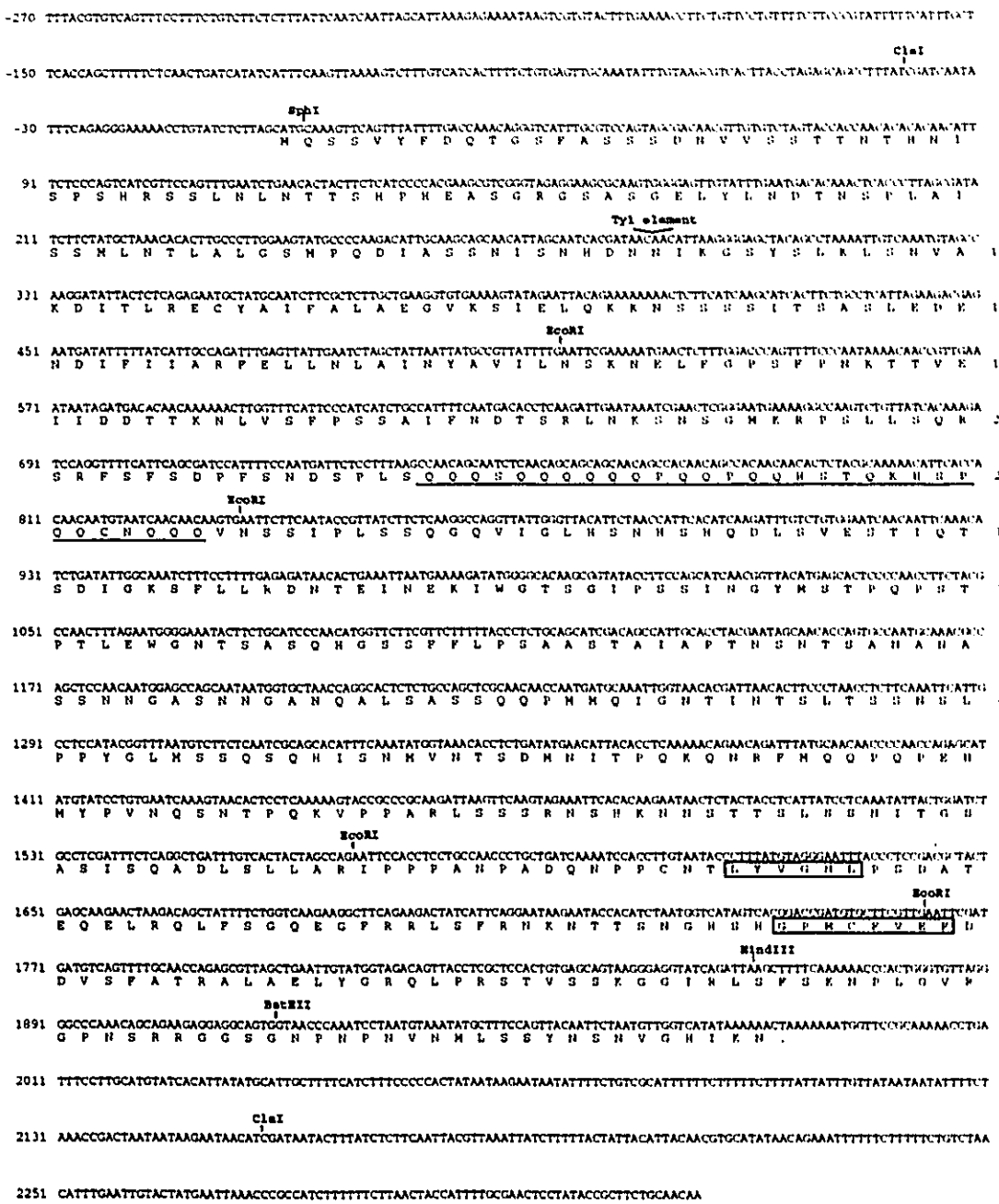

ClaI
SphI
Tyl element
EcoRI
EcoRI
EcoRI
EcoRI
HindIII
BstEII
ClaI

Figure 3.15. Sequence surrounding the site of Ty1 insertion. The sequence surrounding the position of insertion of the Ty1 element was determined using the oligos WHI301 and WHI304. Template DNA was prepared from the 3' flanking sequences subcloned in pUC119 and from the mutant clones 3 and 12. The corresponding wild-type sequence is shown below. The nucleotide position relative to start of the open-reading frame is indicated.

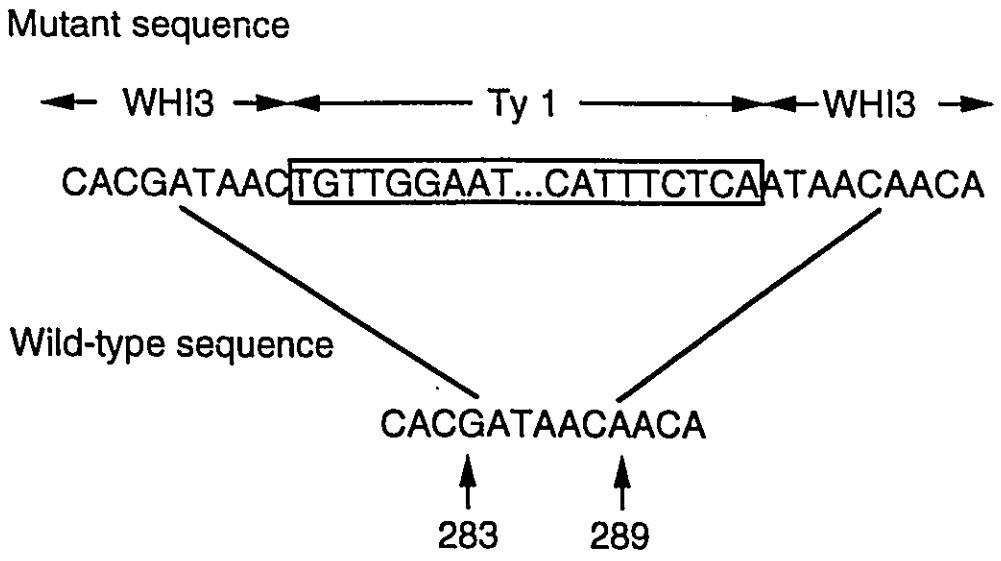
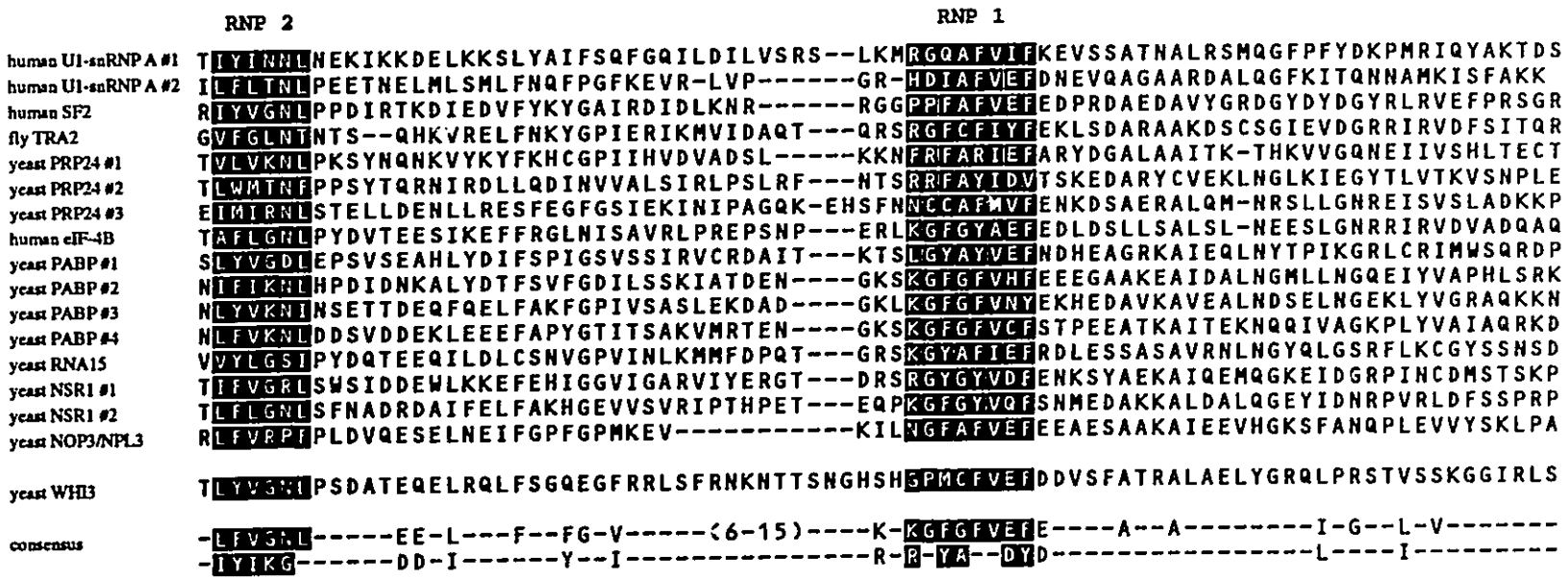

Mutant sequence
WHI3
Ty 1
WHI3
CACGATAACTGTTGGAAT...CATTTCTCAATAACAACA
Wild-type sequence
CACGATAACAACA
283
289

Figure 3.16. Alignment of the WHI3 RNA binding domain with selected RNA recognition motifs. The RRMs shown are from a variety of RRM containing proteins and many of these chosen were from yeast. The alignment is anchored on the highly conserved RNP1 and RNP2 motifs, shown in reverse type and is based on the alignments in Banziulis *et al.* (1989) and Kenan *et al.* (1991). Sequences were obtained from the following sources: Banziulus *et al.* (1989); Kenan *et al.* (1991); Krainer *et al.* (1991); Russell and Tollervey (1992); Bossie *et al.* (1992); Minvielle-Sebastia *et al.* (1991). The RNP consensus is based on that of Banzuilis *et al.* (1989); Takagaki *et al.* (1992); Kim and Baker (1993).


RNP 2
RNP 1
human U1-snRNP A #1
human U1-snRNP A #2
human SF2
fly TRA2
yeast PRP24 #1
yeast PRP24 #2
yeast PRP24 #3
human eIF-4B
yeast PABP #1
yeast PABP #2
yeast PABP #3
yeast PABP #4
yeast RNA15
yeast NSR1 #1
yeast NSR1 #2
yeast NOP3/NPL3
yeast WHI3
consensus

A second feature of interest is a glutamine rich region located between amino acids 247-277 where 19 of the 31 residues are glutamines (61%) (Figure 3.14). In addition, the protein contains 10.6% asparagine residues. A third sequence feature of interest is the overall serine richness of the protein. The WHI3 protein contains 18% serine and 6.35% threonine residues accounting for a total of 24.35% of the protein. The significance of these two features with regard to the biological role of the protein are unknown.

There are numerous potential glycosylation (Asn X Ser/Thr) and phosphorylation sites present in the Whi3 protein. The codon bias of *WHI3* was calculated to determine the predicted level of expression of the gene and was found to be 0.13 (Bennetzen and Hall, 1982). This suggests that the protein is not abundant. For comparison the codon bias for *CLN3*, which encodes a rare protein, is 0.27 and the codon bias for *ADH1*, an abundant protein, is 0.92 (Nash *et al.*, 1988, Bennetzen and Hall, 1982).

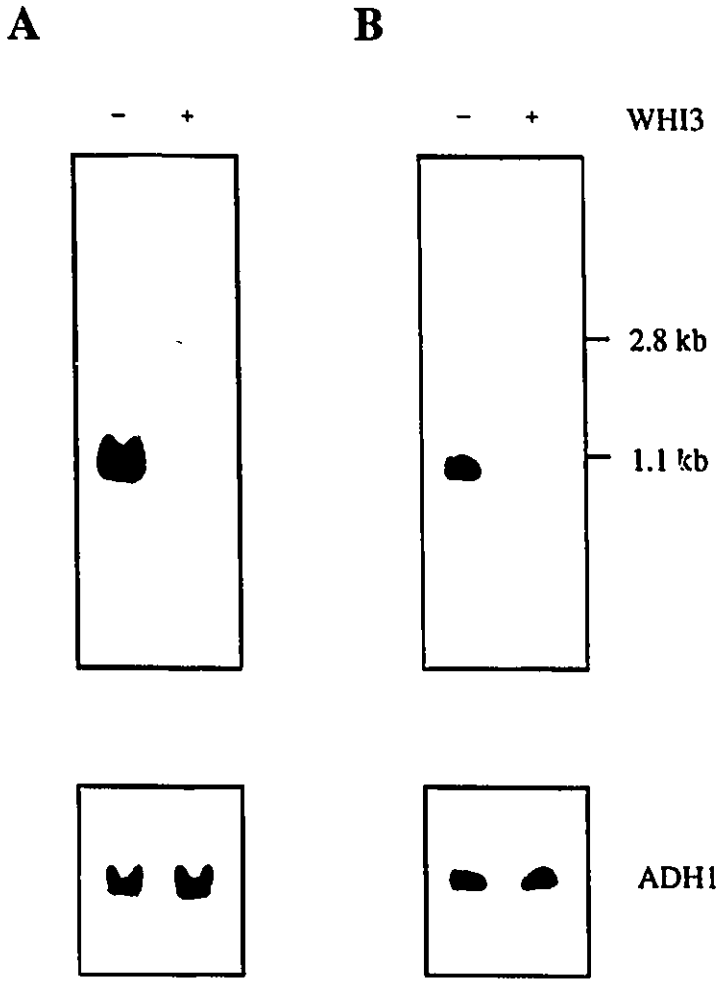
3.8 Analysis of the *WHI3* Transcript.

Northern blots were used to analyze the *WHI3* transcript in mutant and wild-type strains. RNA was prepared from isogenic mutant (T504b-1c) and wild-type (T504b-1d) strains. Gels were transferred and membranes hybridized with the 1.3 kb *EcoRI* probe which flanks the site of Ty insertion. A transcript of about 2.8 to 2.9 kb was identified in *WHI3*$^+$ strains (Figure 3.17A). *whi3*$^-$ mutants contained a much smaller transcript of about 1.1 kb (Figure 3.17A). The truncated mutant transcript was present at much higher levels than the wild-type transcript (Figure 3.17A). Northern blots were normalized by probing with the yeast *ADH1* (alcohol dehydrogenase) gene (Figure 3.17A). Northern

analysis was repeated using RNA prepared from several different spore clones and similar results were obtained (data not shown). When the wild-type message levels were compared to others such as *ADH1* it was evident that the message was not abundant. The insertion of the Ty1 element had affected both the size and abundance of the transcript.

There were two possible explanations for the origin of the shorter mutant transcript. A fusion transcript may have initiated within the 3' LTR of the Ty1 element and continued into the *WHI3* gene or may have initiated at the normal *WHI3* transcriptional start site and proceeded into the Ty element until encountering a transcriptional terminator. To distinguish between these alternatives Ty-WHI3 junction oligos were designed. These oligos were 16 nucleotides long and spanned the junction between *WHI3* and Ty at the 5' or 3' ends of the site of transposition. When used as probes the junction oligo complementary to the fusion transcript should hybridize while the other oligo should not as there are only 8 bp to duplex with. In addition, neither of these oligos should hybridize with the wild-type transcript for the same reason. When the 5' junction was used to probe a Northern strip containing wild-type and mutant RNA a transcript of the appropriate size (1.1 kb) was found in the lane containing mutant RNA while no transcript could be seen in the lane containing wild-type RNA (Figure 3.17B). The 3' junction oligo did not hybridize with either *whi3*$^-$ or *WHI3*$^+$ RNA (data not shown). The 1.3 kb *Eco*RI probe was used as a positive control to prove that hybridizing RNA was present in both lanes (data not shown) and the *ADH1* probe was used as a loading control (Figure 3.17B). These results indicate that the mutant transcript probably initiates at the normal *WHI3* start site and proceeds into the Ty1 element where it must encounter a transcription termination signal. The reasons for the

Figure 3.17. Northern blot analysis of mutant and wild-type transcripts. RNA was isolated fron the *whi3*$^-$ strains T504b-1c (panel A) and RN100-3c (panel B) and the *WHI3*$^+$ strains T504b-1d (panel A) and RN100-3d (panel B). After Northern transfer membranes were probed with the 1.3 kb *Eco*RI fragment of *WHI3* (panel A) and the 5' junction oligo (panel B). Blots were reprobed with the *ADH1* gene. Transcript size was estimated from the position of rRNAs (Linskens and Huberman, 1988).


A
B
-
+
-
+
WHI3
2.8 kb
1.1 kb
ADH1

increased abundance of the mutant fusion transcript are unknown but may result from a higher rate of transcription or an increase in the stability of the fusion transcript.

Attempts were made to map the 5' and 3' ends of the *WHI3* transcript using short gel purified fragments as probes for Northern blot analysis. The transcriptional start site was mapped to a location between -200 and -300 as probes further upstream failed to identify the transcript but did identify the appropriate band on control Southern blots (data not shown). The limits of the 3' end of the transcript could not be readily defined. A probe starting 175 nucleotides 3' of the translational stop and ending 570 nucleotides beyond the TAA hybridized to the transcript. Probes further 3' of this position were not tested. However, the 3' end of the transcript would not be expected to be more than 600 nucleotides downstream of the stop codon assuming the size of the transcript predicted by Northern lot analysis was correct and the message contained a poly (A) tail of average length. To more precisely map the ends of the transcript S1 nuclease protection or primer extension studies would be required.

4. ANALYSIS OF *WHI3* FUNCTION

The experiments presented in the last chapter focused on the isolation of a new mutant called *whi3* that had resulted from the transposition of a tagged Ty element to a locus involved in size control. The wild-type gene was cloned and sequenced. Copy number manipulations with the *WHI3* gene and the phenotype of the gene replacement were consistent with the idea that *WHI3* increased cell size in a dose-dependent manner. The evidence described suggests that *WHI3* may encode a cellular metric used to monitor cell size or mass. An RNA binding motif identified through sequence analysis may indicate that cell size is affected by some aspect of RNA metabolism.

There are a number of critical questions left to be answered regarding Whi3 function. Although Whi3 has been demonstrated to affect cell size, it is not clear whether the changes are due to a specific modulation of the critical cell size required for Start. If the critical cell size has been altered then a corresponding change in the timing of Start might be expected to affect cell cycle distribution. The results of overexpression studies will be presented and are consistent with this interpretation. Is the *WHI3* gene product acting through the *CLN3* pathway, or through an independent and parallel pathway? If *WHI3* and *CLN3* are located in the same pathway then their effects might be epistatic and double mutants would be expected to have the more severe of the two phenotypes. If the mutations define separate pathways then their effects would be expected to be multiplicative or synergistic. Results will be presented which are consistent with the two mutations defining independent pathways.

In addition, *whi3* was crossed to a number of the Start mutants to determine whether this mutation affected the cellular requirements for Start. The *WHI3* transcript was analyzed in more detail and the cycloheximide sensitivity of wild-type and mutant strains was measured to determine whether the protein synthetic requirements of mutant and wild-type strains differed.

4.1 Overexpression of *WHI3* Results in G1 Arrest

If *WHI3* specifically alters the critical cell requirement necessary for the completion of Start then overexpression might affect the ability of cells to pass through Start. If *WHI3* affects cell size and progress through Start in a dose-dependent manner then overexpression might lead to G1 arrest. The complementation studies described earlier indicated that there were toxic effects associated with high copy number *WHI3* vectors. Transformants had slower growth rates and a high rate of plasmid loss when grown non-selectively. We therefore decided to investigate the effects associated with overexpressing *WHI3*. Mutant and wild-type strains were transformed with single (pCGW2 and pCGW5) and high copy number plasmids (pHGW8) containing a fusion between the inducible *GAL1* promoter and the *WHI3* gene (see Section 2.3.6). Transformants were replica plated to galactose containing media for promoter induction and to raffinose containing media where the promoter is essentially turned off. While the mutant strain could tolerate single copy *GAL1-WHI3*, high copy vectors containing the fusion were lethal to mutant strains grown on galactose (Figure 4.1). Overexpression of *WHI3* from either single or high copy number vectors was lethal to *WHI3*$^+$ strains (Figure 4.2). Strains transformed with control plasmids grew on both raffinose and galactose (Figure 4.1 and 4.2).

Figure 4.1. Induction of *GAL-WHI3* on high copy but not *cen* vectors is lethal to *whi3*⁻ strains. The strain RN100-1d #3 was transformed with pCGAL1, a centromere based vector containing the *GAL1* promoter and pCGW2 and pCGW5, *cen* vectors containing the *GAL1-WHI3* constructs. This strain was also transformed with the high copy number (2μm) control vector, pHGAL1, which contains the *GAL1* promoter and pHGW8, which contains the *GAL1-WHI3* construct. Transformants were grown on -ura raffinose plates and replica plated to -ura raffinose, galactose plates for promoter induction. Plates were incubated for two days at 30°C.

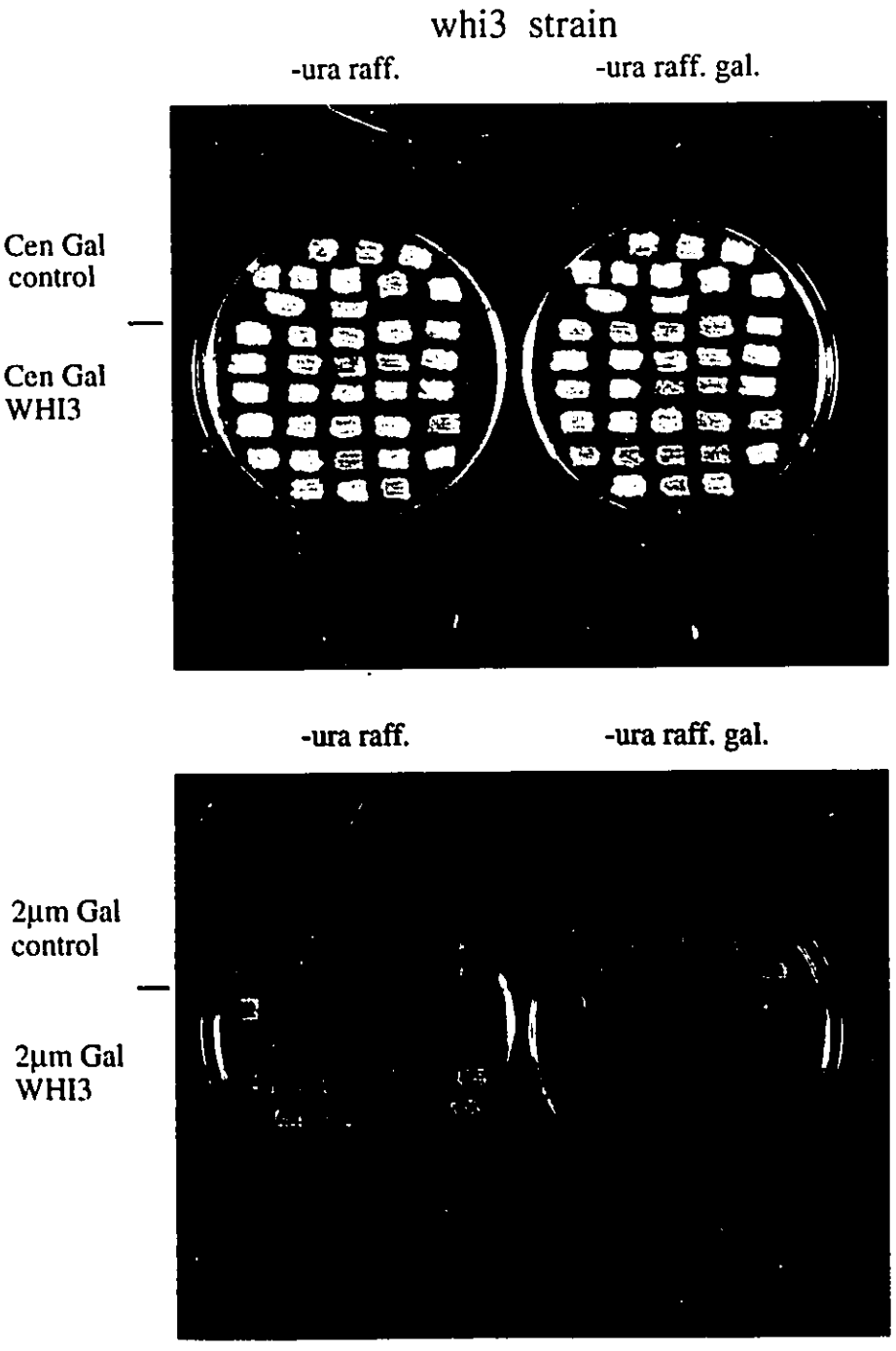
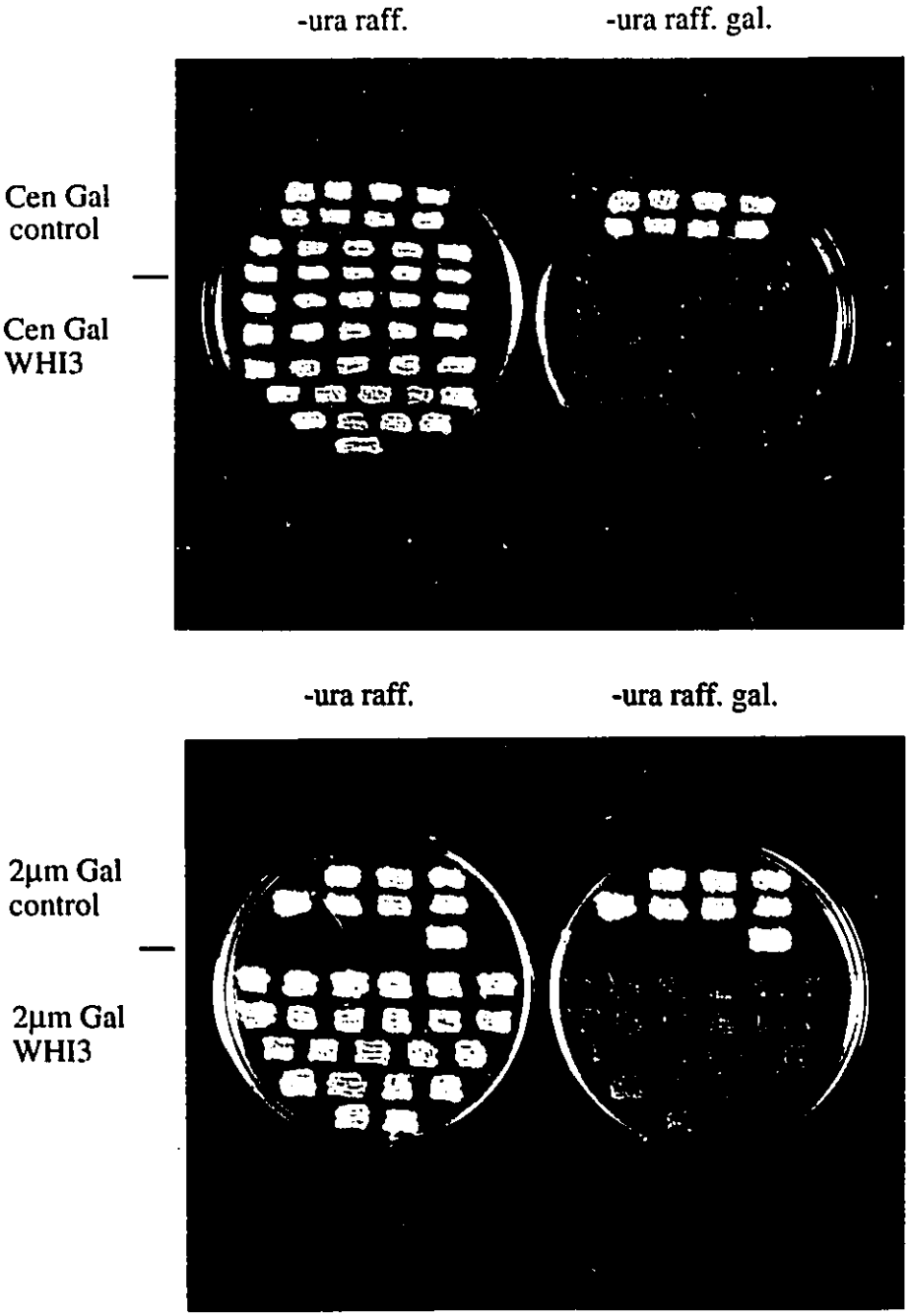

whi3 strain
-ura raff.
-ura raff. gal.
Cen Gal control
Cen Gal WHI3
-ura raff.
-ura raff. gal.
2µm Gal control
2µm Gal WHI3

Figure 4.2. Overexpression of the *WHI3* gene is lethal to wild-type strains when present on single or high copy number vectors. The strain BWG1-7a was transformed with pCGAL1, a centromere based vector which contains the *GAL1* promoter and pCGW2 and pCGW5 which contain the *WHI3* gene under the control of the *GAL1* promoter. This strain was also transformed with the high copy number (2μm) control vector, pHGAL1 and with pHGW8, which contains the *GAL1-WHI3* construct. Transformants were grown on -ura raffinose plates and were then replica plated to -ura raffinose, galactose plates to induce the promoter. Plates were incubated for two days at 30°C.

wild-type strain



To analyze the terminal arrest phenotype in more detail, a *WHI3*$^+$ strain containing pCGW2 was grown to mid-log phase in -ura raffinose, and galactose was added to half of the culture. Samples were removed at various times after induction and cell volume and cell number were measured for both induced and uninduced cultures. Addition of galactose resulted in a significant increase in cell volume and a cessation of cell division (Figure 4.3). Samples were also processed for FACS analysis. FACS analysis was used to measure the relative DNA content of cells as a measure of the proportion of cells in various cell cycle phases. Cells in the G1 phase of the cell cycle have a 1N DNA content and cells with replicated DNA have a 2N DNA content. When galactose was added there was a significant increase in the proportion of cells with a 1N DNA content (Figure 4.4). These results indicated that induced cells were arresting in the G1 phase of the first or second cell cycle. As volume increased cells became more fragile so that procedures such as sonication resulted in considerable cell lysis. This might explain the increase in the fluorescence peak located at the left end of the FACS profiles for induced cultures (Figure 4.4). Samples of galactose induced samples for the 5 and 7.5 hour timepoints were processed for FACS analysis without prior sonication of the cells. This resulted in an additional increase in the proportion of cells in the G1 phase of the cell cycle and a corresponding decrease in the peak at the far left of the FACS profiles (Figure 4.5). This suggests a predominantly G1 arrest associated with *WHI3* overexpression.

Immunofluorescent staining of tubulin was also used to classify the terminal arrest phenotype of cells. Within 2.5 hours of galactose addition there was a large increase in the number of uninucleate cells containing an interphase microtubule array emanating from one microtubule organizing

Figure 4.3. Overexpression of *WHI3* increases cell size and arrests cell division. Wild-type (BWG1-7a) transformants containing pCGW5 were grown to mid-log phase in -ura raffinose at which time galactose was added to half of the culture. Samples were taken over the next 7.5 hours, placed on ice and sonicated. Cell numbers (panel A) and median cell volumes (panel B) of samples were measured with the Coulter Counter and Coulter Channelyzer and plotted.

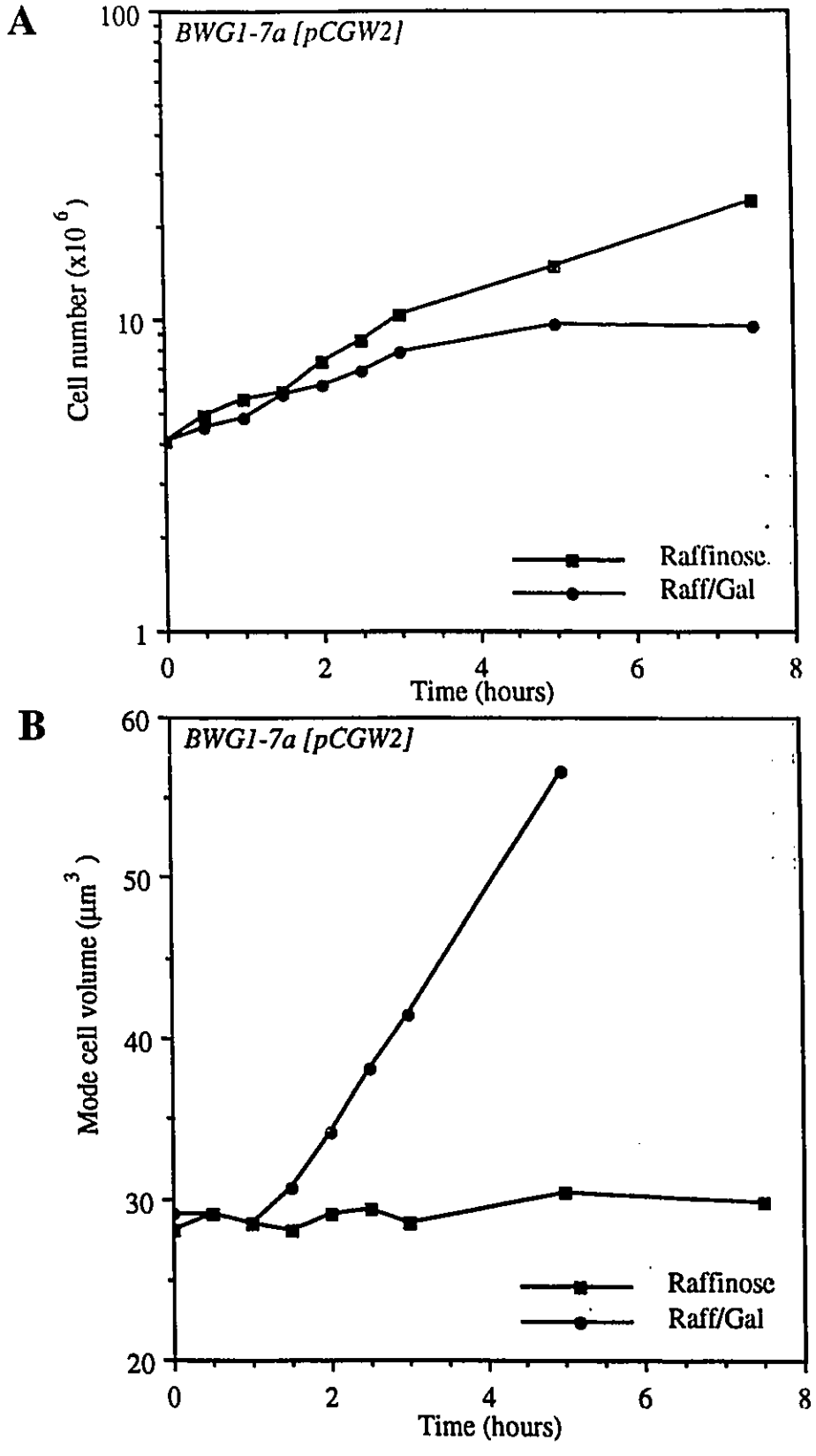

A
BWG1-7a [pCGW2]
Cell number (x10 6)
100
10
1
0
2
4
6
8
Time (hours)
Raffinose
Raff/Gal
B
BWG1-7a [pCGW2]
Mode cell volume (µm 3)
60
50
40
30
20
0
2
4
6
8
Time (hours)
Raffinose
Raff/Gal

Figure 4.4. Overexpression of *WHI3* arrests cells in the G1 phase of the cell cycle. Wild-type (BWG1-7a) transformants containing pCGW5 were grown to mid-log phase in -ura raffinose and galactose was added to half of the culture. Samples were taken over the next 7.5 hours, placed on ice and sonicated. Sonicated samples were processed for FACS analysis.

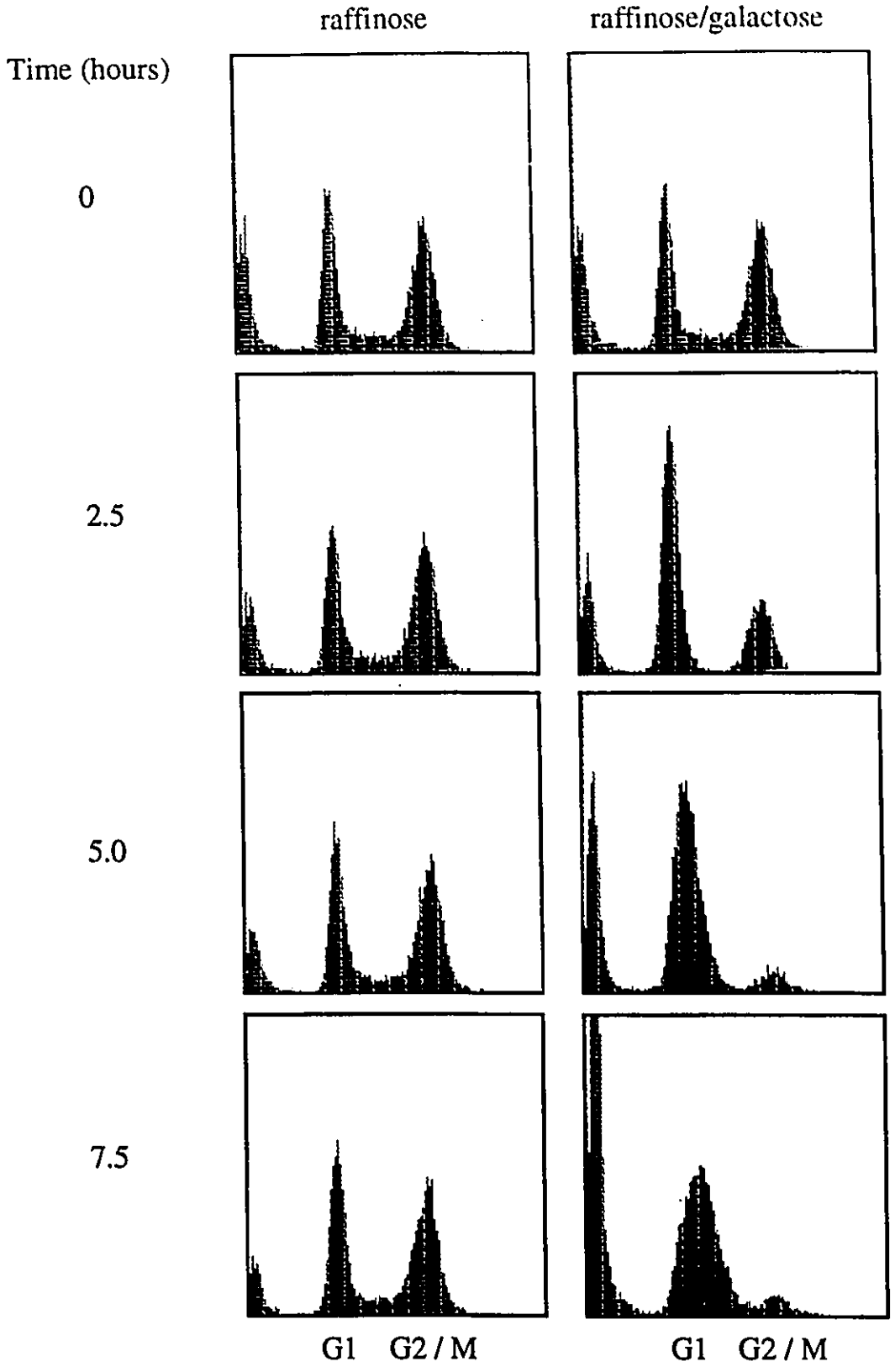

raffinose
raffinose/galactose
Time (hours)
0
2.5
5.0
7.5
G1
G2 / M
G1
G2 / M

Figure 4.5. G1 arrest of unsonicated samples of *GAL1-WHI3* induced cultures. Wild-type (BWG1-7a) transformants containing pCGW5 were grown to mid-log phase in -ura raffinose at which time galactose was added to half of the culture. Samples were taken over the next 7.5 hours and placed on ice. Raffinose control samples were sonicated but samples from galactose induced cultures were not, to prevent cell lysis. Samples were then processed for FACS analysis.

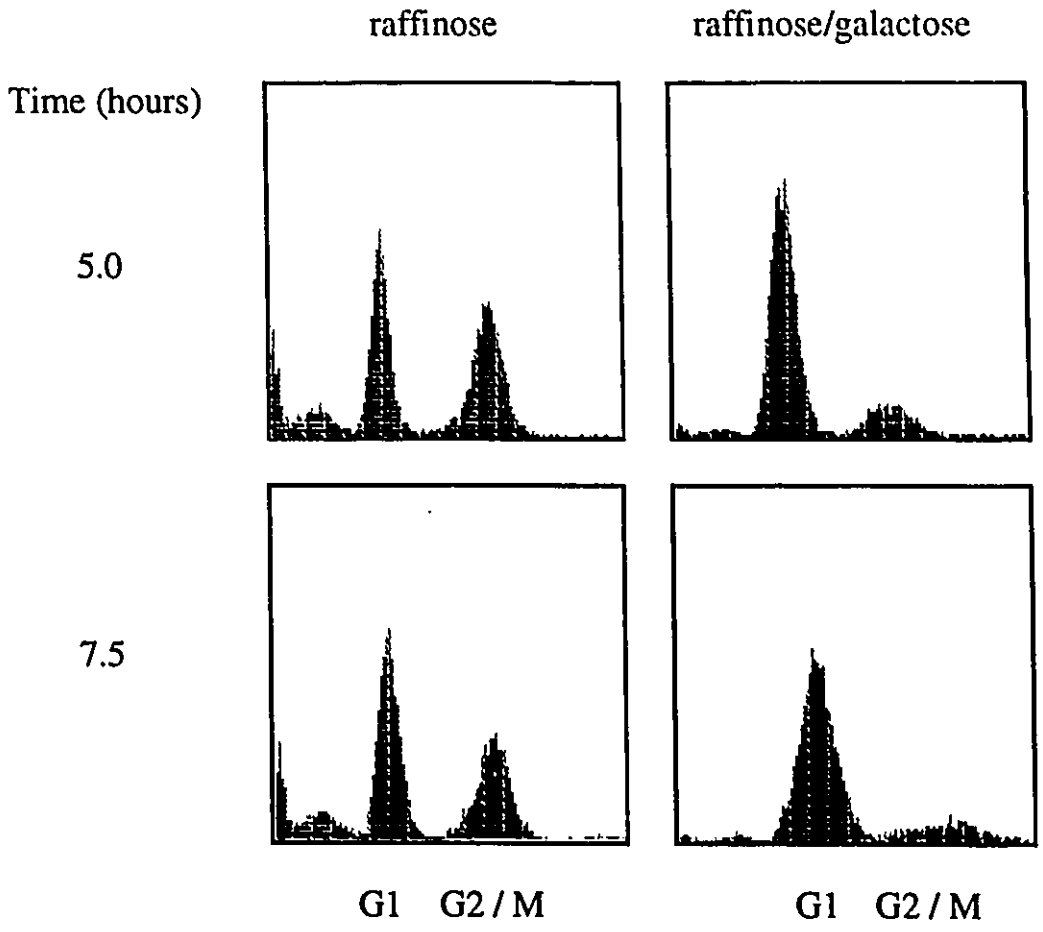
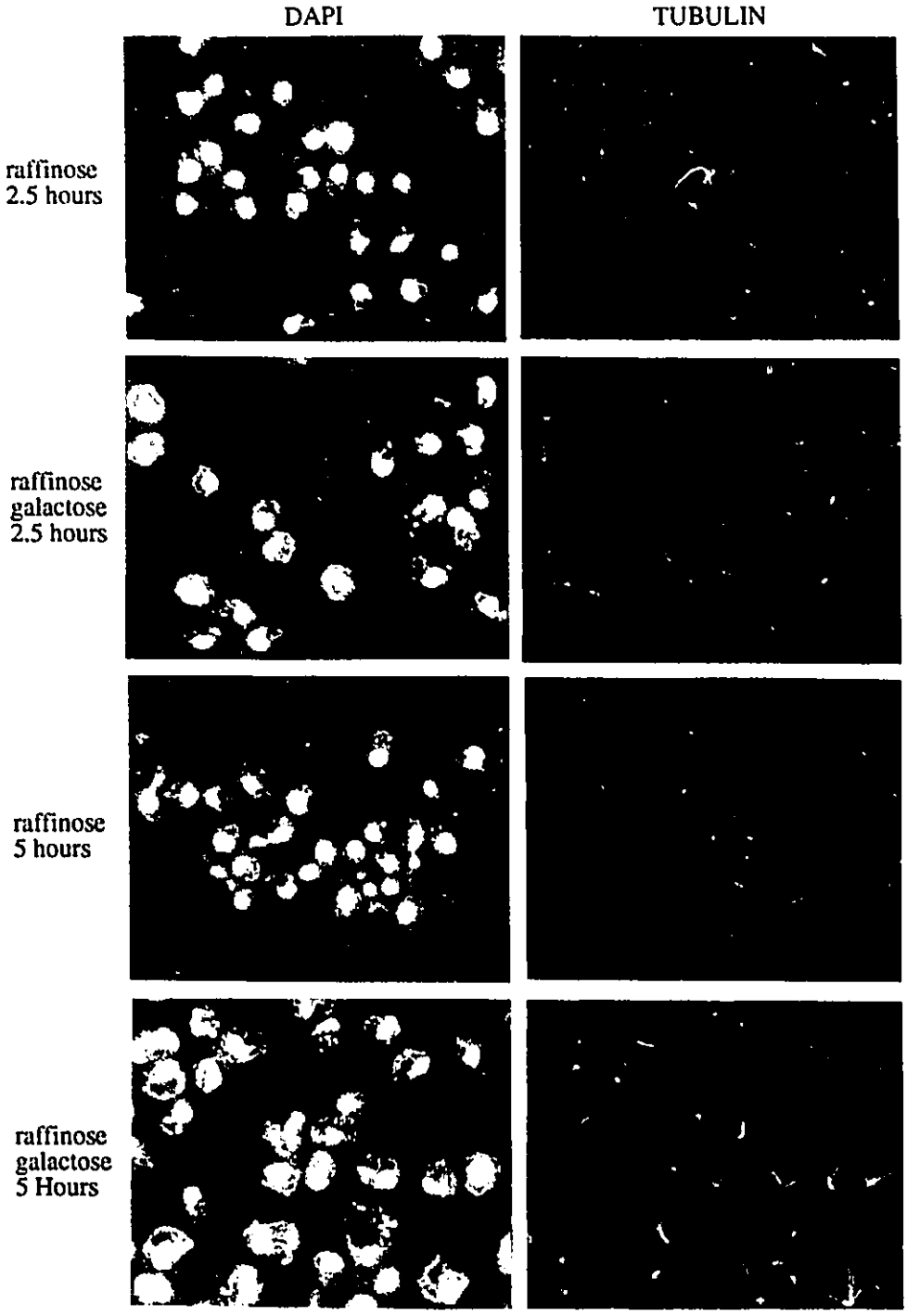

raffinose
raffinose/galactose
Time (hours)
5.0
7.5
G1 G2 / M
G1 G2 / M

Figure 4.6. Tubulin and DNA staining of induced and uninduced cells. Wild-type (BWG1-7a) transformants containing pCGW5 were grown to mid-log phase in -ura raffinose at which time galactose was added to half of the culture. Samples were taken over the next 7.5 hours, sonicated and placed on ice. Samples were concentrated, fixed and stained with DAPI (left) and rat tubulin antibodies followed by FITC conjugated goat anti-rat IgG (right). Stained cells were viewed at a final magnification of 1000X.


DAPI
TUBULIN
raffinose
2.5 hours
raffinose
galactose
2.5 hours
raffinose
5 hours
raffinose
galactose
5 Hours

center (MTOC) (Figure 4.6). After five hours in the presence of galactose all of the *GAL1-WHI3* induced cells had similar microtubule arrays and the lengths of the arrays had increased (Figure 4.6). DNA was also stained with DAPI but was more difficult to interpret as cells were slightly overdigested. DAPI staining was included for orientation. The tubulin distribution was similar to what has been observed for unbudded cells located in the G1 phase of the cell cycle (Kilmartin and Adams, 1984). Reciprocal shifts could not be used to specifically position the arrest with respect to Start as the arrest was not readily reversible. The results are however consistent with a G1 Start arrest. The vast majority of cells displayed homogeneous microtubule arrays indicative of a specific arrest. The homogeneity of the arrays suggests that the small 2N peak present in FACS profiles of arrested cells may represent cells which have not properly separated at cytokinesis but are actually G1 arrested cells. This evidence supports the hypothesis that the product of the *WHI3* gene encodes a G1 specific inhibitor that blocks the progression of cells through Start. In addition, these results are consistent with what would be expected for overexpression of a dose-dependent inhibitor of Start.

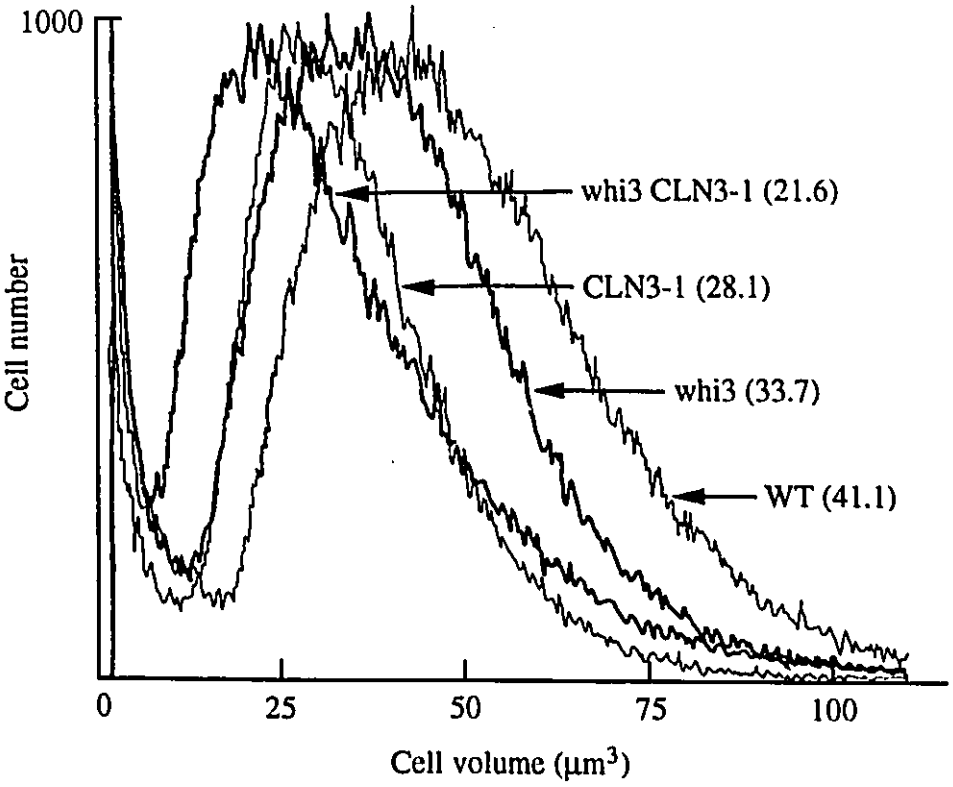
Attempts were made to specifically suppress the arrest by introducing G1 cyclin constructs into the *WHI3* overexpressor. Increasing the level of an activator of Start might be able to overcome the effects of increasing the level of *WHI3* and suppress the arrest. This experiment would also address whether *WHI3* was acting through the *CLN* pathway or an independent pathway. If *WHI3* was acting through the *CLN* pathway then the G1 cyclin constructs might suppress the lethality. A *GAL1* driven *CLN3-1* construct and the *CLN2* gene under the control of the constitutive *ADH1* promoter were transformed into the *GAL1-WHI3* strain but were unable to reverse the arrest

(data not shown). This suggests that the Whi3 protein functions in an independent pathway with respect to the G1 cyclins and may define a separate requirement for Start. A genomic bank was also transformed into the *GAL1-WHI3* overexpressor. Numerous suppressors were identified but appeared to function by negatively regulating the *GAL1* promoter and therefore may decrease the expression of the *WHI3* gene (data not shown).

4.2 Interactions with *CLN3-1*

Whi3 may define a component of an independent pathway required for the completion of Start or may be a component of the Cln3 pathway. To differentiate between these possibilities the *CLN3-1* mutant was crossed to the *whi3* mutant. A *whi3* strain (T504b-1c) was crossed to a *CLN3-1* strain (BF328-5a) to create the diploid T654b. The cell volumes of spore clones from single and double mutants were measured and compared to those of the wild-type spore clones. Cells with a *whi3* mutation were slightly larger than cells with a *CLN3-1* mutation and were more heterogeneous in size (Figure 4.7). Cells containing both mutations (*whi3 CLN3-1*) had median cell volumes of 20-22 μm^3, approximately half the volume of isogenic wild-type strains (Figure 4.7). The size effects of these two mutations were multiplicative as would be expected of mutations which define independent pathways. In addition, double mutants had a slightly longer doubling time than wild-type strains or single mutants, had reduced cell viability and spontaneously diploidized at a low rate (data not shown). The reasons for these phenotypes are unclear but may relate to a selective pressure imposed by the very small size of the double mutant.

Figure 4.7. *whi3* and *CLN3-1* mutants have additive cell size defects. Spore clones of the tetrad T654b-4 were grown to mid-log phase in YEPD. T504b-4a is wild-type, T654b-4d is *whi3*$^-$, T654b-4c contains the *CLN3-1* mutation and T654b-4b is *whi3*$^-$*CLN3-1*. Cultures were sonicated and aliquots were removed for cell sizing with the Coulter Channelyzer. Cell volume distributions were plotted and median cell volumes determined. Median cell volumes (μm^3) are indicated.


1000
Cell number
whi3 CLN3-1 (21.6)
CLN3-1 (28.1)
whi3 (33.7)
WT (41.1)
0
25
50
75
100
Cell volume (μm³)

For the experiments which follow a set of isogenic strains was constructed by gene replacement. These strains were wildtype or deleted for *whi3* and contained either the activated *CLN3-1* allele, a *cln3* deletion or the wild-type gene (see Section 2.3.6 for details). Replacements were confirmed to be correct by Southern analysis and had the appropriate cell volumes (data not shown).

The DNA content of single and double mutants was measured by FACS analysis. If the critical cell size required for commitment had been specifically changed then there should have been a corresponding change in the timing of Start. Compared to wild-type, the *whi3::LEU2* deletion strain had a 3-5% decrease in the proportion of cells with a 1N DNA content (data not shown). As previously reported, the *CLN3-1* mutant has a shorter G1 phase resulting from a relaxed size requirement for the completion of Start (Nash *et al.*, 1988, Cross, 1988). The proportion of cells with a 1N DNA content was reduced by about 10% in *CLN3-1* mutants. The *whi3 CLN3-1* double mutant had 13-15% fewer cells in the G1 phase of the cell cycle compared to wild-type controls (data not shown). This suggests that the timing of Start was slightly advanced in the *whi3* single mutant and advanced even further in the double mutant suggesting that the effects were additive.

The dominant *CLN3-1* mutant is α-factor resistant, arresting transiently but recovering more rapidly than wild-type strains while a *CLN3* deletion is α-factor sensitive (Nash *et al.*, 1988; Cross *et al.*, 1988). These results coupled with difficulties encountered obtaining diploids in crosses involving *whi3* and *CLN3-1* mutants led us to measure the relative α-factor resistance of single and double mutants. Deletion of *whi3* increased the α-factor resistance of RN100-13d so that the upper limit of growth increased from

1 μM to 3 μM α-factor (Figure 4.8). A similar result was obtained when a spore clone containing the tagged Ty *whi3* allele was compared to an isogenic *WHI3*$^+$ strain (Nash *et al.*, 1988). The *whi3 CLN3-1* double mutant was slightly more resistant than the *CLN3-1* single mutant on plates containing 100 mM α-factor, the highest concentration tested (Figure 4.8). The absolute resistance of the strain could not easily be measured because of the insolubility of α-factor at the concentration required for such a determination. Strains deleted for *CLN3* were more sensitive than wild-type strains (Nash et al., 1988) but less sensitive than strains deleted for both *whi3* and *CLN3* (Figure 4.8).

To analyse the α-factor response in more detail, log phase cultures of single and double mutants were treated with 3 μM α-factor. Samples were taken every hour and cell number, cell volume and cell cycle distributions were measured. Nomarski images of cells were taken with the light microscope at 0 and 2 hours so that the morphological changes associated with the addition of mating pheromone could be followed. The wild-type control strain arrested cell division within 1-2 hours and continued to increase in cell size throughout the course of the experiment (Figure 4.9 and 4.10). The percentage of cells in the G1 phase of the cell cycle increased from 30% to greater than 80% within 2 hours and then decreased as cells started to recover (Figure 4.9C). Single mutants deleted for *whi3* or containing the *CLN3-1* mutation showed an intermediate level of response at this concentration of α-factor. The single mutants displayed moderate increases in cell size concurrent with the formation of schmoos and an intermediate increase in the percentage of cells with a 1N DNA content (Figure 4.9 and 4.10). The *CLN3-1* mutant seemed to recover more rapidly than wild-type or *whi3* strains as judged by cell number and DNA content (Figure 4.9A and 4.9C). The *whi3 CLN3-1* double mutant was

completely resistant to treatment with this concentration of α-factor. Cells containing both mutations continued to divide, maintained a constant cell size throughout the course of the experiment with only infrequent examples of schmooing cells and displayed no noticeable changes in the percentage of cells with a 1N DNA content (Figure 4.9 and 4.10). To summarize, there was a transient partial arrest displayed by single mutants in response to treatment with $3x10^{-6}$ M α-factor as assayed by cell number, cell volume, percent of cells in the G1 phase of the cell cycle and by morphological criteria. In addition, the *CLN3-1* mutant displayed a more rapid recovery. The *whi3 CLN3-1* double mutant was totally unresponsive to treatment with this concentration of α-factor and did not arrest even transiently.

An additional response to treatment with mating pheromone is the rapid transcriptional induction of α-factor inducible genes. This response was compared in single and double mutants after treating cells with 3 μM α-factor. Samples were taken every 20 minutes after α-factor addition and RNA was isolated for Northern blot analysis. Northern blots were hybridized with probes isolated from two α-factor inducible genes, *FUS1* and *FUS3*. Wild-type cells treated with 3 μM α-factor induced both *FUS1* and *FUS3* transcripts. This response was rapid and occurred within 20 minutes of treatment (Figure 4.11, data not shown). *whi3* and *CLN3-1* single mutants displayed a normal transcriptional response inducing both transcripts within 20 minutes of α-factor addition (Figure 4.11, data not shown). The double mutant displayed a very weak almost negligible induction of both *FUS1* and *FUS3* transcripts (Figure 4.11, data not shown). Northern blots were also probed with a fragment of the *CLN2* gene. The levels of the *CLN2* transcript decreased when wild-type strains were treated with α-factor as expected (data not shown). Although

Figure 4.8. α-factor resistance of single and double mutants. Mid-log phase cultures of the *whi3* deletion strain RN210-13d and isogenic *CLN3-1* or *CLN3* deletion derivatives (see section 2.3.6) were sonicated and 1000 cells were spotted onto plates containing increasing concentrations of α-factor. Plates were incubated for 2-3 days at 30°C and the highest concentration at which cells were able to form colonies was recorded and plotted.

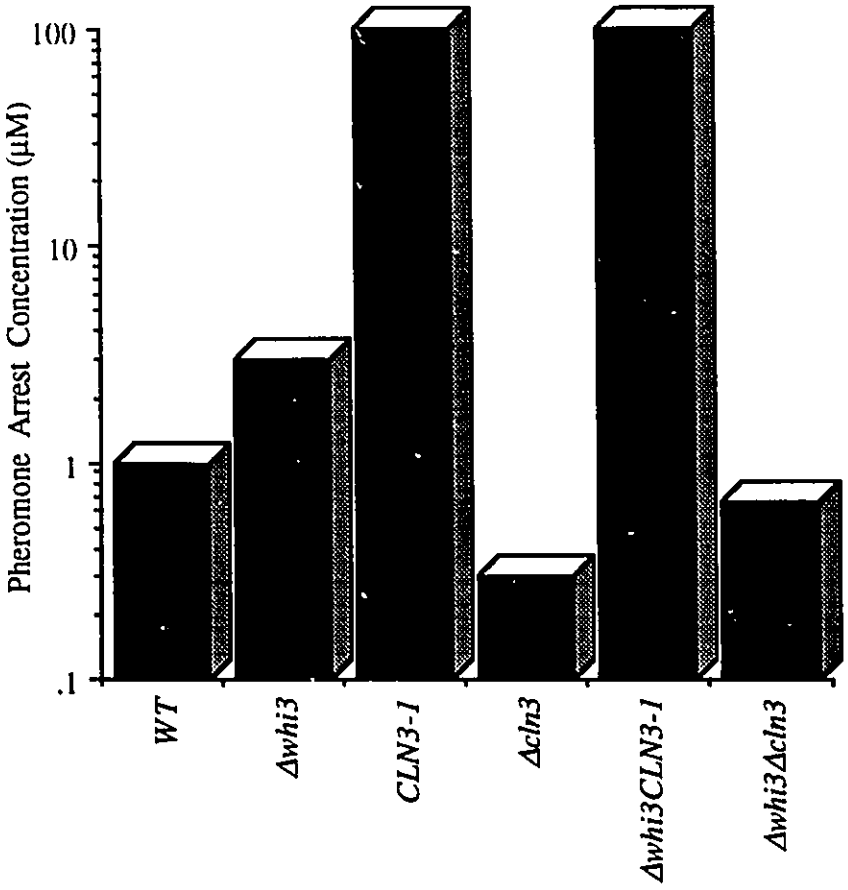

Pheromone Arrest Concentration (μM)
100
10
1
.1
WT
Δwhi3
CLN3-1
Δcln3
Δwhi3CLN3-1
Δwhi3Δcln3

Figure 4.9. The affect of *whi3* and *CLN3-1* mutations on the α-factor response. Mid-log phase cultures of the *whi3* deletion strain RN210-13d and isogenic *CLN3-1* derivatives (see section 2.3.6) were treated with 3 μM α-factor and samples were removed every hour. Cell number (panel A) and median cell volumes (panel B) were measured with the Coulter Counter and Channelyzer after sample sonication. Sonicated samples were stained with propidium iodide for FACS analysis. The percentage of cells with a 1N DNA content (panel C) was calculated after FACS analysis.

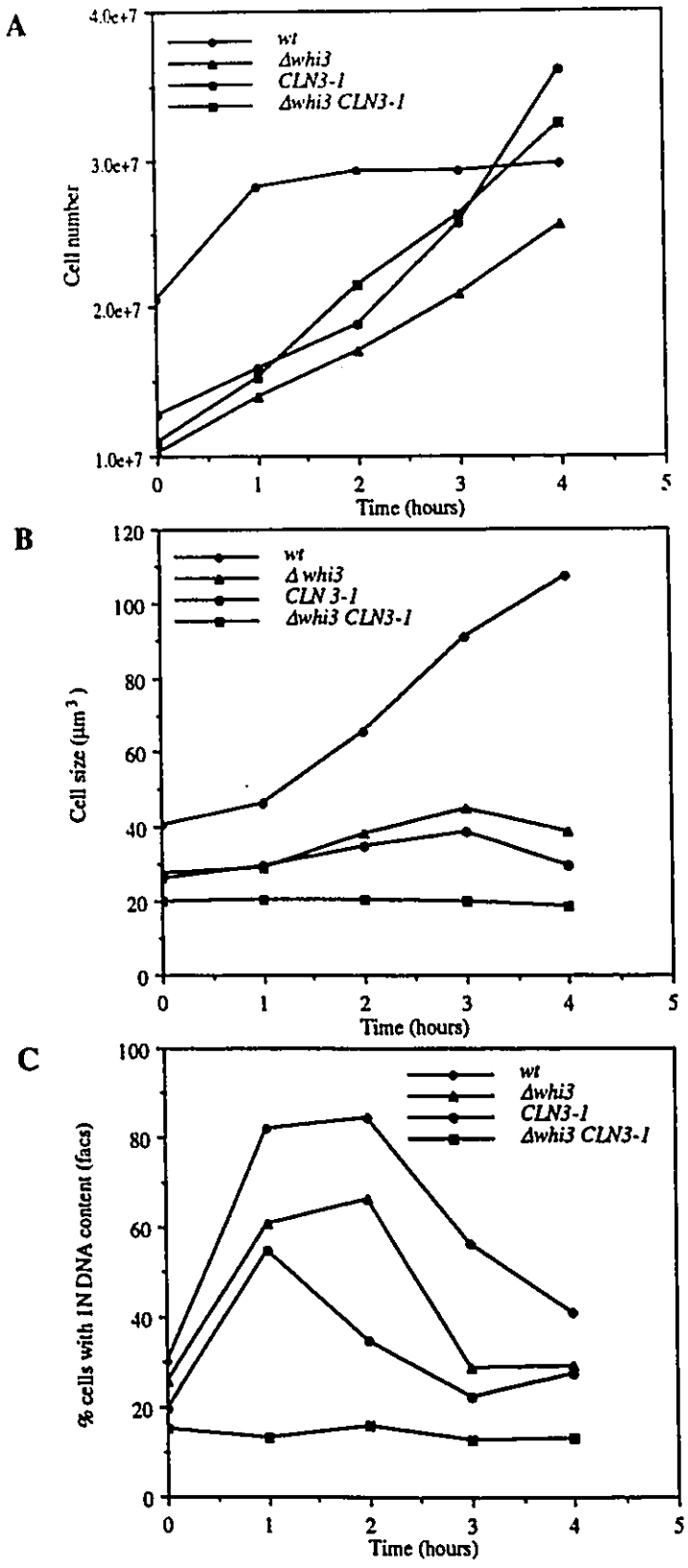

A
4.0e+7
3.0e+7
2.0e+7
1.0e+7
Cell number
wt
Δwhi3
CLN3-1
Δwhi3 CLN3-1
0
1
2
3
4
5
Time (hours)
B
120
100
80
60
40
20
0
Cell size (μm 3)
wt
Δ whi3
CLN 3-1
Δwhi3 CLN3-1
0
1
2
3
4
5
Time (hours)
C
100
80
60
40
20
0
% cells with 1N DNA content (facs)
wt
Δwhi3
CLN3-1
Δwhi3 CLN3-1
0
1
2
3
4
5
Time (hours)

Figure 4.10. Morphological changes associated with α-factor treatment in *whi3* and *CLN3-1* single and double mutants. Mid-log phase cultures of the *whi3* deletion strain RN210-13d and isogenic *CLN3-1* derivatives (see section 2.3.6) were treated with 3 μM α-factor and samples were taken zero and two hours. Samples were sonicated, concentrated and placed on slides. Photographs were taken under Nomarski optics at a final magnification of 1000X.

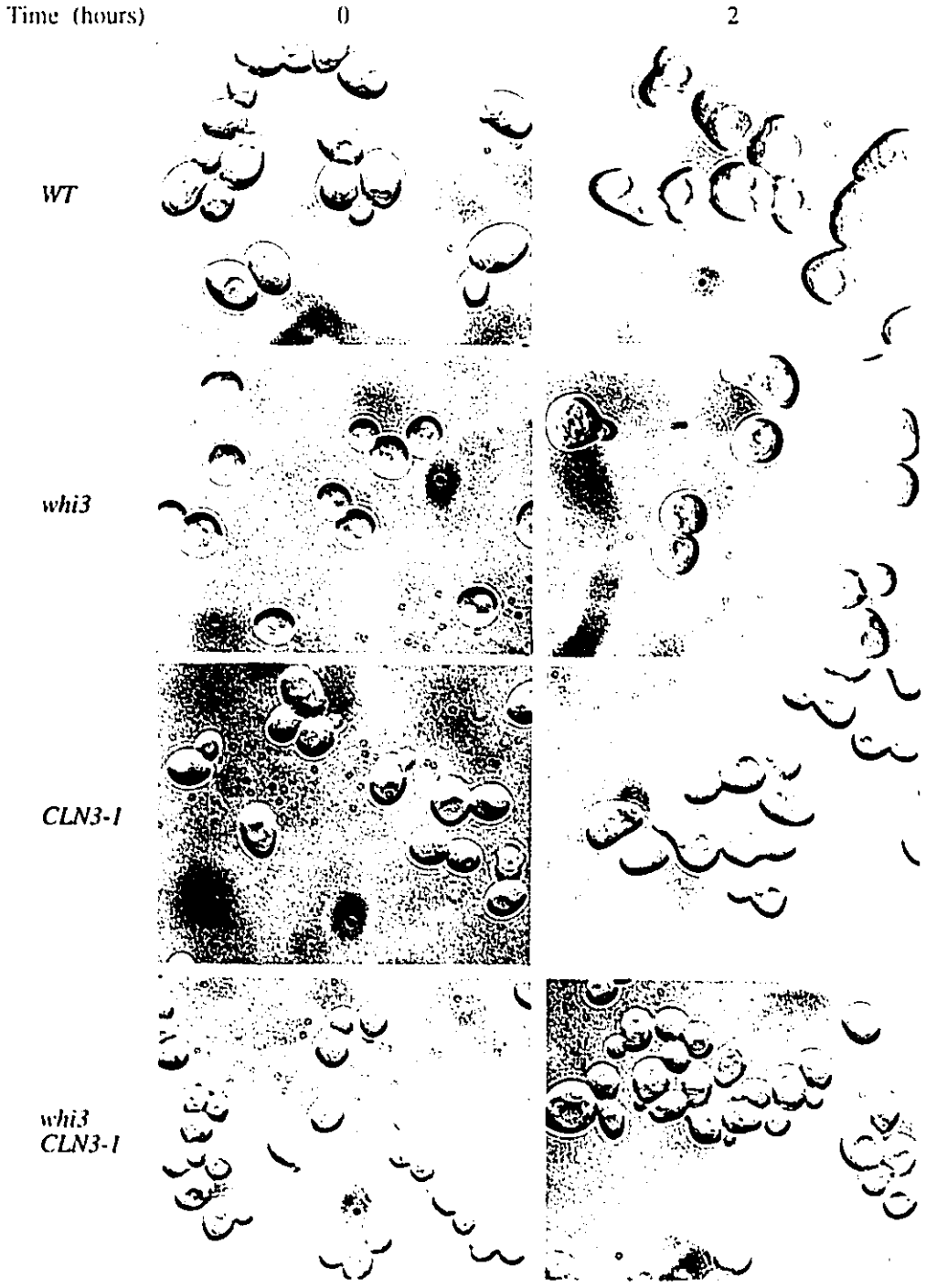
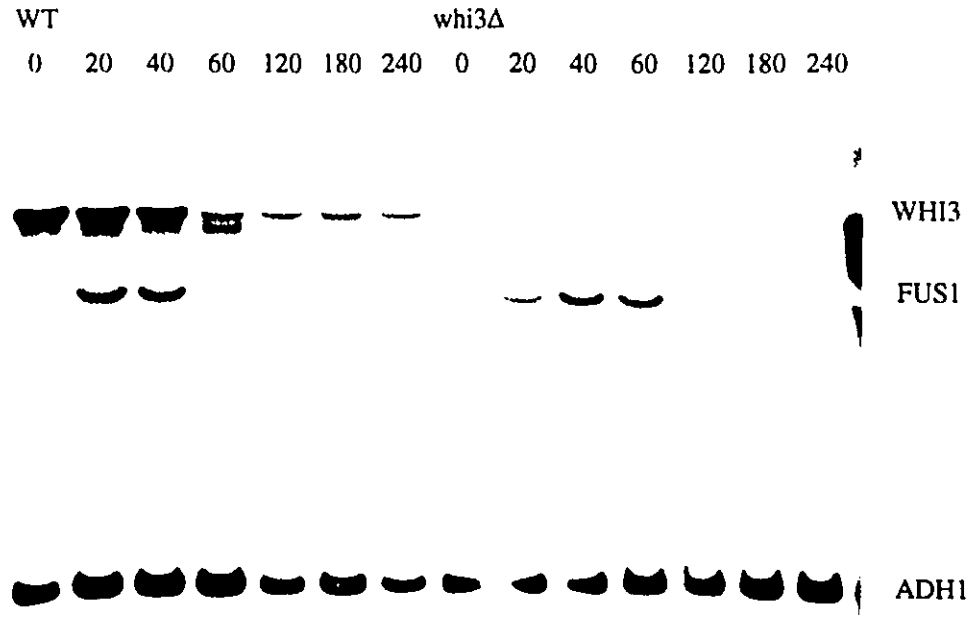
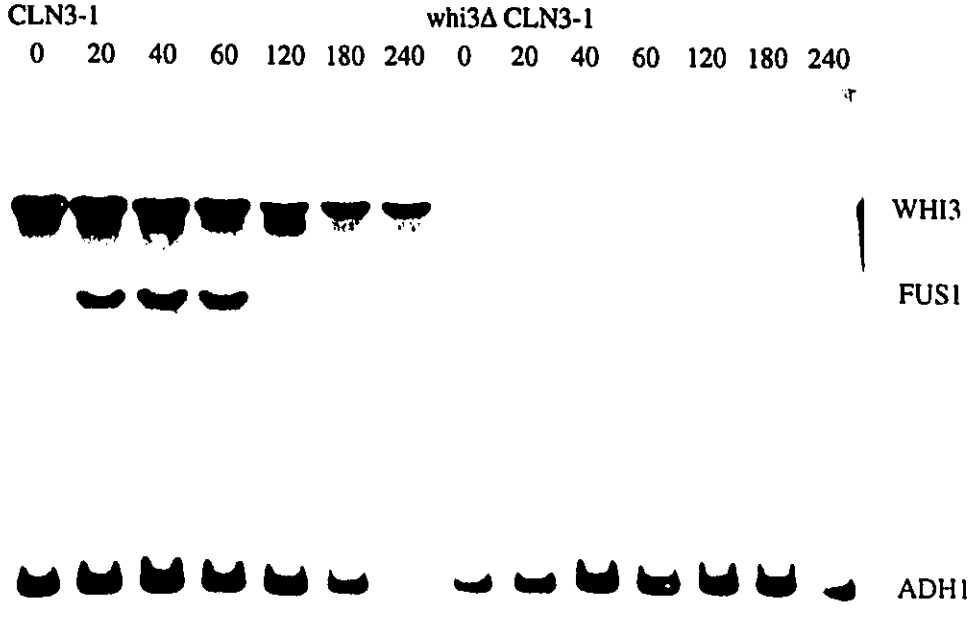

Time (hours)
0
2
WT
whi3
CLN3-1
whi3
CLN3-1

Figure 4.11. Northern analysis of the α-factor transcriptional induction in *whi3* and *CLN3-1* single and double mutants. Mid-log phase cultures of the *whi3* deletion strain RN210-13d and isogenic *CLN3-1* derivatives (see section 2.3.6 for details) were treated with 3 μM α-factor and samples were taken at the indicated times. RNA was prepared, electrophoresed and transferred. Northern blots were probed with a 1.1 kb *Bst*XI-*Kpn*I fragment of the *FUS1* gene, 1.9 kb *Bst*EII-*Kpn*I fragment of the *WHI3* gene and the *ADH1* gene (linearized pJD14).


WT
whi3Δ
0 20 40 60 120 180 240 0 20 40 60 120 180 240
WHI3
FUS1
ADH1


CLN3-1
whi3Δ CLN3-1
0 20 40 60 120 180 240 0 20 40 60 120 180 240
WHI3
FUS1
ADH1

single mutants maintained higher then normal levels of the *CLN2* transcript. the double mutant had very high levels of the *CLN2* transcript throughout the time course (data not shown). It is possible that the lack of cell cycle arrest and the weak transcriptional induction observed in the double mutant occurred as a direct result of the elevation in *CLN2* transcript levels. Alternatively, the elevated *CLN2* levels may be an effect rather then a direct cause of the inability of the double mutant to arrest and be more of an indication of the cycling state. The combined effects of these two mutations on the α-factor response is consistent with the interpretation that these two mutations reside in independent pathways as the double mutants had a very severe phenotype compared to the single mutant.

Northern blots were also probed with a fragment purified from the *WHI3* gene. As expected *whi3* deletion strains have no detectable *WHI3* transcript. In wild-type and *CLN3-1* strains a decrease in the abundance of the full length transcript occurred within one hour of α-factor addition and was accompanied by the transient appearance of a shorter transcript (Figure 4.11). This shorter transcript was only observed at one timepoint and was coincident with a decrease in the level of the *WHI3* full length transcript (Figure 4.11). This shorter transcript may represent a specific cleavage product and may be an intermediate required for *WHI3* message turnover. The remaining full length transcript was stable throughout the rest of the time course (Figure 4.11). The decrease in the level of the full-length *WHI3* transcript may represent part of the α-factor recovery response. In an independent block-release experiment wild-type cells were arrested with α-factor for three hours and then cells were released from the block by washing out the α-factor. Samples were prepared for Northern blot analysis and the filter was probed with the *WHI3* gene. The

level of the *WHI3* transcript was lower then the asynchronous control at the 3 hour time point (block) and started to increase within 40 minutes of the removal of α-factor (release) reaching a steady state level within 60 minutes of release (data not shown). For comparison the *CLN2* message was absent at the block and had reached a high within 40 minutes of the release (data not shown). These results also indicated that the *whi3* message was down regulated in response to α-factor treatment. In summary, a reduction in the levels of the *WHI3* transcript occurs after the addition of α-factor and the timing of the reduction is consistent with a recovery response. A reduction in the levels of an inhibitor of Start may be required to promote cell cycle reentry after arrest. After recovery the *WHI3* transcript levels return to normal.

A quantitative mating assay was used to measure the efficiency with which single and double mutants mated in comparison to a wild-type strain. Haploid cells whose mating efficiency was being measured were mixed with an excess of tester cells of the opposite mating type, incubated to allow mating to occur and then diluted and plated. Auxotrophic markers were used to calculate the ratio of haploids to diploids. Relative to a wild-type strain both *whi3::LEU2* deletion and *CLN3-1* mutant strains mated with a four to five fold lower efficiency (Table 4.1). *whi3 CLN3-1* double mutants mated very poorly with a relative mating efficiency which was about 500 fold lower than the wild-type control (Table 4.1). Thus, the *whi3 CLN3-1* double mutant has a synergistic mating defect which may be due to the double mutants' inability to respond to α-factor. The synergistic defect was not a direct result of the small cell size of the double mutant as spontaneously diplodized double mutants, which were twice the size of haploid double mutants had a comparable mating defect (Table 4.1). Strains deleted for *CLN3* appear to mate slightly better than

Table 4.1 Results of the quantitative mating assay

Relevant Genotype	Relative Mating Efficiency[a]	Normalized Efficiency[b]
WHI3^{+} *CLN3*$^{+}$	0.79	1.00
whi3::LEU2	0.17	0.22
CLN3-1	0.18	0.22
whi3::LEU2 CLN3-1	0.0016	0.0020
whi3::LEU2 CLN3-1 dip.	0.0016	0.0020
Cln3::URA3	0.96	1.22
whi3::LEU2 cln3::URA3	0.47	0.60

[a] The quantitative mating assay and the formula used to calculate the relative mating efficiency have been described in Section 2.3.1 and Sprague, 1991.

[b] Normalized mating efficiencies were calculated assuming that the wild-type strain mated with an efficiency of one.

wild-type strains while the introduction of the *whi3* deletion into this background reduced the efficiency (Table 4.1). The synergistic mating defect observed in the double mutant was also consistent with the proposal that Whi3 and CLN3 are in separate pathways.

If the *whi3* mutant inhibits cell cycle progression by affecting the accumulation or stability of G1 cyclin mRNA then differences in the levels of these mRNAs might be expected. Since the *CLN1* and *CLN2* mRNAs are cell cycle regulated, it is possible that Whi3 affects passage through Start by influencing cell cycle oscillations in these mRNAs. The levels of *CLN1*, *CLN2*, *CLN3* and *SWI6* transcripts were compared by Northern analysis using RNA obtained from *whi3* and *WHI3* strains. No differences were observed in the relative levels of these transcripts in mutant and wild-type strains (data not shown). These results suggest that Whi3 does not alter the levels of the *CLN* transcripts. It should be noted that RNA for these Northerns had been prepared from asynchronously growing cells. If Whi3 functioned to inhibit the periodic accumulation of *CLN1* or *CLN2* mRNA then the mutant strain might have higher overall levels of these transcripts. However, *whi3* mutants also have a shorter G1 phase than wild-type cells and this might therefore mask such an effect.

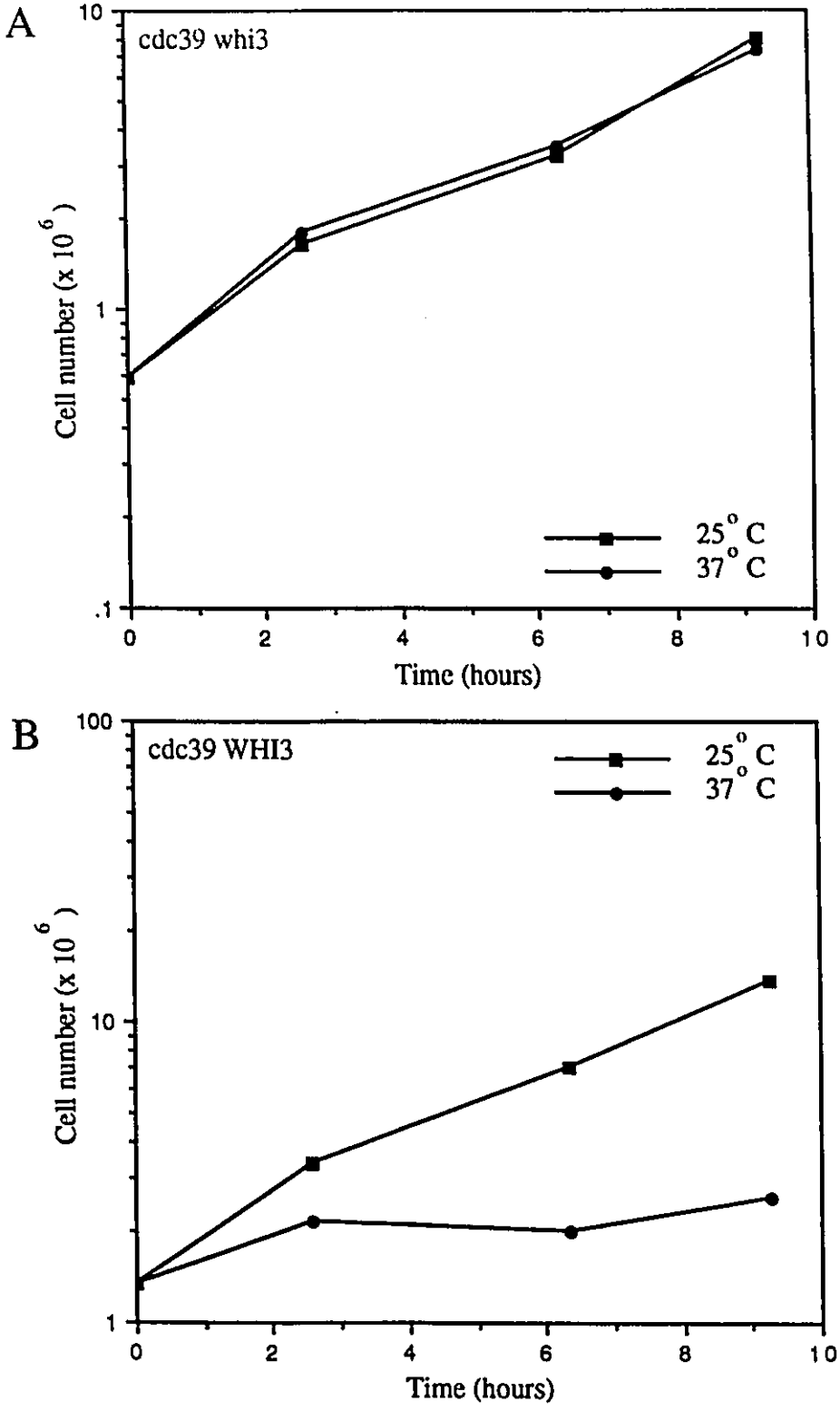
4.3 Genetic Interactions Between *whi3* and the Start Mutants

The *whi3* mutant was crossed to many of the Start mutants to investigate the possibility of genetic interactions. Since the *whi3* mutation affects the critical cell size required for commitment during the G1 phase of the cell cycle it is possible that other requirements are also relaxed. If

requirements are relaxed then the *whi3* mutation might suppress the temperature sensitive arrest associated with the Start mutations. *whi3*$^-$ strains were crossed to the following alleles of the class I Start mutants: *cdc28-13* (MDMy256a), *cdc36-16* (SR661-2a), *cdc37-1* (SR672-1) and *cdc39-1* (SR665-1). The *whi3* mutant was also crossed to an allele of a class II Start mutant, *cdc25-1*. Spore clones resulting from these crosses were analyzed at the restrictive temperature. The *whi3* mutation was unable to suppress the temperature sensitive defects in any of the Start mutants with the exception of the *cdc39* mutant where a weak suppression of the temperature sensitive defect was observed on plates.

To investigate this suppression in more detail, *whi3*$^-$ *cdc39*$^-$ and *cdc39*$^-$ spore clones were inoculated into liquid media and grown to mid-log phase. The cultures were split and half was shifted to the non-permissive temperature. Time points were taken and cell number and cell size were measured. For all but one of the liquid cultures there was a correlation between the genotype of the spore clone and its ability to suppress the temperature sensitive defect. The exception was a *cdc39* single mutant which did not arrest at the restrictive temperature. Spore clones which were *whi3*$^-$ *cdc39*$^-$ were able to grow at 37°C while those which were *cdc39*$^-$ and *WHI3*$^+$ arrested. An example of this effect is shown in Figure 4.12. At 37°C the *whi3* *cdc39* double mutants increased in cell number similar to the control culture (Figure 4.12A). In contrast, the *cdc39* single mutant arrested very rapidly at 37°C (Figure 4.12B). In addition, the *cdc39* spore clone continued to increase in cell size at the restrictive temperature while the *whi3* *cdc39* double mutant became even smaller at 37°C than at 25°C. For example, at the last time point in the experiment the median cell volume of the *whi3* *cdc39* mutant was 25.1

Figure 4.12. The *whi3* mutation suppresses the *cdc39* defect. RN101-1d, a *whi3 cdc39* double mutant (panel A) and RN101-2b, a *cdc39* single mutant (panel B) were grown to mid-log phase and half of the cultures were shifted to 37°C. Samples were taken at the indicated times, sonicated and accurately diluted into isoton. Cell numbers were measured with the Coulter Counter.


A
cdc39 whi3
Cell number (x 10^6)
10
1
.1
0
2
4
6
8
10
Time (hours)
25° C
37° C
B
cdc39 WHI3
Cell number (x 10^6)
100
10
1
0
2
4
6
8
10
Time (hours)
25° C
37° C

μm^3 at 25°C compared to 46 μm^3 for the *cdc39* mutant. At 37°C the median cell volume of the *whi3 cdc39* mutant had decreased to 20.1 μm^3 while the size profile of the *cdc39* mutant became very heterogeneous as cell size increased. At the last timepoint the percentage of budded versus unbudded cells was also calculated. The *cdc39* mutant showed an increase in the percentage of unbudded cells from 56% at the permissive temperature to 86% at the restrictive temperature. The *whi3 cdc39* double mutant displayed only a moderate increase from 44% to 56% under the same conditions. The *CDC39* gene product appears to function as a negative element in the mating factor pathway as *cdc39* mutants constitutively activate this pathway (Barros Lopes *et al.*, 1990; Neiman *et al.*, 1990). The suppression may occur if *whi3* mutants are more permissive for Start or may occur if the *whi3* mutant is defective in α-factor induced signal transdution and can therefore suppress the constitutive mating defect of the *cdc39* mutant.

4.4 Additional Transcriptional Analysis

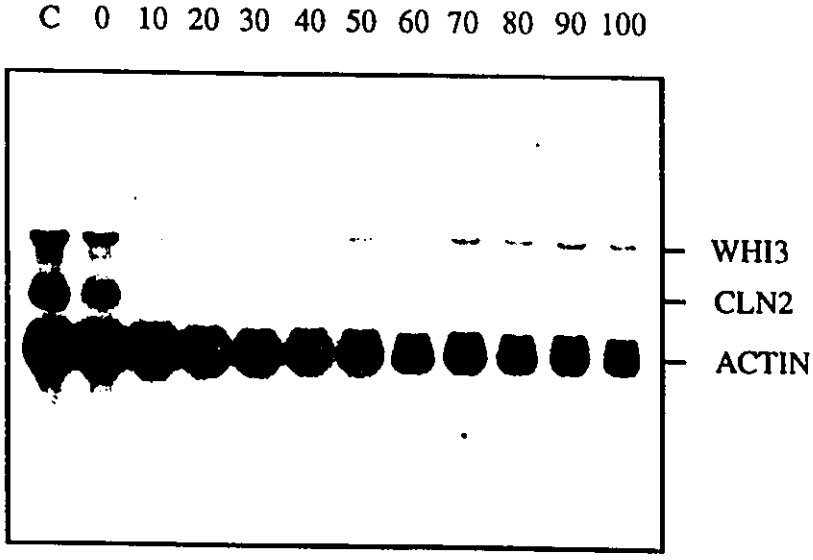
Many activities are cell cycle regulated by periodic fluctuations in the level of the transcript. For, example both *CLN1* and *CLN2* have cell cycle regulated transcripts which peak in the G1 phase of the cell cycle (Wittenberg *et al.*, 1990). To investigate the possibility that the *WHI3* transcript was cell cycle regulated two Northerns provided by G. Tokiwa were probed with a fragment of the *WHI3* gene. Both of these experiments utilized block-release techniques to synchronize cells. *cdc15* mutants arrest in late anaphase were incubated at the restrictive temperature. When shifted back to the permissive temperature the cells entered G1 in a highly synchronous manner. In the

second block-release, cells were arrested in the G1 phase of the cell cycle with α-factor and then released from the block in a highly synchronous manner by washing out the α-factor. Samples were taken at the block and at various times after release and RNA was prepared. Although control probings indicated a strong periodicity associated with *CLN2* these Northerns failed to reveal any significant cell cycle periodicity associated with the *WHI3* transcript (data not shown).

The stability of the *WHI3* transcript was investigated using the yeast strain Y260, which contains a temperature sensitive allele of the yeast RNA pol II (*rpb1-1*) gene. At the non-permissive temperature the *rpb1-1* mutation shuts off all RNA polymerase II dependent transcription (Nonet *et al.*, 1987). This strain has proven to be very useful for the investigation of mRNA half-lives (Herrick *et al.*, 1990). A Northern blot prepared by G. Tokiwa with RNA isolated at 10 minute intervals after shifting Y260 to the restrictive temperature was hybridized with *WHI3*, *CLN2* and actin probes. The majority of the *WHI3* transcript was very labile disappearing within 10 minutes of the shift to the non-permissive temperature (Figure 4.13). The initial half life was very similar to that of the *CLN2* transcript which has a half life of 10 minutes or less (Figure 4.13). A small subpopulation of the transcript was very stable and remained throughout the rest of the timecourse. This residual transcript had a stability like that of the actin transcript (Figure 4.13).

The addition of the protein synthetic inhibitor cycloheximide results in the transcriptional induction of the many serum inducible early-response genes in Swiss 3T3 cells (Lau and Nathans, 1987; Almendral *et al.*, 1988). To investigate whether the *WHI3* message was induced by cycloheximide treatment RNA was prepared from a wild-type strain treated with 50 μg/ml cycloheximide

Figure 4.13. The majority of the *WHI3* transcript is unstable. A temperature sensitive RNA polymerase II mutant, Y260, was grown to mid-log phase at 24°C. The mutant was shifted to the non-permissive temperature (36°C) by the adding an equal volume of prewarmed media (48°C). Samples were removed and prepared at 10 minute intervals. Northern blots were probed sequentially with a 600 bp *Hind*III fragment of *CLN2*, a 600 bp *Nco*I-*Spe*I fragment of the actin gene and a 1.9 kb *Sph*I-*Bst*EII fragment of the *WHI3* gene.


C 0 10 20 30 40 50 60 70 80 90 100
WHI3
CLN2
ACTIN

(0.178 M) for 20 minutes. The levels of the *WHI3* transcript increased significantly (data not shown). This induction may be a consequence of polysome stabilization but similar increases were not observed when blots were reprobed with *ADH1*, actin, *CLN2* or *CLN3* (data not shown; M. Tyers, personal communication).

Many of the RNA recognition motif containing proteins discovered to date play an important role in splicing. In yeast only a small percentage of the genes sequenced to date contain introns. To investigate the possibility that *WHI3* encodes a splicing factor, northern blots containing RNA prepared from wild-type and mutant strains were probed with the actin gene and *SOS1*, the yeast homolog of the rat L35 ribosomal protein. If the Whi3 protein played an essential role in the splicing process then a difference in the size of the transcript might be expected. However, when the level and size of these transcripts was compared on Northern blots prepared with mutant and wild-type RNA there were no differences in either the size or the abundance of the transcripts (data not shown).

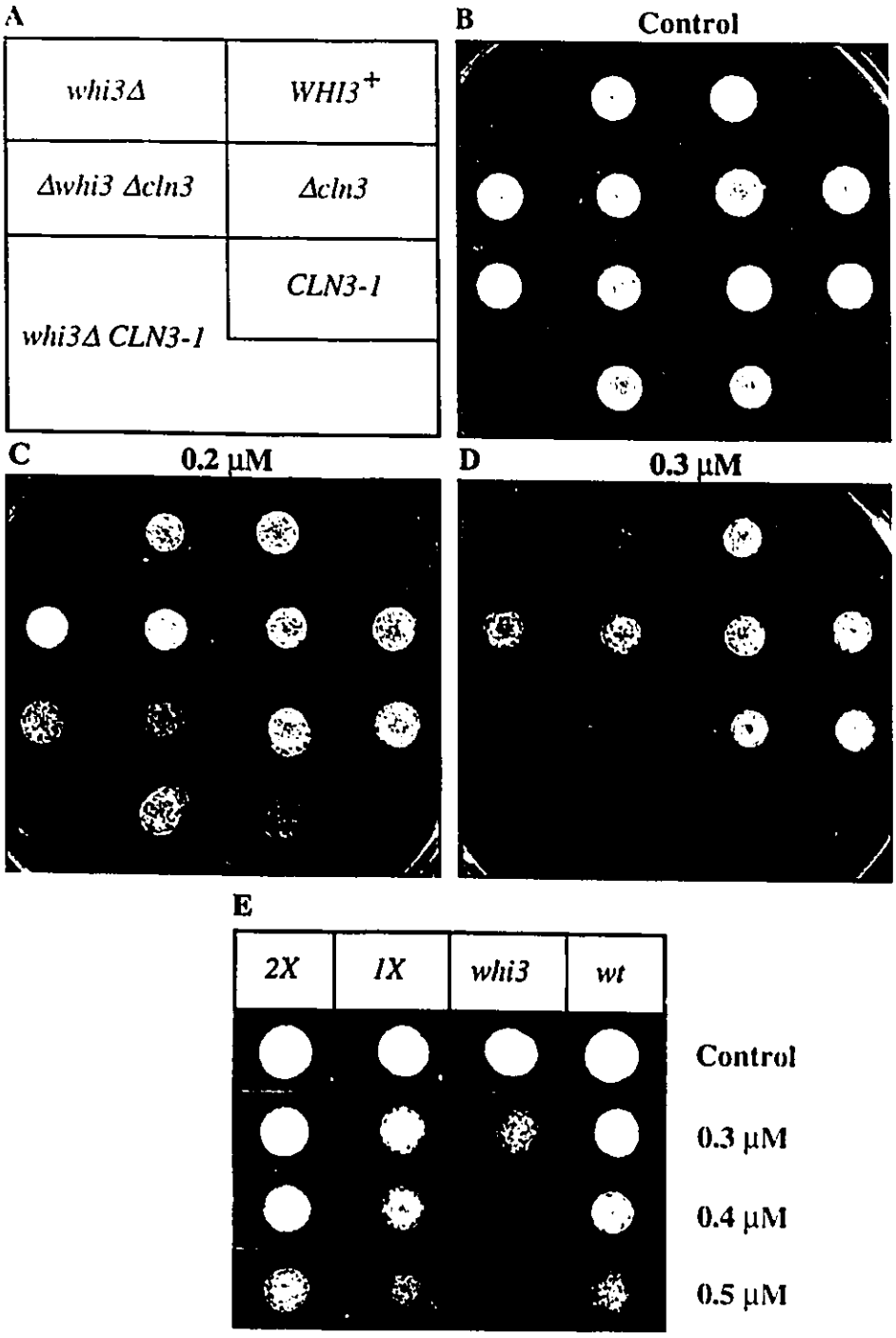
4.5 The *whi3* Mutant is Cycloheximide Sensitive.

The identification of a cycloheximide sensitive prestart step in yeast may define the cellular requirement for a critical rate of protein synthesis (Ko and Moore, 1990). A critical rate of protein synthesis may be required specifically for the accumulation of G1 cyclins so that a critical concentration can be reached and Start can be completed (Ko and Moore, 1990). If the Whi3 protein inhibits the G1/S phase transition by affecting some aspect of G1 cyclin accumulation then the *whi3* mutant might display an altered sensitivity to

cycloheximide. Since the *WHI3* transcript is induced by cycloheximide and low doses of cycloheximide result in a G1 delay it is possible that the Whi3 inhibitor is an important component of this delay. If a transient G1 arrest is necessary for recovery from the effects of this protein synthetic inhibitor then *whi3* mutants may be more sensitive.

Isogenic wild-type and *WHI3* deleted strains were plated onto YEPD plates containing increasing concentrations of cycloheximide. The growth of *WHI3*$^{+}$ strains was not affected until concentrations of 0.5-0.6 μM cycloheximide were used (data not shown and Figure 4.14E). The growth of *whi3* deletion strains was inhibited on plates containing 0.3-0.4 μM cycloheximide (Figure 4.14A-D). A similar difference in sensitivity to cycloheximide was observed when the *whi3* mutant strain (RN100-1d) was compared to an isogenic wild-type control strain (RN100-1b) (Figure 4.14E). Strains containing extra copies of the *WHI3* gene integrated at the *ura3* locus were tested but these were no more resistant than wild-type (Figure 4.14E). The sensitivity of the *CLN3-1* mutant and a *CLN3* deletion allele were also tested alone or in combination with a *whi3* deletion. There was no substantial change in the level of cycloheximide sensitivity although a *CLN3-1 whi3* double mutant appeared slightly more sensitive than the *whi3* deletion on its own (Figure 4.14A-D). When higher concentrations of cycloheximide (3.33 μM) were used *CLN3-1* mutants papillated resistant colonies more frequently than other mutant or wild-type strains (data not shown). The appearance of these rare resistant colonies required long periods of incubation and may suggest that *CLN3-1* mutants can survive for longer periods of time at these concentrations of cycloheximide allowing for rare resistant colonies to emerge.

Figure 4.14. *whi3* mutants are cycloheximide sensitive. Mid-log phase cultures of the *whi3* deletion strain RN210-13d and isogenic *CLN3-1* or *CLN3* deletion derivatives (see section 2.3.6) (panels A-D) were sonicated and 2000 cells were spotted onto plates containing increasing concentrations of cycloheximide. Plates were incubated for 2-3 days and the resistance of these strains was compared. In panel E, the wild-type strain RN100-1b, the mutant strain RN100-1d and derivatives containing one or two copies of the *WHI3* gene integrated at *URA3* were grown to mid-log phase and plated as above.


A
whi3Δ
WHI3+
Δwhi3 Δcln3
Δcln3
CLN3-1
whi3Δ CLN3-1
B
Control
C
0.2 μM
D
0.3 μM
E
2X
1X
whi3
wt
Control
0.3 μM
0.4 μM
0.5 μM

These results may indicate that the *whi3* mutant is specifically defective in some aspect of protein synthesis. Alternatively, the increased sensitivity may occur because the *whi3* mutant enters into a new cell cycle when conditions are inappropriate. The *whi3* mutant or the *whi3* null allele were crossed with a number of conditional mutants defective in protein synthesis but no strong suppression was observed (data not shown). There did appear to be a weak suppression of a poly (A) binding protein deletion (data not shown).

DISCUSSION

5.1 Ty1 Mutagenesis and the Isolation of *whi3*

Entry into the yeast cell cycle is controlled in the G1 phase of the cell cycle at a point called Start. The ability to complete Start and enter a new cell cycle is influenced by growth, nutrient availability, events of the previous cycle and mating pheromone (Pringle and Hartwell, 1981). The process of growth is rate limiting for cell cycle progression and it is during the G1 phase of the cell cycle where growth requirements are satisfied (Johnston *et al.*, 1977a). Once cells have obtained a critical cell size and satisfied the growth requirement, Start is completed and cells become committed to a full round of cell division. To investigate the controls which regulate the commitment event we used a screen designed to enrich for small cell size mutants. It was hoped that these mutants might define components that regulate critical cell size. The study of these mutants might provide insight into the controls regulating Start.

A mutagenesis procedure based on the transposition of a *neo* tagged Ty1 element to novel chromosomal locations was used to isolate *whi3* (Garfinkel and Strathern, 1991; Garfinkel *et al.*, 1988). This procedure was chosen because of difficulties that we had experienced cloning the *CLN3* gene. In contrast to selection schemes based on the complementation of conditionally lethal defects, screening individual transformants for size complementation is difficult and time consuming. The transposon mutagenesis procedure is advantageous as it generates physically and genetically tagged mutations

(Garfinkel and Strathern, 1991). That is, because the tagged Ty1 element has a selectable marker for both yeast and bacteria, the tagged Ty1 locus can be followed in genetic crosses and DNA flanking the site of Ty insertion element can be identified. This facilitates the isolation of yeast DNA flanking the site of insertion. The flanking DNA can be used as a probe to isolate the wild-type gene. The chromosomal position of the tagged Ty element can also be mapped because yeast chromosomes are readily separated by electrophoresis and the resulting chromoblots can be hybridized with the tagging gene. In addition, Ty1 elements can transpose into both coding and non-coding regions of genes resulting in both activating and inactivating transposition events (Boeke, 1989). Although transposition events preferentially occur within the 5' ends of genes, an alignment of target site sequences failed to identify a consensus sequence for insertion (Gafner and Philippsen, 1980; Eibel and Philippsen, 1984; Natsoulis *et al.*, 1989). Therefore, this procedure should be useful for the induction of semi-random tagged transposition events which might activate or inactivate a size control gene.

The tagged Ty1 element used in this procedure was placed under the control of the *GAL1* promoter so that transposition was inducible. After completing the mutagenesis, size selection was used to enrich for small cell size mutants. The unit gravity sedimentation device had been used extensively in our lab to size select yeast cells and worked quite effectively. In fact, after completing the size enrichment, relatively little screening was required to identify numerous small cell size mutants.

Once mutants had been identified and backcrossed, many segregated small cell size in a 2:2 fashion but this phenotype was linked to the G418 resistance phenotype in only one mutant. Small cell size segregated 4:0

in crosses between different mutants suggesting that the mutations had occurred at the same locus or were very tightly linked. There are three possibilities to explain the occurance of the untagged small cell size mutations at the *whi3* locus. They may have occurred as a result of a spontaneous mutation, an endogenous chromosomal element might have been induced to transpose or a tagged Ty might have recombined after transposition. Although these first two possibilities cannot be excluded, they seem less likely as this class of untagged mutants would have occurred independently of the tagged *whi3* Ty1 transposition event. The later possibility seems more likely as all of the mutants could be explained by the same initial transposition event. In addition, the most frequently occurring recombination event involving Ty elements results from a single crossover event between the long terminal repeats or δ elements (Boeke, 1989). Most of the Ty element including the marker would be lost but the mutant phenotype would remain because a solo δ element (LTR) would continue to interrupt the locus. When a galactose inducible Ty element was used to induce transpositions at the *URA3* locus, solo δ insertions were identified in some of the 5-FOA resistant mutants (Natsoulis, 1989). To explain these events the authors proposed that any DNA damage resulting from the transposition process might stimulate homologous recombination in the region of the terminal δ elements (Natsoulis, 1989). The untagged *whi3* mutants were not studied further because a small size mutant with an associated tagged Ty element had been discovered. Linkage between the small cell size phenotype and the G418 resistance, coupled with Southern blot analysis indicated that the tagged Ty1 element had transposed near, or within, a gene involved in size control.

Evidence obtained through the course of this study suggested that the tagged Ty1 element was responsible for the *whi3* mutation. Clones which contained the mutant *whi3* locus were identified and this DNA was used to replace the *WHI3* locus in a wild-type strain. The resulting G418 resistant transformants had a small cell size phenotype. Wild-type clones which spanned the site of Ty transposition were able to complement the size defect of the *whi3* mutant. Replacing the *WHI3* open-reading frame with the *LEU2* gene resulted in a small cell size phenotype. These results indicated that the tagged Ty element had caused the phenotype by transposition into a size control gene.

5.2 The *WHI3* Gene Encodes a Dose-Dependent Inhibitor of Start

The small cell size of the *whi3* mutant could be explained by an increase in either the expression or stability of an activator of commitment (like *CLN3-1*) or by the inactivation of an inhibitor. Heterozygous diploids had intermediate median cell volumes suggesting that *WHI3* was partially dominant. This was the first indication that the gene-product might have a dose-dependent activity. The cell size of a *WHI3*$^+$ strain was not affected when the mutant allele was present on a plasmid. This was consistent with a recessive loss of function mutation within an inhibitor. The small cell size phenotype of the null allele suggested that *WHI3* inhibited cell cycle progression by increasing the critical cell size requirement. In the absence of the Whi3 protein cells were able to enter into a new cell cycle at a reduced cell size.

When wild-type cells are grown in media containing various carbon sources there is a correlation between the growth rate supported by the carbon source and the size at which cells initiate a bud (Johnston *et al.*, 1979). Slower

growth rates result in a reduction in the critical cell size requirement and a lengthening of the G1 phase (Jagadish and Carter, 1977; Johnston *et al.*, 1979). When *whi3* mutants were grown in glycerol or ethanol containing media there was reduction in cell size proportional to that observed for wild-type cultures. Therefore, the *whi3* mutant was defective in basic cell size control rather than in the nutritional modulation of the critical cell size.

Plasmid constructs containing DNA from the *WHI3* locus were able to complement the size defect of the mutant strain and increased the size of a wild-type strain. This indicated that the *WHI3* gene had been cloned and that the gene functioned in a dose-dependent manner. Consistent with this, extra copies of the *WHI3* gene integrated into a *whi3* strain increased cell size in proportion to the number of copies integrated. When *WHI3* was overexpressed under the control of the *GAL1* promoter there was a large increase in cell size. The dose-dependence of the cell size effect was significant as it suggested that the cell was using the Whi3 protein as a metric to measure some parameter such as the accumulation of size or mass.

Although the evidence indicated that the *WHI3* gene encodes a dose-dependent inhibitor it did not indicate whether the inhibitory function was specific for Start. Earlier work indicated that both the critical cell size and the timing of Start were altered by a mutation, *CLN3-1*, that stabilized an activator of Start (Nash *et al.*, 1988; Cross, 1988). *CLN3* deletion strains had an increased critical cell size requirement and spent a longer period of time in the G1 phase of the cell cycle (Nash *et al.*, 1988; Cross, 1988). If the Whi3 protein was inhibiting Start by specifically affecting the critical cell size then loss of this inhibitor should advance cells through the cell cycle and extra copies should increase the proportion of cells in G1. This appeared to be the case but the

effects were not large. *whi3* mutant and *whi3* deletion strains typically had about 5% fewer cells in the G1 phase of the cell cycle. Increasing the copy number had a small effect in the anticipated direction but this effect was not always reproducible. Limitations in the resolution of the technique coupled with the size of the effect may account for these difficulties. It is also possible that an additional defect at the cryptic G2/M control point masked the effects by advancing the cells through both transitions. This would require that mutant strains have a shorter generation time. If the Whi3 protein were inhibiting growth processes in general then the doubling time might be affected but the critical cell size requirement should not. The *whi3 CLN3-1* double mutant was advanced through the cell cycle more than either of the single mutants. The most logical interpretation of these results is that Whi3 affects the critical cell size at which cells enter into a new cell cycle and has a small effect on the timing of Start.

The most conclusive demonstration that Whi3 was specifically affecting the completion of Start was obtained when the *WHI3* gene was fused to the *GAL1* promoter. Induction of *GAL1-WHI3* resulted in a dramatic increase in cell size and cell cycle arrest. The arrest occurred within one to two cell cycles and was accompanied by a large increase in the proportion of cells with a 1N DNA content. The tubulin staining pattern observed at the arrest point was consistent with what had been observed for unbudded cells in the G1 phase (Kilmartin and Adams, 1984). The tubulin staining pattern was identical to that observed for *cdc28-4* and *sit4-102* mutants when arrested at 37°C (Surana *et al.*, 1991; Sutton *et al.*, 1991). *Cdc28-4* mutants arrest specifically at Start and reciprocal shift experiments with *sit4-102* mutants positioned the arrest in G1 at Start (Pringle and Hartwell, 1981; Sutton *et al.*, 1991).

The Whi3 protein appears to inhibit passage through Start by affecting critical cell size. The dose-dependence suggests that the relative level of Whi3 is monitored by the cell and influences the size requirement. Passage through Start may therefore require the dilution, inactivation or destruction of the Whi3 protein. The *whi3* mutant and null allele would allow cells to complete Start at a smaller cell size due to a lack of the inhibitor. Cells with increased levels of the protein would be unable to complete Start until they reached a larger critical cell size. Cells overexpressing *WHI3* may arrest in G1 because of the high levels of this inhibitor and an inability to achieve the critical cell size correlated with these levels. This is the first described dose-dependent inhibitor of Start.

5.3 Whi3 and Cln3 Probably Function in Independent Pathways

With respect to the small cell size phenotype of the *whi3* mutant, it was intriguing that the *whi3 CLN3-1* double mutant displayed a further reduction in cell size. *CLN3* encodes one of the G1 cyclins responsible for promoting the G1/S phase transition through activation of the Cdc28 protein kinase (Tyers *et al.*, 1992). The *CLN3-1* mutation alters the critical cell size requirement and the timing of the commitment event (Cross, 1988; Nash *et al.*, 1988). Cells containing extra copies of the *CLN3-1* allele are not much smaller than cells containing a single copy of the activated allele suggesting that there is a limit to this effect, yet the *whi3 CLN3-1* double mutant is smaller than the *CLN3-1* mutant or a 2X *CLN3-1* mutant (Cross, 1988; Nash *et al.*, 1988). If the mutations were affecting the same pathway then a further reduction in cell size would not have been expected. Yet the reduction in cell size in the double

mutant was multiplicative and was present in all carbon sources tested. This is consistent with mutations in two independent pathways. Similar arguments could be made based on the results of the mating assays and α-factor response of single and double mutants. The *whi3 CLN3-1* double mutant had a synergistic mating defect consistent with mutations in different pathways. In addition, if these proteins were acting in opposition in the same pathway then the *ADH1-CLN2* construct or the *GAL1-CLN3-1* construct would have been expected to suppress the lethality associated with *WHI3* overexpression. However, neither of these constructs were able to suppress the lethality. If Whi3 were specifically inhibiting CLN mRNA accumulation or increasing mRNA turnover then differences in the abundance of these transcripts might have been expected when mutant and wild-type strains were compared. Northern analysis failed to reveal differences, suggesting that the G1 cyclin mRNAs were not targets of the RNA binding motif of the Whi3 protein. These results favor the interpretation that Whi3 and Cln3 are influencing cell size by independent mechanisms. To extend these observations it would be interesting to determine whether the *CLN2-1* mutation caused a further reduction in cell size when combined with *whi3* and *CLN3-1* mutations.

Strains with both *whi3* and *CLN3-1* mutations had a lower growth rate and lower viability than single mutants or wild-type strains and spontaneously diplodized. This suggested that the combined size effects were deleterious. The small cell size resulting from the combination of the two mutations may impose a selective pressure to increase cell size. One way to achieve this is through diplodization. The increase in cell volume appeared to give these cells a growth advantage as the population eventually became fully

diploid. The mechanism of diplodization is unknown but may result if cells bypass mitosis. Cells containing many copies of the *CLN3-1* allele spend a larger proportion of time between DNA synthesis and nuclear division and may be coordinating the processes of growth and division at the normally cryptic G2/M control (Cross, 1988). *whi3 CLN3-1* mutants had a shorter G1 phase and therefore also spend a larger proportion of time between DNA synthesis and nuclear division. This may enable cells to diplodize.

5.4 Mating Defects in *whi3* and *CLN3-1* Mutants

Addition of the α-factor to **a** cells activates a signal transduction pathway and results in cell cycle arrest and the transcriptional induction of genes required for the mating process (reviewed in Marsh *et al.*, 1991). This leads to cellular fusion followed by nuclear fusion and results in diploid formation. Cell cycle arrest requires the inactivation and turnover of specific activators of Start. The addition of α-factor induces a decrease in the levels of *CLN1* and *CLN2* mRNAs, proteins and associated kinase activities (Wittenberg *et al.*, 1990; Tyers *et al.*, 1992). Activating mutations in *CLN3* makes cells α-factor resistant (Cross, 1988; Nash *et al.*, 1988). Inactivating mutations in two negative cell cycle regulators, required for α-factor induced G1 arrest, *FUS3* and *FAR1*, also results in α-factor resistance (Elion *et al.*, 1990; Chang and Herskowitz, 1990).

The α-factor resistance of *whi3* mutant and deletion strains was measured since the lack of an inhibitor might make cells more permissive for Start and therefore less likely to arrest in the presence of α-factor. Both the *whi3* mutant and the deletion strain were more resistant then wild-type strains

but less resistant than *CLN3-1* mutants (Nash *et al.*, 1988). In addition, the *whi3* mutation seemed to increase the relative resistance of a *CLN3-1* mutant and increased the level of resistance of an α-factor sensitive *CLN3* deletion strain. The lack of this inhibitor made cells less responsive to α-factor.

A quantitative mating assay was used to measure the mating defects associated with *whi3* and *CLN3-1* single mutants and the *whi3 CLN3-1* double mutant. Both single mutants had a small reduction in mating ability but the double mutant had a synergistic mating defect and was very nearly sterile. This mating defect involved more than the cumulative effects of these mutations on cell size as diplodized double mutants were twice the size of haploids yet had a severe mating defect like that of the haploid.

Further investigation of the α-factor response in single and double mutants indicated that both *whi3* and *CLN3-1* single mutants displayed an intermediate response. The percentage of cells with a G1 DNA content increased, both mutants increased in cell size and formed schmoos. The arrest of the *CLN3-1* mutant was more transient then the *whi3* mutant or the wild-type control. This fits with the proposal that Cln3 plays an important role in the recovery process (Nash *et al.*, 1988). In contrast, the *whi3 CLN3-1* double mutant continued to divide, did not increase in cell size even transiently and only very rarely schmooed. The percentage of cells in the G1 phase of the cell cycle did not change during the α-factor timecourse. Furthermore, both of the single mutants displayed a relatively normal transcriptional induction of both the *FUS1* and *FUS3* messages while the double mutant displayed a very low level of induction. These results suggested that *whi3* and *CLN3-1* single mutants had a weak mating defect and were more resistant than wild-type strains. However, both single mutants displayed a normal α-factor response.

The *whi3 CLN3-1* double mutant had a severe mating defect and did not display a normal α-factor response. Each of these mutants altered the critical cell size requirement and the timing of commitment. As a result the double mutant spends very little time in the G1 phase of the cell cycle and cells may require a longer period of time in G1 to react to α-factor. The additive effects of these two mutations on the length of the G1 phase may eliminate a window of α-factor responsiveness. However, signal transduction, as measured by the expression of pheromone inducible genes, can still occur throughout the cell cycle even though there is some overlapping cell cycle control affecting the expression of these genes (Zanolari and Riezman, 1991). In other words, even though the double mutant spends little time in G1 these cells should still undergo a transcriptional induction. The lack of response displayed by the double mutant may therefore be indicative of a specific signal transduction defect. However, there has been no indication that *CLN3* or *WHI3* plays a role in signal transduction. It is possible that the combined effects of the two mutations enhance the recovery process so much that the signal transduction pathway is turned on but is then rapidly shut-off.

When α-factor was added to a *whi3 CLN3-1* double mutant, the levels of the *CLN2* transcript remained high throughout the timecourse. In wild-type cells the expression of *CLN2* decreased upon treatment with α-factor (Wittenberg *et al.*, 1990). In *CLN3-1* mutants the level of *CLN1* and *CLN2* mRNAs remain elevated (Cross and Tinklenberg, 1991; Dirick and Nasmyth, 1991). The levels of *CLN2* mRNA observed in the *whi3 CLN3-1* double mutant were even higher than those observed in the *CLN3-1* single mutant. This may be a direct consequence of the double mutant's inability to arrest and therefore may be indicative of the cycling state of the cells. If there is a specific defect in

the signal transduction pathway then the levels of *CLN1* and *CLN2* mRNAs might not be expected to decline.

The *whi3* mutation was able to partially suppress the *cdc39* defect. *CDC39* encodes a nuclear protein with two glutamine rich regions and functions as a negative transcriptional regulatory protein (Collart and Struhl, 1993). It was originally isolated as a class I Start mutant as cells continued to grow at the block and remained mating competent (Reed, 1980). It has since been shown that the G1 arrest of both *cdc36* and *cdc39* mutants is a secondary consequence of constitutive activation of the pheromone response pathway (Connolly *et al.*, 1983; Barros Lopes *et al.*, 1990; Neiman *et al.* , 1990). It has been proposed that these gene products function as negative regulators of the signal transduction pathway and may function at the level of the G proteins (Neiman *et al.*, 1990; Barros Lopes, *et al.*, 1990). The *whi3* mutation suppressed the temperature sensitive G1 arrest and reduced cell size at the normally non-permissive temperature. The Start arrest of *cdc39* mutants has also been suppressed non-specifically by secondary mutations that make cells α-factor resistant (Connolly *et al.*, 1983). The inactivation of an inhibitor of cell division may non-specifically suppress the loss of a negative regulator of the pheromone response pathway by returning these cells to a cycling state. Alternatively, the interaction may be specific because the *whi3* mutation did not suppress a *cdc36* mutant and this mutant arrests in G1 for similar reasons. If the *whi3* mutant was defective in some aspect of signal transduction it might be expected to suppress the constitutive activation of this pathway. It is also intriguing that both the Whi3 and the Cdc39 proteins have glutamine rich regions and these regions have been shown to be involved in protein-protein interactions (Pascal and Tjian, 1991; Hoey *et al.*, 1993).

5.5 The Sequence of *WHI3* and Functional Implications

After sequencing the *WHI3* gene the amino acid sequence derived from the open-reading frame was compared to the contents of the database. The C-terminus of *WHI3* had homology to an 80-90 amino acid motif termed the RNA recognition motif (RRM) (also called the consensus type RNA binding domain, RNP-80 and the RNP motif) (reviewed in Bandziulis *et al.*, 1989; Kenan *et al.*, 1991; Haynes, 1992). The most highly conserved sequences of this motif are the RNP-1 octapeptide and the RNP-2 hexapeptide (Bandziulis *et al.*, 1989; Kenan *et al.*, 1991; Haynes, 1992). RNA recognition motifs have been found in a large number of proteins involved in various aspects of RNA metabolism including: capping, splicing, polyadenylation, storage, transport and translation. In addition, some RNA binding proteins have important regulatory roles and are required for processes such as sexual and neuronal differentiation (Bandziulis *et al.*, 1989). Many RNA binding proteins contain multiple copies of this motif. The yeast poly (A) binding protein contains four of these motifs and only one is required to maintain RNA binding activity (Kenan *et al.*, 1987; Sachs *et al.*, 1987). The three-dimensional structure of the U1 small nuclear ribonucleoprotein A RRM has recently been determined (Nagai *et al.*, 1990; Hoffman *et al.*, 1991). It contains a four-stranded β sheet flanked by two α helices (Nagai *et al.*, 1990; Hoffman *et al.*, 1991). The RNP-1 and the RNP-2 sequences are located side by side in the middle two β strands forming a flat surface for binding RNA (Nagai *et al.*, 1990; Hoffman *et al.*, 1991). There appear to be structural and topological similarities with the ribosomal proteins suggesting an evolutionary relationship between the RNA-protein complexes involved in splicing and protein synthesis (Hoffmann *et al.*, 1991).

While the RNA binding domain of the Whi3 protein contains many of the conserved residues found in this group of proteins it displays a considerable amount of variability at other conserved positions. The RNP-2 hexapeptide is highly conserved but the RNP-1 motif is poorly conserved at three out of the first four positions of the octapeptide. The RNP-1 residues form part of one of the most highly conserved regions of the RNA recognition motif. Variance at conserved positions may lower the binding affinity to accommodate a mechanism for rapid turnover of the associated RNA (Kenan *et al.*, 1991). While the finding of an RNA binding motif is important in terms of understanding the role of the Whi3 protein in cell cycle control, the size of this family of proteins and the diversity of function that is represented makes it difficult to postulate as to the specific function of Whi3 in RNA metabolism.

Most of the RNA binding proteins described to date are modular in nature and contain auxiliary domains that may be important for protein-protein interactions (Bandzuilis *et al.*, 1989; Haynes, 1992). In this regard there are additional interesting features in Whi3. The Whi3 protein is serine rich throughout, contains a glutamine rich region located in the middle of the protein and a higher than average asparagine content.

A number of proteins have been described with glutamine rich regions. Some transcription factors including the mammalian proteins Sp1 and TAF110 have glutamine rich regions and the yeast transcription factor MCM1 or PRTF (Courey and Tijan, 1988; Hoey *et al.*, 1993; Passmore *et al.*, 1988; Ammerer, 1990). In the Sp1 protein these glutamine rich regions have been demonstrated to function in transcriptional activation (Courney and Tjian, 1988). The Sp1 protein interacts with the coactivator TAF110, a factor tightly associated with TFIID, through a glutamine rich region (Hoey *et al.*, 1993). In

addition to glutamine rich regions, both of these proteins also contain regions rich in serine and threonine. If the function of glutamine rich regions is to activate processes through protein-protein interactions it is possible that these regions of Whi3 serve similar purposes. There are other classes of proteins with glutamine rich regions. Several RNA binding proteins have an auxilliary domain rich in glutamine residues. The yeast *RNA15* gene which is involved in controlling the poly (A) tail status and stability of mRNA has a glutamine and asparagine rich stretch (Minvielle-Sebastia, 1991). A poly (A) binding protein localized to the granules of cytolytic lymphocytes plays a role in apoptosis and has a glutamine rich region (Tian *et al.*, 1991). Two *Drosophilia* neuronal RNA binding proteins, couch potato and embryonic lethal abnormal vision, have glutamine rich regions (Bellen *et al.*, 1992; Robinow *et al.*, 1988). In addition, two yeast proteins involved in cell cycle control, Cdc39 and Cks1, have glutamine rich regions (Collart and Struhl, 1993; Hadwiger *et al.*, 1989a). Finally, a glutamine rich region located in the two yeast casein kinase-1 homologs may function as a flexible tether between the catalytic core and the membrane lipid anchor (Wang *et al.*, 1992).

As mentioned above, Sp1 and TAF110 proteins both have serine/threonine rich regions (Courey and Tjian, 1988; Hoey *et al.*, 1993). The yeast protein kinases Ste20 and Bck1 have serine/threonine rich domains (Leberer *et al.*, 1992; Lee and Levin, 1992). In addition, several yeast cell surface proteins are rich in serine and threonine and many of these are O-glycosylated (Yamashita *et al.*, 1985; Kuranda and Robbins, 1991; Lipke *et al.*, 1989; Boone *et al.*, 1990; McCaffrey *et al.*, 1987; Roy *et al.*, 1991; Bender and Pringle, 1992). These proteins can be separated into two groups, one with ser/thr residues concentrated in discrete domains and a second group with

ser/thr residues located throughout (Bender and Pringle, 1992). A number of nuclear pore complex proteins have a repeated motif separated by spacer regions that are rich in glutamine, asparagine, serine and threonine (Wente *et al.*, 1992; Wimmer *et al.*, 1992). The *RCS1* gene, which has been implicated in size control, is rich in serine and threonine residues and has a high percentage of glutamine and asparagine residues (Gil *et al.*, 1991). The Whi3 protein is rich in all of these residues.

To explain both the phenotype of the mutant and the sequence homologies, the Whi3 protein may modulate the mRNA levels of a protein which functions in coordinating cell size at commitment. The Whi3 protein may affect the processing, transport or translation of this mRNA or affect its halflife. Whi3 may contact other proteins through its glutamine rich region or via its N-terminus. Recently, an RNP-1 like motif was identified in a class of DNA-binding proteins that function as transcription factors (Landsman, 1992). One of these has dual specificity binding both RNA and DNA (Murray *et al.*, 1992; Deschamps *et al.*, 1992). By analogy, it is possible that Whi3 binds to DNA and functions as a transcription factor as well as binding to RNA. This would explain the glutamine and serine rich regions found in the protein. However, the RNP homology indicates that it is more likely an RNA binding protein.

5.6 Transcriptional Analysis

A low abundance transcript of about 2.8 kb was identified in wild-type strains. The codon bias was calculated and was very low suggesting that *WHI3* encodes a low abundance protein. A strong correlation has been observed between the calculated codon bias index and the cellular mRNA levels

for a number of genes (Bennetzen and Hall, 1982). The low *WHI3* mRNA levels and the predicted abundance of the protein may indicate that Whi3 has a regulatory role. Block-release experiments indicated that the message did not oscillate in abundance but was present at a low constant level throughout the cell cycle. Much of the *WHI3* transcript was unstable, and disappeared rapidly with a half-life of 10 minutes or less. Instability may facilitate the rapid modulation of transcript abundance in response to changing growth conditions. A small subpopulation of transcript appeared to be quite stable. This may indicate that there are two subpopulations of mRNA existing in different subcellular compartments or that the message exists in two forms with one form more susceptible to degradation.

An abundant transcript of 1.1 kb was identified in the *whi3* mutant. The qualitative difference resulted from the insertion of the Ty1 element creating a fusion transcript between *WHI3* and Ty1. The reasons for the quantitative differences were not investigated. The increased abundance of the transcript could be due to an increase in the stability of the message or could result from increased expression of the message. Enhancer like sequences capable of increasing gene expression have been identified in the δ sequences (LTRs) of Ty elements (Boeke, 1989). To differentiate between these two possibilities, the RNA polymerase II mutant or α-amanitin, could be used to shut off transcription so that the stability of the mutant transcript could be measured and compared to that of the wild-type.

Addition of the protein synthetic inhibitor cycloheximide resulted in the transcriptional induction of the *WHI3* message. This effect has been observed for the serum inducible early-response genes in Swiss 3T3 cells. When serum treated cells were treated with cycloheximide the early-response

genes were superinduced as a result of prolonged transcription and stabilization of the mRNAs (Lau and Nathans, 1987; Almendral *et al.*, 1988). In the case of c-myc, a sequence located within the protein coding region coupled the turnover of this message to its translation and this cotranslational degradation was inhibited by cycloheximide (Wisdom and Lee, 1991).

The *WHI3* transcript was negatively regulated by α-factor. When cells were treated with α-factor, a decrease in the level of the *WHI3* transcript was observed within 1-2 hours of treatment. For comparison, the *CLN1* and *CLN2* messages decreased within 15 minutes of treatment and may constitute part of the arrest process (Wittenberg *et al.*, 1990). A pheromone responsive transcript that was turned off within 30-60 minutes of α-factor addition has also been described (Stetler and Thorner, 1984). The timing of the dissapearance of the *WHI3* transcript correlated more closely with a recovery rather than an arrest response. The decrease in abundance of the *WHI3* message coincided with the transient appearance of a shorter transcript that may represent an intermediate in message turnover. The remaining transcript could be resistant to degradation or could represent newly synthesized message. The α-factor induced modulation of the *WHI3* transcript may represent an important step in the recovery process as decreasing the levels of an inhibitor of Start may allow cells to reenter the cell cycle after arrest.

5.7 Cycloheximide Effects

A cycloheximide sensitive prestart step has been described in yeast and may define the cellular requirement for a critical rate of protein synthesis (Ko and Moore, 1990). The critical rate of protein synthesis would facilitate the

net accumulation of unstable molecules like the G1 cyclins until a critical concentration triggered the completion of Start (Ko and Moore, 1990). If the *whi3* mutants small cell size resulted from a reduction in the critical cell size requirement necessary for passage through Start then mutants might display an altered sensitivity to cycloheximide. The *whi3* mutant and deletion strains were tested and both were found to be more cycloheximide sensitive than wild-type controls. These effects were small but reproducible. If the Whi3 protein were functioning at Start by inhibiting G1 cyclin accumulation then *whi3* mutants might be expected to be more cycloheximide resistant than wild-type strains. Since the opposite result was obtained, this was more consistent with what would be expected if *WHI3* functioned independently of the G1 cyclins.

It is possible that the transient G1 arrest which occurs when cells are treated with limiting doses of cycloheximide requires the Whi3 protein. This might explain why the *WHI3* message is induced when cells are treated with cycloheximide as it might facilitate the G1 delay. The mutant might be more sensitive because it can no longer pause in G1 to overcome the effects of cycloheximide but enters into the cell cycle inappropriately. The *whi3* mutant's increased sensitivity may also be affected by the mutants reduced cell size requirement. For the *CLN3-1* mutant, the small size has been correlated with decreased levels of both RNA and protein per cell (Carter and Sudbery, 1980; Cross, 1989). If the *whi3* mutant's small cell volume correlates with a reduced protein and RNA content per cell then the cellular protein synthetic apparatus may not be able to tolerate the effects of cycloheximide as well as wild-type strains. In contrast, although *CLN3-1* mutants have reduced protein and RNA content per cell, these mutants still display a G1 delay when treated with limiting doses of cycloheximide (Cross, 1989). The increased stability of this

normally labile G1 cyclin might allow *CLN3-1* mutants to survive long enough to papillate resistant colonies at higher concentrations while other mutant and wild-type strains did not.

Many of the cycloheximide sensitive mutants isolated previously have defective ribosomal subunits or defective translation initiation factors (Singh and Manney, 1974; Sachs and Davis, 1989, Kawai *et al.*, 1992; Kitaoka and Miyazaki, 1990; T. Zhong, personal communication). A number of cycloheximide sensitive mutants were isolated as suppressors of a temperature sensitive poly (A) binding protein mutant (Sachs and Davis, 1989). The poly (A) binding protein has been postulated to play a role in ensuring that only full length poly $(A)^+$ mRNAs will be translated efficiently. Conditional mutants were defective in poly (A) tail shortening and the initiation of translation (Sachs and Davis, 1989). The cycloheximide sensitive suppressors suppressed the translational initiation defect by altering the amount of the 60S ribosomal subunit but did not alter the length of poly (A) tail (Sachs and Davis, 1989). The *whi3* deletion partially suppressed the poly (A) binding protein deletion. This observation coupled with the cycloheximide sensitivity of the *whi3* mutant might suggest that the Whi3 protein is involved in some aspect of translational initiation. However, the cycloheximide effect was small and the other phenotypes of the *whi3* mutant make it more likely that the cycloheximide sensitivity was a secondary consequence of the primary defect.

5.8 Conclusions and Future Directions

A novel small cell size mutant called *whi3* was isolated using a Ty mutagenesis procedure and size enrichment. The phenotype of the mutant and

the replacement was consistent with the proposal that *WHI3* encodes an inhibitor of cell division. The inhibitory effects were dose-dependent and correlated with an increased cell size requirement. The size effects of the *whi3* mutant were additive with those of the *CLN3-1* mutant, a stabilized variant of a G1 cyclin. This may indicate that Whi3 is affecting cell size through an independent pathway. A potential RNA binding domain identified through sequence homology suggests that Whi3 is an RNA binding protein. The RNA binding domain was located in the carboxyl terminus of the protein. The rest of the protein may mediate protein-protein interactions or may regulate the activity and subcellular localization of the protein.

In addition to the size defect, the *whi3* mutant was α-factor resistant but did undergo a normal transcriptional response and cell cycle arrest. The *whi3 CLN3-1* mutant had a severe mating defect, did not undergo cell cycle arrest in the presence of α-factor and did not display a significant transcriptional induction. This may indicate that the combination of the two mutations results in a signal transduction defect or alternatively may result from the double mutants altered requirements for passage through Start.

Although this work suggests that Whi3 functions in the coordination of growth and division by its effect on the critical cell size and completion of Start, additional studies will be required to understand the precise role of the Whi3 protein in this process. There are a number of logical experimental avenues to follow in attempting to understand the function of Whi3. For example, if the Whi3 protein binds to a specific RNA it would be very informative to determine the identity of the RNA. The most straightforward way to accomplish this would be to tag the protein with a triple copy of the HA1 epitope. This epitope has been used by others in the lab to tag the yeast G1

cyclins and has been particularly useful for studying the rare Cln3 protein. A high affinity monoclonal antibody which specifically recognizes the HA epitope is available. Once tagged, the Whi3 protein can be identified and it may be possible to coimmunoprecipitate an associated RNA. UV crosslinking has proven to be a useful technique for the study of hnRNPs and the 70K U1 snRNP protein (Dreyfuss *et al.*, 1988; Query *et al.*, 1989). If an RNA is not precipitated with the tagged protein then UV light could be used to covalently crosslink the protein to its RNA substrate. By labelling cellular RNA with [^{3}H]uracil, specific binding could be visualized on protein gels. In addition, unlabeled RNA and DNA could be used as competitor to determine the specificity of the interaction. If a sufficient quantity of RNA could be obtained, cDNA synthesis could be used to make the complementary DNA for the purposes of sequencing or the RNA could be sequenced directly. If enough RNA could be obtained a radioactive probe could be synthesized using reverse transcriptase and random primers or oligo (dT) primers. This probe could be used to identify the gene and the chromosomal location on the lambda filter library. To determine whether the Whi3 protein can bind to DNA a filter binding assay could be used. It might also be possible to use *in vitro* translated protein in gel retardation studies. The ability of the Whi3 protein to alter the mobility of a random mix of oligonucleotides could then be tested.

In addition, epitope tagging would facilitate studies of the Whi3 protein. The abundance, half life and the levels of the protein throughout the cell cycle could be determined. The role of post-translational modifications such as phosphorylation and glycosylation could be investigated. Subcellular fractionation could be used to determine the cellular distribution. Immunofluorescent staining using the monoclonal antibody could also be used

to determine the intracellular localization of the tagged Whi3 protein.

Overexpression of Whi3 results in a lethal G1 arrest and this phenotype could be used as the basis for a suppressor screen. Initial attempts to isolate high copy number suppressors were successful but rescuing clones were suppressing the lethality by reducing the expression of the *GAL1-WHI3* construct. A continuation of this suppressor analysis might identify the gene encoding the RNA target of the Whi3 protein, genes encoding negative regulators of Whi3 function or activators of Start that overcome the inhibitory effects of Whi3. Mutagenesis could also be used to isolate chromosomal mutations that reverse the lethality of the GAL overexpressor. This may identify positive regulators of Whi3 function, downstream components in the Whi3 pathway, or interacting components required for Whi3 function.

In a separate screen the mating deficiency of a *whi3 CLN3-1* double mutant could be used to identify suppressors that restore mating. This approach might not be as direct because high copy plasmids or mutations that increase the length of the G1 phase might nonspecifically reverse the mating defect. However, if the double mutant has a specific defect in the signal transduction pathway then it may be possible to restore mating by affecting components in the pathway. If mating competent suppressors can be isolated then these could be investigated to see if α-factor results in transcriptional induction and cell cycle arrest. This might provide information that would prove useful for dissecting the specific defect associated with the *whi3 CLN3-1* double mutant. If the mating defect in the double mutant occurred because of a short G1 phase and relaxed requirements for commitment then G1 could be lengthened by growth in glycerol. The α-factor responsiveness of the strain could then be measured.

The Whi3 protein may function in the Cdc28/G1 cyclin pathway by regulating the accumulation or turnover of the G1 cyclins or other components of the positive feedback loop. Alternatively, it may function as an inhibitor in a parallel pathway. To address these possibilities it would be interesting to determine whether the Cdc28/Cln kinase activity is high or low when *GAL1-WHI3* strains are induced. This might differentiate between these possibilities as low kinase activity would suggest that Whi3 is exerting its effects through the Cdc28/Cln pathway. If the Cdc28/Cln kinase activity was high this might indicate that the strain is arrested at Start due to a block in an independent parallel pathway. The levels of the Cln1, Cln2 and Cln3 protein and associated kinase activities could also be compared in *whi3* and wild-type strains to see whether differences exist.

Northern analysis was used to address the possibility that the Whi3 RNA binding protein regulates the accumulation or enhances the turnover of G1 cyclin mRNAs. The levels of *CLN1*, *CLN2*, *CLN3* and *SWI6* mRNAs present in wild-type and *whi3* mutant strains were compared and found to be similar. A difference in the level of a cell cycle regulated transcript that peaks in G1 might not be observed if the mutant strain spends less time in G1. That is, if message levels peak in G1 but mutants spend a shorter time in this phase of the cell cycle then differences might not be apparent in samples prepared from asynchronous cells. To overcome this problem, RNA could be isolated from wild-type and mutant strains after synchronization. The RNA levels present in G1 phase cells could then be directly compared. It is interesting in this respect that the levels of the *CLN2* message were high in the α-factor treated double mutant. To investigate the possibility that Whi3 effects the stability or half life of the *CLN2* message, RNA could be prepared from *GAL1-CLN2* strains grown

on galactose and then shifted to glucose to repress transcription. The stability of the *CLN2* message could then be compared in mutant and wild-type strains. Alternatively, the temperature sensitive RNA polymerase II mutant could be used to measure the half-life of the *CLN2* message in *whi3* strains.

To investigate the reasons for the increased cycloheximide sensitivity of *whi3* mutant strains sucrose gradient sedimentation could be used to measure the polyribosome content and to determine the relative content of 40S and 60S ribosomal subunits. These gradients could be used to compare the ribosomal profiles of *whi3* and wild-type strains. In addition, the ribosome profiles of a *GAL1-WHI3* strain which has been induced could be analyzed. It might also be informative to see whether the mutant strain experiences a G1 delay like wild-type strains. This could be measured by quantitating the percentage of unbudded cells after the addition of cycloheximide and by FACS analysis. If the mutant strain does not experience a G1 delay then this would support the idea that the mutant is more sensitive because it enters into a new cell cycle inappropriately.

Although these studies have not defined the precise role of the Whi3 protein it is hoped that the work presented will provide the necessary frame work for future studies aimed at understanding the role of Whi3 in the coordination of growth and division.

BIBLIOGRAPHY

Almendral, J.M., Sommer, D., Macdonald-Bravo, H., Burckhardt, J., Perera, J. and Bravo, R. (1988) Mol. Cell. Biol. *11*, 2140-2148.

Ammerer, G. (1990) Genes Dev. *4*, 299-312.

Andrews, B.J. and Herskowitz, I. (1989) Cell *57*, 21-29.

Bandziulis, R.J., Swanson, M.S. and Dreyfuss, G. (1989) Genes Dev. *3*, 431-437.

Baroni, M.D., Martegani, E., Monti, P. and Alberghina, L. (1989) Mol. Cell. Biol. *9*, 2715-2723.

Baroni, M.D., Monti, P., Marconi, G. and Alberghina, L. (1992) Exp. Cell Res. *201*, 299-306.

Barros Lopes, M., Ho, J.Y. and Reed, S.I. (1990) Mol. Cell. Biol. *10*, 2966-2972.

Becker, D.M. and Guarente, L. (1991) Methods Enzymol. *194*, 182-187.

Bedard, D., Johnston, G.C. and Singer, R.A. (1981) Current Genetics 4, 205-214.

Bellen, H.J., Kooyer, S., D'Evelyn, D. and Pearlman, J. (1992) Genes Dev. *6*, 2125-2136.

Bender, A. and Pringle, J.R. (1992) Yeast *8*, 315-323.

Bennetzen, J.L. and Hall, B.D. (1982) J. Biol. Chem. *257*, 3026-3031.

Boeke, J.D. (1989) In: Mobile DNA Berg, D.E. and Howe, M.M., eds. American Society of Microbiology, Washington, D.C. pp 335-374.

Boeke, J.D., Xu,H. and Fink, G.R. (1988) Science *239*, 280-282.

Boone, C., Sommer, S.S., Hensel, A. and Bussey, H. (1990) J. Cell Biol. *110*, 1833-1843.

Bossie, M.A., Deltoratius, C., Barcelo, G. and Silver, P. (1992) Mol. Biol. Cell *3*, 875-893.

Boutelet, F., Petitjean, A. and Hilger, F. (1985) EMBO J. *4*, 2635-2641.

Brenner, C., Nakayama, N., Goebl, M., Tanaka, K., Toh-e, A. and Matsumoto, K. (1988) Mol. Cell. Biol. *8*, 3556-3559.

Brewer, B.J., Chlebowicz-Sledziewska, E. and Fangman, W.L. (1984) Mol. Cell. Biol. *4*, 2529-2531.

Broach, J.R. (1991) Trends Genetics *7*, 28-33.

Cairns, B.R., Ramer, S.W. and Kornberg, R.D. (1992) Genes and Dev. *6*, 1305-1318.

Calvert, G.R. and Dawes, I.W. (1984) Nature *312*, 61-63.

Camonis, J.H., Kalekine, M., Gondre, B., Garreau, H., Boy Marcotte, E. and Jacquet, M. (1986) EMBO J. *5*, 375-380.

Carter, B.L.A. and Jagadish, M.N. (1978) Exp. Cell Res. *112*, 15-24.

Carter, B.L.A. and Sudbery, P.E. (1980) Genetics *96*, 561-566.

Chang, F. and Herskowitz, I. (1990) Cell *63*, 999-1011.

Church, G.M. and Gilbert, W. (1984) Proc. Nat. Acad. Sci. *81*, 1991-1995.

Collart, M.A. and Struhl, K. (1993) EMBO J. *12*, 177-186.

Connolly, B.M., Bugeja, V.C., Piggot, J.R. and Carter, B.L.A. (1983) Curr. Genetics *7*, 309-312.

Courey, A.J. and Tjian, R. (1988) Cell *55*, 887-898.

Cross, F.R. (1988) Mol. Cell Biol. *8*, 4675-4684.

Cross, F.R. (1989) J. Cell Sci. Suppl. *12*, 117-127.

Cross, F.R. (1990) Mol. Cell Biol. *10*, 6482-6490.

Cross, F.R., Roberts, J. and Weintraub, H. (1989) Ann. Rev. Cell Biol. 5, 341-395.

Cross, F.R. and Tinkelenberg, A.H. (1991) Cell *65*, 875-883.

Deschamps, S., Viel, A., Garrigo, M., Denis, H. and Maire, M. (1992) J. Biol. Chem. *267*, 13799-13802.

Dirick, L., Moll, T., Auer, H. and Nasmyth, K. (1992) Nature *357*, 508-513.

Dirick, L. and Nasmyth, K. (1991) Nature *351*, 754-757.

Dreyfuss, G., Swanson, M.S. and Pinol Roma, S. (1988) Trends Biol. Sci. *13*, 86-91.

Eibel, H. and Philippsen, P. (1984) Nature, *307*, 386-388.

Eichinger, D.J. and Boeke, J.D. (1990) Yeast *6*, 139.

Elion, E.A., Brill, J.A. and Fink, G.R. (1991) Proc. Nat. Acad. Sci. USA *88*, 9392-9396.

Elion, E.A., Grisafi, P.L. and Fink, G.R. (1990) Cell *60*, 649-664.

Epstein, C.B. and Cross F. (1992) Genes Dev. *6*, 1695-1706.

Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. *132*, 6-13.

Fernandez-Sarabia, M.J., Sutton, A., Zhong, T. and Arndt, K.T. (1992) Genes Dev. *6*, 2417-2428.

Fitch, I., Dahmann, C., Surana, U., Amon, A., Nasmyth, K., Goetsch, L., Byers, B. and Futcher, B. (1992) Mol. Cell. Biol. *3*, 805-818.

Frolich, K.U., Fries, H.W., Rudiger, W., Erdmann, R., Botstein, D. and Mecke, D. (1991) J. Cell Biol. *114*, 443-453

Gafner, J. and Philippsen, P. (1980) Nature *286*, 414-418.

Garfinkel, D.J., Mastrangelo, M.F., Sanders, N.J., Shafer, B.K. and Strathern, J.N. (1988) Genetics *120*, 95-108.

Garfinkel, D.J. and Strathern, J.N. (1991) Methods Enzymol. *194*, 342-361.

Gartner, A., Nasmyth, K. and Ammerer, G. (1992) Genes Dev. *6*, 1280-1292.

Gerring, S.L., Connelly, C. and Hieter, P. (1991) Methods Enzymol. *194*, 57-77.

Ghiara, J.B., Richarson, H.E., Sugimoto, K., Henze, M., Lew, D.J., Wittenberg, C. and Reed, S.I. (1991) Cell *65*, 163-174.

Gil, R., Zueco, J., Sentandreau, R. and Herrero, E. (1991) Yeast *7*, 1-14.

Haber, J.E. and Halvorson, H.O. (1972) J. Bact. *109*, 1027-1033.

Hadwiger, J.A., Wittenberg, C., Mendenhall, M.D. and Reed, S.I. (1989a) Mol. Cell. Biol. *9*, 2034-2041.

Hadwiger, J.A., Wittenberg, C., Richardson, H., Barros Lopes, M. and Reed, S.I. (1989b) Proc. Nat. Acad. Sci. USA *86*, 6255-6259.

Hanahan, D.H. (1983) J. Mol. Biol. *166*, 557-580.

Hanic-Joyce, P.J., Singer, R.A. and Johnston, G.C. (1987) J. Biol. Chem. *262*, 2845-2851.

Hartwell, L.H. (1973) J. Bact. *115*, 966-974.

Hartwell, L.H. (1974) Bacteriol. Rev. *38*, 164-198.

Hartwell, L.H. (1976) J. Mol. Biol. *104*, 803-817.

Hartwell, L.H., Culotti, J., Pringle, J.R. and Reid, B.J. (1974) Science *183*, 46-51.

Hartwell, L.H., Culotti, J. and Reid, B. (1970) Proc. Nat. Acad. Sci. USA *66*, 352-359.

Hartwell, L.H., Mortimer, R.K., Culotti, J. and Culotti, M. (1973) Genetics *74*, 267-286.

Hartwell, L.H. and Unger, M.W. (1977) J. Cell Biol. *75*, 422-435.

Haynes, S.R. (1992) The New Biologist *4*, 421-429.

Helms, C., Dutchik, J.E. and Olson, M.V. (1987) Methods Enzymol. *153*, 69-82.

Herrick, D., Parker, R. and Jacobson, A. (1990) Mol. Cell. Biol. *10*, 2269-2284.

Hindley, J. and Phear, G.A. (1984) Genes *31*, 129-134.

Hoey, T., Weinzierl, R.O.J., Gill, G., Chen, J.L., Dynlacht, B.D. and Tjian, R. (1993) Cell *72*, 247-260.

Hoffman, D.W., Query, C.C., Golden, B.L., White, S.W. and Keene, J.D. (1991) Proc. Nat. Acad. Sci. USA *88*, 2495-2499.

Holm, C., Meeks-Wagner, D.W., Fangman, W.L. and Botstein, D. (1986) Gene *42*, 169-173.

Ito, H., Fukura, Y., Murata, K. and Kimura, A. (1983) J. Bact. *153*, 163-168.

Jagadish, M.N. and Carter, B.L.A. (1977) Nature *269*, 145-147.

Jahng, K.Y., Ferguson, J. and Reed, S.I. (1988) Mol. Cell. Biol. *8*, 2484-2493.

Johnston, G.C., Ehrhardt, C.W., Lorincz, A. and Carter, B.L.A. (1979) J. Bact. *137*, 1-5.

Johnston, G.C., Pringle, J.R. and Hartwell, L.H. (1977a) Exp. Cell. Res. *105*, 79-98.

Johnston, G.C. and Singer, R.A. (1978) Cell *14*, 951-958.

Johnston, G.C., Singer, R.A. and McFarlane, E.S. (1977b) J. Bact. *132*, 723-730.

Kawai, S., Murao, S., Mochizuki, M., Shibuya, I., Yano, K. and Takagi, M. (1992) J. Bact. *174*, 254-262.

Kenan, D.J., Query, C.C. and Keene, J.D. (1991) Trends Biol. Sci. *16*, 214-220.

Kilmartin, J.V. and Adams, A.E.M. (1984) J. Cell Biol. *98*, 922-933.

Kim, Y.J. and Baker, B.S. (1993) Mol. Cell. Biol. *13*, 174-183.

Kitaoka, Y. and Miyazaki, M. (1990) Nuc. Acids Res. Symp. Series 22, 69-70.

Ko, H.A. and Moore, S.A. (1990) J. Biol. Chem. *265*, 21652-21663.

Kohrer, K. and Domdey, H. (1991) Methods Enzymol. *194*, 398-405

Kozak, M. (1986) Cell *44*, 283-292.

Krainer, A.R., Mayeda, A., Kozak, D. and Binns, G. (1991) Cell *66*, 383-394.

Kuranda, M.J. and Robbins, P.W. (1991) J. Biol. Chem. *266*, 19758-19767.

Landsman, D. (1992) Nuc. Acids Res. *20*, 2861-2864.

Lau, L.F. and Nathans, D. (1987) Proc. Nat. Acad. Sci. USA *84*, 1182-1186.

Leberer, E., Dignard, D., Harcus, D., Thomas, D.Y. and Whiteway, M. (1992) EMBO J. *11*, 4815-4824.

Lee, K.S. and Levin, D.E. (1992) Mol. Cell Biol. *12*, 172-182.

Linskens, M.H.K. and Huberman, J.A. (1988) Mol. Cell Biol. *8*, 4927-4935.

Lipke, P.N., Wojciechowicz, D. and Kurjan, J. (1989) Mol. Cell. Biol. *9*, 3155-3165.

Lord, P.G. and Wheals, A.E. (1981) J. Cell Sci. *50*, 361-376.

Lorincz, A. and Carter, B.L.A. (1979) J. Gen. Micro. *113*,287-295.

Lorincz, A. and Carter, B.L.A. (1983) J. Gen. Micro. *129*, 1599-1605.

Lorincz, A.T. and Reed, S.I. (1984) Nature *307*, 183-185.

Mann, C., Micouin, J.Y., Chiannilkulchai, N., Treich, I., Buhler, J.M. and Sentenac, A. (1992) Mol. Cell. Biol. *75*, 422-435.

Marsh, L., Neiman, A.M. and Herskowitz, I. (1991) Ann. Rev. Cell Biol. *7*, 699-728.

Matsumoto, K., Uno, I. and Ishikawa, T. (1983) Exp. Cell Res. *146*, 151-161.

Matsumoto, K., Uno, I., Oshima, Y. and Ishikawa, T. (1982) Proc. Nat. Acad. Sci. USA *79*, 2355-2359.

McCaffrey, G., Clay, F.J., Kelsay, K. and Sprague Jr., G.F. (1987) Mol. Cell Biol. *7*, 2680-2690.

Minvielle-Sebastia, L., Winsor, B., Bonneaud, N. and Lacroute, F. (1991) Mol. Cell Biol. *11*, 3075-3087.

Moore, S.A. (1988) J. Biol. Chem. *263*, 9674-9681.

Morgan, A.R., Lee, J.S., Pulleyblank, D.E., Murray, N.L. and Evans, D.H. (1979) Nuc. Acids Res. *7*, 547-569.

Mountain, H.A. and Sudbery, P.E. (1990) J. Gen. Micro. *136*, 733-737.

Murray, A.W. and Kirschner, M.W. (1989) Science *246*, 614-621.

Murray, M.T., Schiller, D.L. and Franke, W.W. (1992) Proc. Nat. Acad. Sci. USA *89*, 11-15.

Nagai, K., Oubridge, C., Jessen, T.H., Li, J. and Evans, P.R. (1990) Nature *348*, 515-520.

Nash, R., Tokiwa, G., Anand, S., Erickson, K. and Futcher, A.B. (1988) EMBO *7*, 4335-4346.

Nasmyth, K. and Dirick, L. (1991) Cell *66*, 995-1013.

Natsoulis, G., Thomas, W., Roghmann, M.C., Winston, F. and Boeke, J.D. (1989) Genetics *123*, 269-279.

Neiman, A.M., Chang, F., Komachi, K. and Herskowitz, I. (1990) Cell Regulation *1*, 391-401.

Nonet, M., Scafe, C., Sexton, J. and Young, R. (1987) Mol. Cell. Biol. *7*, 1602-1611.

Nurse, P. (1990) Nature *344*, 503-508.

Ogas, J., Andrews, B.J. and Herskowitz, I. (1991) Cell *66*, 1015-1026.

Olson, M.V., Dutchik, J.E., Graham, M.Y., Brodeur, G.M., Helms, C., Frank, M., MacCollin, M., Scheinman, R. and Frank, T. (1986) Proc. Nat. Acad. Sci. USA *83*, 7826-7830.

Paquin, C.E. and Williamson, V.M. (1984) Science *226*, 53-55.

Pascal, E. and Tjian, R. (1991) Genes Dev. *5*, 1646-1656.

Passmore, S., Maine, G.T., Elble, R., Christ, C. and Tye, B.K. (1988) J. Mol. Biol. *204*, 593-606.

Pearson, W.R. and Lipman, D.J. (1988) Proc. Nat. Acad. Sci. *85*, 2444-2448.

Peter, M., Gartner, A., Ammerer, G. and Herskowitz, I. (1993) Cell, *73*, 747-760.

Peterson, E.A. and Evans, W.H. (1967) Nature *214*, 824-825.

Piggott, J.R., Rai, R. and Carter, B.L.A. (1982) Nature *298*, 391-393.

Popolo, L., Vanoni, M. and Alberghina, L. (1982) Exp. Cell Res. *142*, 69-78.

Prendergast, J.A., Murray, L.E., Rowley, A., Carruthers, D.R., Singer, R.A. and Johnston, G.C. (1990) Genetics *124*, 81-90.

Primig, M., Sockanathan, S., Auer, H. and Nasmyth, K. (1992) Nature *358*, 593-597.

Pringle, J.R. and Hartwell, L.H. (1981) In: The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, Strathern, J.N., Jones, E.W. and Broach, J.R., eds. Cold Spring Harbor Labs, Cold Spring Harbor, N.Y. pp 97-142.

Query, C.C., Bentley, R.C. and Keene, J.D. (1989) Cell *57*, 89-101.

Reed, S.I. (1980) Genetics *95*, 561-577.

Reed, S.I., Hadwiger, J.A. and Lorincz, A. (1985) Proc. Nat. Acad. Sci. USA *82*, 4055-4059.

Reed, S.I., Hadwiger, J., Richardson, H. and Wittenberg, C. (1989) J. Cell Sci. Suppl. *12*, 29-37.

Richardson, H.E., Lew, D.J., Henze, M., Sugimoto, K. and Reed, S.I. (1992) Genes Dev. *6*, 2021-2034.

Richardson, H.E., Wittenberg, C., Cross, F. and Reed S.I. (1989) Cell *59*, 1127-1133.

Robinow, S., Campos, A.R., Yao, K.W. and White, K. (1988) Science *242*, 1570-1572.

Rogers, S., Well, R. and Rechsteiner, M. (1986) Science *234*, 363-368.

Rose, M.D., Winston, F. and Hieter, P. (1990) Laboratory Course Manual for Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Rowley, A., Singer, R.A. and Johnston, G.C. (1991) Mol. Cell. Biol. *11*, 5718-5726.

Roy, A., Lu, C.F., Marykwas, D.L., Lipke, P.N. and Kurjan, J. (1991) Mol. Cell. Biol. *11*, 4196-4206.

Russell, I.D. and Tollervey, D. (1992) J. Cell Biol. *119*, 737-747.

Sachs, A.B. and Davis, R.W. (1989) Cell *58*, 857-867.

Sachs, A.B., Davis, R.W. and Kornberg, R.D. (1987) Mol. Cell. Biol. *7*, 3268-3276.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Nat. Acad. Sci. USA *74*, 5463-5467.

Saul, D.J., Walton, E.F., Sudbery, P.E. and Carter, B.L.A. (1985) J. Gen. Micro. *131*, 2245-2251.

Shilo, B., Riddle, V.G.H. and Pardee, A.B. (1979) Exp. Cell Res. *123*, 221-227.

Sidorova, J. and Breeden, L. (1993) Mol. Cell Biol. *13*, 1069-1077.

Sikorski, R.S. and Hieter, P. (1989) Genetics *122*, 19-27.

Singer, R.A., Johnston, G.C. and Bedard, D. (1978) Proc. Nat. Acad. Sci. USA *75*, 6083-6087.

Singh, A. and Manney, T.R. (1974) Genetics *77*, 651-659.

Song, O., Dolan, J.W., Yuan, Y.O. and Fields, S. (1991) Genes Dev. *5*, 741-750.

Sprague Jr., G.F. (1991) Methods Enzymol. *194*, 77-93.

Stetler, G.L. and Thorner, J. (1984) Proc. Nat. Acad. Sci. USA *81*, 1144-1148.

Stevenson, B.J., Rhodes, N., Errede, B. and Sprague Jr., G.R. (1992) Genes Dev. *6*, 1293-1304.

Struhl, K. (1985) Proc. Nat. Acad. Sci. USA *82*, 8419-8423.

Sudbery, P.E., Goodey, A.R. and Carter, B.L.A. (1980) Nature *288*, 1401-1404.

Surana, U., Robitsch, H., Price, C., Schuster, T., Fitch, I., Futcher, A.B. and Nasmyth, K. (1991) Cell *65*, 145-161.

Sutton, A., Immanuel, D. and Arndt, K.T. (1991) Mol. Cell. Biol. *11*, 2133-2148.

Takagaki, Y., MacDonald, C.C., Shenk, T. and Manley, J.L. (1992) Proc. Nat. Acad. Sci., USA *89*, 1403-1407.

Tian, Q., Streuli, M., Saito, H., Schlossman, S.F. and Anderson, P. (1991) Cell *67*, 629-639.

Toda, T. Cameron, S., Sass, P., Zoller, M., Scott, J.D., McMullen, B., Hurwitz, M., Krebs, E.G. and Wigler, M. (1987a) Mol. Cell. Biol. *7*, 1371-1377.

Toda, T., Cameron, S., Sass, P., Zoller, M., Wigler, M. (1987b) Cell *50*, 277-287.

Tyers, M., Fitch, I., Tokiwa, G., Dahmann, C., Nash, R., Linskens, M. and Futcher, B. (1991) Cold Spring Harbor Quantitative Symposia on Quantitative Biology *LVI*, 21-32.

Tyers, M. and Futcher, B. (1992) Mol. Cell. Biol., in press.

Tyers, M., Tokiwa, G., Nash, R. and Futcher, B. (1992) EMBO J. *11*, 1773-1784.

Tyers, M., Tokiwa, G. and Futcher, B. (1993) EMBO J. *12*, 1955-1968.

Unger, M.W. and Hartwell, L.H. (1976) Proc. Nat. Acad. Sci. USA *73*, 1664-1668.

Verdier, J.M. (1990) Yeast *6*, 271-297.

Viera, J. and Messing, J. (1987) Methods Enzymol. *154*, 3-83.

Wang, P-C., Vancura, A., Micheson, T.G.M. and Kuret, J. (1992) Mol. Biol. Cell *3*, 275-286.

Wente, S.R., Rout, M.P. and Blobel, G. (1992) J. Cell Biol. *119*, 705-723.

Wimmer, C., Doye, V., Grandi, P., Nehrbass, U. and Hurt, E.C. (1992) EMBO J. *11*, 5051-5061.

Wisdom, R. and Lee, W. (1991) Genes Dev. *5*, 232-243.

Wittenberg, C. and Reed, S.I. (1988) Cell *54*, 1061-1072.

Wittenberg, C., Sugimoto, K. and Reed, S.I. (1990) Cell *62*, 225-237.

Yamashita, I., Suzuki, K. and Fukui, S. (1985) J. Bact. *161*, 567-573.

Zanolari, B. and Reizman, H. (1991) Mol. Cell. Biol. *11*, 5251-5258.

LIST OF PUBLICATIONS

Papers

Nash, R., Tokiwa, G., Anand, S., Erickson, K., and Futcher, A.B. (1988). The *WHI1*$^+$ gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. EMBO J. *7*, 4335-4346.

Tyers, M., Fitch, I., Tokiwa, G., Dahmann, C., Nash, R., Linskens, M. and Futcher, B. (1991). Characterization of G1 and mitotic cyclins of budding yeast. Cold Spring Harbor Symposia on Quantitative Biology *LVI*, 21-32.

Tyers, M., Tokiwa, G., Nash, R. and Futcher, B. (1992). The Cln3-Cdc28 kinase complex of *S.cerevisiae* is regulated by proteolysis and phosphorylation. EMBO J. *11*, 1773-1784.

Nash, R. and Futcher, A.B. (in preparation). Isolation of the *whi3* cell size mutant whose gene product encodes an inhibitor of Start.

Abstracts

Nash, R.S. and Futcher, A.B. (1986). Characterization of the *whi1* mutation of *S. cerevisiae*. 13th International Conference on Yeast Genetics and Molecular Biology, Banff, Alberta, Canada. Yeast *2*, S262.

Nash, R., Tokiwa, G. and Futcher, A.B. (1987). Characterization of a new whi mutant of *S. cerevisiae*. Yeast Cell Biology Meeting, Cold Spring Harbor Laboratory, New York.

Nash, R., Tokiwa, G., Anand, S., Stojcic, C., Hazlett, M., Erickson, K. and Futcher, B. (1988). Cloning and partial characterization of the *WHI1* gene of *S. cerevisiae*. 14th International Conference on Yeast Genetics and Molecular Biology, Espoo, Finland. Yeast 4, S51.

Tokiwa, G., Nash, R., Erickson, K., Anand, S., Tyers, M. and Futcher, B. (1989). The *WHI1* gene tethers cell division to cell size and is a cyclin homolog. Yeast Genetics and Molecular Biology, Atlanta, Georgia.

Nash, R., Tokiwa, G., Erickson, K., Tyers, M. and Futcher, B. (1989). The *CLN3* gene of *S. cerevisiae* is a cyclin homologue and helps regulate start. Yeast Cell Biology Meeting, Cold Spring Harbor Laboratory, New York.

Futcher, B., Tokiwa, G., Erikson, K., Tyers, M. and Nash, R. (1989). The *CLN3* gene of *S. cerevisiae* is a cyclin homologue and regulates the G1/S transition. Eukaryotic DNA Replication Meeting, Cold Spring Harbor Laboratory, New York.

Tyers, M., Tokiwa, G., Fitch, I., Nash, R. and Futcher, B. (1990). Biochemistry of Cln3, a G1 cyclin of *S. cerevisiae*. FASEB. Summer Conference on Yeast Chromosome Structure and Replication. Vermont.

Tyers, M., Tokiwa, G., Nash, R. and Futcher, A.B. (1990). Biochemical analysis of cln3, a G1 cyclin of *S. cerevisiae*. 15th International Conference on Yeast Genetics and Molecular Biology, The Hague, Netherlands.

Yeast 6, S156.

Tyers, M., Tokiwa, G., Nash, R., Fitch, I., and Futcher, B. (1991). Biochemical characterization of the G1 cyclins of *S. cerevisiae*. Symposium: The Cell Cycle, Cold Spring Harbor Laboratory, New York.

Nash, R. and Futcher, B. (1991). Characterization of *whi3*, a new cell size mutant. Yeast Cell Biology Meeting, Cold Spring Harbor Laboratory, New York.