The *GCS1* Gene of *Saccharomyces cerevisiae*

by

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For my father, Professor Kenneth F. Ireland (1937-1991)

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ABSTRACT

Mutations in the *GCS1* gene of *Saccharomyces cerevisiae* produce a novel reentry-mutant phenotype. At a restrictive temperature, reentry-mutant cells are specifically impaired for resumption of proliferation from stationary phase in response to fresh growth medium. In contrast, the ability of actively dividing mutant cells to maintain ongoing proliferation is not impaired at the temperature that is restrictive for stationary-phase mutant cells. Thus, the reentry-mutant phenotype genetically defines requirements for resumption of proliferation from stationary phase that are distinct from those for maintenance of ongoing proliferation. In this thesis, a molecular and genetic characterization of the *GCS1* gene has extended our characterization of the reentry-mutant phenotype. The *GCS1* gene sequence predicts a 39-kd polypeptide containing a CxxC(x_{16})CxxC putative Zn-finger motif. Either deletion of the *GCS1* gene or amino-acid substitution of the 2nd cysteine in the Zn-finger motif produces the same reentry-mutant phenotype, suggesting that the Zn-finger domain is important for Gcs1 protein function.

ABBREVIATIONS AND SYMBOLS

ATP	adenosine 5'-triphosphate
cAMP	adenosine 3',5'-cyclic-monophosphate
A-kinase	cAMP-dependent protein kinase
eIF-4E	eucaryotic initiation factor-4E
RNA	ribonucleic acid
RNAase A	ribonuclease A
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ORF	open reading frame
C-terminal	carboxyl-terminal
N-terminal	amino-terminal
PEG	polyethylene glycol
DTT	Dithiothreitol
SDS	sodium dodecyl sulfate
dH_2O	distilled water
EMS	ethyl methane sulfonate
PBS	phosphate-buffered saline
bp	base pairs
kbp	kilobase pairs
sec	second
min	minute
h	hour
rpm	revolutions per minute
cM	centiMorgans
PD	parental ditype
NPD	non-parental ditype
TT	tetratype

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I. INTRODUCTION

1. Introductory remarks

Like other unicellular organisms, the budding yeast *Saccharomyces cerevisiae* proliferates in response to nutrients and ceases to proliferate upon nutrient depletion. Nonproliferating cells that maintain viability for long periods under conditions of nutrient insufficiency are generally thought to be in a distinct physiological state, termed stationary phase. These stationary-phase cells differ both physiologically and genetically from actively proliferating cells (Hartwell, 1974; Pringle and Hartwell; 1981; Werner-Washburne *et al.*, 1993), and resumption of cell proliferation from this nonproliferating stationary-phase state occurs in response to renewed supplies of nutrients. Since stationary-phase cells differ from actively proliferating cells, one could predict that resumption of proliferation from stationary phase has requirements that are distinct from those for maintenance of ongoing proliferation. However, this prediction was not confirmed genetically until the discovery of a novel mutant phenotype (Drebot *et al.*, 1987).

Mutant phenotypes that are imposed only under certain conditions, are particularly useful for the study of cell proliferation, if only because mutations that prevent cell proliferation under all conditions are necessarily lethal. Such conditional mutations frequently impose mutant phenotypes only at a particular temperature (termed the restrictive temperature). In general, cold-sensitive mutants are impaired at temperatures below 23°C, while temperature-sensitive mutants are impaired at temperatures above 29°C. However, conditions other than temperature can also impose conditional-mutant phenotypes. Of particular interest to this lab, was the discovery of a mutant in which cell proliferation was impaired at a restrictive temperature of 14°C

(cold-sensitive), but only when cells were in a certain physiological state. This physiological state was met by stationary-phase cells but not by actively proliferating cells. Thus, this novel conditional mutation specifically impaired the resumption of cell proliferation by stationary-phase cells at 14°C in response to fresh growth medium, without simultaneously affecting the ability of actively dividing cells to maintain ongoing proliferation at the same temperature (Drebot, 1987).

This novel mutant phenotype has been called a "reentry-mutant" phenotype (Drebot *et al.*, 1987). Stationary-phase reentry-mutant cells are cold-sensitive for the resumption of cell proliferation. In contrast, actively proliferating reentry-mutant cells maintain ongoing proliferation at a temperature that is restrictive for stationary-phase cells. Thus, the reentry-mutant phenotype genetically defines requirements for resumption of proliferation from stationary phase that are distinct from those for maintenance of ongoing cell proliferation. This thesis describes a molecular approach that has extended our analysis of the reentry-mutant phenotype.

2. Cell morphology, cell division, growth status and the life cycle in *S. cerevisiae*

The budding yeast *S. cerevisiae* is a particularly useful organism with which to study cell proliferation. As the name "budding yeast" implies, cell division in this yeast proceeds by the formation of a protrusion, or bud, on the cell surface which gradually enlarges to form a new cell. The process of cell division is completed by separation of the newly-formed "daughter" cell from the original "mother" cell. Thus, in this organism the progress of cell division can be monitored by observation of cell morphology.

Cell division in *S. cerevisiae* requires the duplication and movement of nuclear and cellular constituents into the growing bud. Early investigators observed that the process of cell division is temporally organized so that DNA replication occurs within a

discrete interval of time, beginning at approximately the same time as the initiation of a bud, and ending well before nuclear division (Williamson, 1965). This temporal organization which is such that chromosome duplication always precedes segregation of duplicated chromosomes between mother and daughter cell, can be represented by the concept of a cell cycle (Michison, 1971). The cell cycle in *S. cerevisiae*, as in higher eukaryotes, can be divided into 4 phases (Hartwell, 1974). Initiation of DNA replication, which coincides morphologically with initiation of a bud (Williamson, 1965; Hartwell, 1974; Johnston *et al.*, 1980), occurs in S phase. The completion of DNA replication marks the end of S phase and the beginning of the G2 interval that separates the completion of DNA replication from nuclear division. During nuclear division, or M phase, duplicated chromosomes are segregated between mother and daughter cells. The completion of nuclear division marks the end of M phase and the beginning of the G1 interval. Cells in G1 that have completed cytokinesis and cell separation show an unbudded morphology and contain unreplicated nuclear DNA (Pringle and Hartwell, 1981).

Cell morphology is also a useful indicator of stationary phase for nonproliferating cells. As previously described, nonproliferating cells that have ceased cell proliferation through nutrient depletion and yet continue to maintain viability are thought to be in a distinct physiological state, termed stationary phase (reviewed in Werner-Washburne *et al.*, 1993). These viable, nonproliferating cells are almost invariably found with a uniform cell morphology of single, unbudded cells (reviewed in Hartwell, 1974). This morphology indicates that these cells ceased proliferation from the G1 phase of the cell cycle. This regulated arrest of cell proliferation in the G1 phase of the cell cycle is correlated with maintenance of viability in nonproliferating cells, since starvation conditions that result in a mixture of budded and unbudded cells fail to produce a population of viable cells. For example, cells that are starved for magnesium

arrest proliferation in all phases of the cell cycle, and these cells rapidly lose viability (Pringle and Hartwell, 1981). In contrast, cells that are starved for glucose or ammonia undergo a regulated arrest of cell proliferation in G1 resulting in a population of unbudded, viable cells (Hartwell, 1974). This "nutrient-arrest" of cell proliferation in the G1 interval of the cell cycle occurs in a number of different starvation conditions including depletion of a carbon or nitrogen source and starvation for sulphur, phosphate, biotin, or potassium (Hartwell 1974; Johnston *et al.*, 1977a).

Arrest of cell proliferation in the G1 or unbudded interval of the cell cycle also occurs as a function of the life cycle of this budding yeast (reviewed in Herskowitz, 1988). The life cycle of *S. cerevisae* consists of interconvertible diploid and haploid forms. Haploid cells can fuse and form diploid cells; diploid cells can undergo meiosis and sporulate to form 4 haploid spores. Haploid cells coordinate their cell cycles for fusion and diploid formation by mutual arrest of cell proliferation in G1 (Hartwell, 1973a). Fusion of haploid cells in G1 ensures the production of diploid cells instead of the triploid or tetraploid cells that could (theoretically) be produced by fusion of cells in other phases of the cell cycle. Haploid cells exhibit one of two mating types a or α, and arrest in G1 in response to mating pheromone secreted by haploid cells of the opposite mating type (Wilkinson and Pringle, 1974). This G1 arrest is mediated by a signal-transduction pathway that ultimately results in the fusion of haploid cells of opposite mating types and the formation of a/α diploid cells (reviewed in Sprague and Thorner, 1993). The G1 arrest of cell proliferation mediated by mating pheromones clearly differs from the G1 arrest mediated by starvation. Cells that are arrested in G1 by mating pheromones continue to grow (enlarge) and are competent for conjugation and diploid formation. In contrast, stationary-phase cells arrested in G1 by starvation (nutrient arrest) do not continue to grow and are competent for maintenance of viability in the absence of nutrients.

3. Regulation of cell cycle initiation occurs in G1

One explanation for the observed arrest of cell proliferation as unbudded cells in response to nutrient limitation is that initiation of new buds (coinciding with S phase) is extremely sensitive to nutrient-limiting conditions. This explanation has been verified experimentally by transfer of actively dividing cells to a starvation medium lacking nitrogen and observation by time-lapse photo-microscopy. Budded cells that were in S, G2 and M phases were all able to complete their cell cycles and cease cell proliferation as unbudded cells in G1, producing extremely small daughter cells in the process. Thus for cells that have already initiated new buds, completion of the formation of daughter cells occurs even under severe nutrient limitation. However, unbudded cells that are in G1 at the time of shift to a starvation medium require growth beyond a critical size to initiate new buds (Johnston, 1977; Johnston *et al.*, 1977a). Thus, new buds are initiated only when sufficient mass has already been accumulated so that the cell cycle can be completed even under severe nutrient limitation (Pringle and Hartwell, 1981).

Further evidence that the regulation of initiation of a new cell cycle occurs in the G1 interval has come from examination of growth conditions that increase the length of time required for completion of the cell cycle. Under optimal growth conditions on rich medium at 29°C, the length of the cell cycle in *Saccharomyces cerevisiae* is approximately 90 min. Under less optimal nutrient conditions or in the presence of inhibitors of protein synthesis the length of the cell cycle can be prolonged, and most of the increase in the length of the cell cycle occurs by delay of initiation of a new cell cycle. This delay results in a prolonged G1 interval (Shilo *et al.*, 1978; Johnston et al., 1980) without affecting the time for completion of S phase, G2 and M phase (Carter and Jagadish, 1978). Thus the commitment to initiation of a new cell cycle occurs in

the G1 interval, and takes into account factors that include cell size, rate of protein synthesis, and the nutrient status of the cell (Hartwell, 1974; Moore 1983).

Early models postulated a requirement for the accumulation of a labile protein in G1 to explain the sensitivity of cell cycle initiation to the rate of protein synthesis (Shilo *et al.*, 1978; Moore, 1988). These ideas have recently been substantiated by the discovery of a requirement for certain labile proteins that peak in abundance just prior to the initiation of S phase (Richardson *et al.*, 1989). These labile proteins, named G1-cyclins, activate a protein kinase that is critical for progression from G1 to S phase (Wittenburg *et al.*, 1990). This protein kinase, encoded by the *CDC28* gene, is referred to as either p34 or Cdc28 kinase. The substrates of Cdc28 kinase are unknown, but they are thought to include transcription factors for genes required for DNA replication (Nasmyth, 1993).

Conditional mutations in *CDC28* arrest cells in G1 as unbudded cells at the same step in the G1 interval (termed Start) as haploid cells blocked by the presence of mating pheromones (Reid and Hartwell, 1977). Cells that are nutritionally deprived arrest at or before the step in the cell cycle that is mediated by *CDC28*, probably because nutritional deprivation prevents accumulation of G1 cyclins and activation of Cdc28 kinase (Hadwiger *et al.*, 1989; Mendenhall *et. al*, 1987; Reed *et al.*, 1988). Thus resumption of cell proliferation from stationary phase ultimately requires the accumulation of labile G1-cyclin proteins to a concentration sufficient to activate Cdc28 protein kinase and initiate a new cell cycle.

4. Growth phase and accumulation of reserve carbohydrates

One essential characteristic of stationary-phase cells is functionally defined by maintenance of viability in the absence of nutrients (Werner-Washburne *et al.*, 1993). This maintenance of viability requires more than arrest of cell proliferation in G1; it

also involves physiological changes that occur in response to decreasing nutrient levels while cells are still engaged in ongoing cell proliferation. For example, stationary-phase cells are frequently produced through exhaustion of rich glucose-based medium (YEPD); under these conditions cell proliferation is ultimately arrested through depletion of the carbon source (Lillie and Pringle, 1980). To describe the physiological adaptation to carbon-source depletion, it is helpful to briefly outline how yeast cells utilize fermentable carbon sources such as glucose. In the presence of glucose as a carbon source, yeast cells derive most of their ATP from the fermentation of glucose to ethanol, and delay the metabolism of ethanol until glucose levels can no longer support the high level of glycolysis required for growth in the absence of respiration (Gancedo and Serrano, 1989). This preference for growth on fermentable carbon sources results in two phases of exponential growth, the first being rapid exponential growth supported by a prodigious rate of glycolysis, and the second phase allowing a much slower rate of proliferation, for a few more generations, during which the ethanol produced during fermentation is consumed. The shift from fermentative to respiratory growth is termed the diauxic shift, and the slow growth on ethanol following the diauxic shift is termed the post-diauxic phase (Kappelli, 1986). Finally, upon depletion of the ethanol cells cease proliferation in stationary phase.

Physiological adaptation to carbon-source depletion includes the synthesis of the reserve carbohydrates glycogen and trehalose (Lillie and Pringle, 1980). Glycogen accumulation in yeast cells proliferating on rich medium with glucose as a carbon source begins approximately one generation time before glucose exhaustion at the diauxic shift (Lillie and Pringle, 1980). Thus adaptation to decreasing glucose levels begins while cells are still engaged in rapid exponential growth. Some degradation of accumulated glycogen is observed at the diauxic shift when glucose is virtually exhausted, and thus may supply energy to cells during the transition from fermentative

to respiratory growth (Lillie and Pringle, 1980). Despite the decrease in glycogen levels at the diauxic shift, glycogen is still maintained at high levels (4-6-fold higher than early log-phase cells) during the postdiauxic phase and during early stationary phase (Lillie and Pringle, 1980), only decreasing after prolonged incubation of stationary-phase cells.

Accumulation of trehalose during depletion of rich glucose-based medium coincides with glucose exhaustion at the diauxic shift and continues during the post-diauxic phase, reaching levels in stationary-phase cells that are 20-fold higher than the levels of trehalose in cells engaged in fermentative growth. Levels of trehalose gradually decline during prolonged incubation of stationary-phase cells, and depletion of trehalose below certain levels correlates with the eventual loss of viablity in stationary-phase cells (Lillie and Pringle, 1980). Thus the accumulation of reserve carbohydrates may be important for the maintenance of viability under starvation conditions.

Cells also enter stationary phase as a result of starvation for other essential nutrients such as nitrogen, phosphorus, sulfur, or biotin (Hartwell, 1974). Transfer of proliferating cells to medium lacking these essential nutrients allows yeast cells to continue to proliferate for a limited number of cell divisions (using intracellular reserves of the essential nutrient) during which stationary-phase properties are acquired. The accumulation of storage carbohydrates in yeast cells transferred to medium lacking either nitrogen or sulfur occurs without a significant depletion of glucose, and thus without a shift from fermentative to respiratory growth. Trehalose and glycogen accumulate at different rates and to different levels in cells starved for nitrogen or sulfur compared to cells allowed to enter stationary-phase through exhaustion of glucose during growth on rich medium (Lillie and Pringle, 1980). Thus the physiological

characterization of stationary-phase cells may also reflect the type of starvation that leads to the cessation of cell proliferation (Werner-Washborne *et al.*, 1993).

5. Stationary phase is a distinct physiological state

Stationary-phase nutrient-arrested cells differ physiologically, biochemically, and morphologically both from actively proliferating cells and from cells arrested in G1 by mating pheromones. In addition to the accumulation of reserve carbohydrates, stationary-phase cells show alterations in cell-wall structure that correlate with increased resistance to cell-wall-degrading enzymes found in the snail gut preparation called glusulase (Deutch and Parry, 1974). This altered cell-wall structure may also account for the refractile, phase-bright appearance of stationary-phase cells (Bujega *et al.*, 1982) when observed by phase-contrast microscopy (actively proliferating cells are not refractile). Increased resistance to cell-wall-digesting enzymes and accumulation of carbohydrates is correlated with increased resistance to other stresses such as brief exposures to high temperatures (Plesset *et al.*, 1987; Iida and Yahara, 1984a). This ability to survive brief exposures to high temperatures (~50°C), a property called thermotolerance, may be related to either the increased levels of carbohydrate (Attfield, 1992) or the preferential synthesis of heat-shock proteins in stationary-phase cells (discussed below).

Overall, transcription and translation are decreased approximately 20-fold in stationary-phase cells (Choder, 1991; Boucherie, 1985). Decreased rates of protein synthesis during stationary phase are observed for 95% of the proteins present in proliferating cells (Boucherie, 1985; E. Braun, personal communication), and this decreased protein synthesis may result in part from an overall decrease in transcription during stationary phase (Choder, 1991). This general transcriptional repression during stationary phase requires a functional topoisomerase I (Choder, 1991), and since

topoisomerase I affects DNA supercoiling, this transcriptional repression may involve altered chromatin structure. Further evidence for altered chromatin structure in stationary-phase cells has come from experiments showing that the chromatin from stationary-phase cells has a sedimentation coefficent in sucrose gradients distinct from that of chromatin from proliferating cells, (Pinon, 1978). However, the importance of altered chromatin structure or decreased transcription for maintenance of viability in stationary phase is unclear. *top1* mutant cells that fail to repress transcription in stationary phase still maintain long-term viability (Choder, 1991). Thus, the relationship of the numerous physiological and molecular changes observed in stationary-phase cells to the fundamental stationary-phase characteristic of viability in the absence of proliferation is not well understood.

6. Heat-shock gene expression in stationary phase

As previously described, both transcription and translation are decreased 20-fold overall in stationary phase. However, some mRNAs are synthesized at the same levels in proliferating and stationary-phase cells, while other mRNAs that are virtually undetectable in proliferating cells are extremely abundant during stationary phase (Choder, 1991). These stationary-phase-specific genes include members of the heat-shock gene family (Bataille *et al.*, 1991; Werner-Washburne *et al.*, 1989). Heat-shock genes include genes whose expression is increased by a variety of stimuli including exposure to elevated temperatures, ethanol, amino-acid analogs, and inhibitors of oxidative phosphorylation (Craig, 1985, 1993). A small number of heat-shock genes including *HSP82*, *SSA3*, *HSP104* , and *HSP12* are abundantly transcribed in stationary-phase cells, but are not expressed in unstressed proliferating cells (Craig, 1993; Bataille , 1991). Some of these heat-shock genes, including *HSP82* and *SSA3*, may encode proteins that function as "molecular chaperones" that are involved in the stabilization of

protein conformation or the refolding of damaged proteins (Gething and Sambrook, 1992). Such chaperone proteins could be envisaged as playing a role both in thermotolerance and in maintenance of long-term viability in stationary phase through stabilization of essential proteins against denaturation and subsequent degradation. However, experimental support for a requirement for individual heat-shock proteins in thermotolerance and maintenance of viability in stationary phase is marginal. No single heat-shock protein has been shown to be indispensable for thermotolerance or maintenance of viablity in stationary phase (Praekelt and Meacock, 1990). This lack of experimental evidence for a role for heat-shock proteins in thermotolerance and maintenance of viability in stationary phase may be due to overlapping functions of heat-shock proteins (Praekelt and Meacock, 1990).

7. Protein turnover in stationary phase

Protein turnover or degradation of preexisting proteins may supply most of the precursor requirements for protein synthesis in stationary-phase cells (Sumrada and Cooper, 1978; Johnston *et al.* 1977b). Since the rate of protein turnover in stationary-phase cells is increased relative to that in proliferating cells (Halvorson, 1958; Johnston *et al.*, 1977b), it is not surprising that at least two genes involved in protein turnover, *UBI4* and *UBC5*, are highly expressed in stationary-phase cells (Finley *et al.*, 1987; Seufert and Jentsch, 1990). The *UBI4* and *UBC5* gene products function in ubiquitin-mediated protein turnover in stationary-phase cells. The importance of the *UBI4* gene product for maintenance of viability in stationary phase is demonstrated by the phenotype of *ubi4* mutant cells, which fail to arrest in G1 upon starvation for nutrients and rapidly lose viability (Tanaka *et al.*, 1988). Thus some of the requirements for G1 arrest and subsequent entry into stationary phase are associated with protein turnover. Mutations in another gene involved in ubiquitin-mediated protein turnover, *UBC1*, lead

to the converse effect, a delay in resumption of cell proliferation from stationary phase (Seufert *et al.*, 1990). Thus protein turnover is involved both in entrance into and resumption of cell proliferation from stationary phase. The elevated expression of genes involved in protein turnover in stationary phase, together with genes involved in chaperone functions, may reflect increased protein turnover of non-essential proteins in combination with increased protection of essential proteins in stationary-phase cells.

8. Stationary-phase properties are not unique to stationary-phase cells

Stationary-phase cells can be described as viable cells that have ceased cell proliferation in the G1 phase of the cell cycle in response to nutrient depletion. This maintenance of viability in the absence of nutrients is associated with accumulation of reserve carbohydrates, thermotolerance, resistance to cell-wall digesting enzymes, and a 20-fold overall decrease in transcription and translation that is manifested as a cessation of growth or mass accumulation (Pringle and Hartwell, 1981; Werner-Washburne *et al.*, 1993). However, with the exception of the G1 arrest and cessation of mass accumulation, most of the properties of stationary-phase cells are also found in slowly proliferating cells that are in all phases of the cell cycle. Increased thermotolerance, accumulation of storage carbohydrates, and glusulase resistance can also be acquired by cells that are proliferating slowly on sub-optimal carbon or nitrogen sources (Elliot and Futcher, 1993; Plesset *et al.*, 1987; Bujega *et al.*, 1982). Thus, the properties of thermotolerance and accumulation of carbohydrates are part of the normal cellullar response to nutrient stress, and do not in themselves define characteristics that are specific only to stationary-phase cells. Thus, stationary-phase properties may be more accurately described as indications of nutrient stress.

9. Interpretation of viability-loss phenotypes

Investigation of fundamental properties of stationary-phase cells can be approached genetically. In this regard, the requirements for maintenance of viability upon starvation are beginning to be defined genetically by various mutations that affect this phenotype (reviewed in Werner-Washbourne *et al.*, 1993). However, mutant cells may lose viability under starvation conditions for very different reasons. Cells may be unable to arrest cell proliferation in G1 upon nutrient depletion; these cells frequently fail to accumulate carbohydrates or acquire any other stationary-phase properties and show a phenotype of rapid viability loss upon starvation. This phenotype was described for *ubi4* mutant cells (Tanaka *et al.*, 1988), and can be interpreted as an inability to respond to decreasing nutrient levels by aquisition of stationary-phase properties. Secondly, cells may undergo a normal arrest of cell proliferation with indications of adaptation to nutrient stress manifested by thermotolerance and carbohydrate accumulation, but still be unable to maintain long-term viability in the absence of nutrients. This phenotype could be due either to a defect in maintenance of viability during stationary phase, or to a defect in resumption of cell proliferation from stationary phase. That is, since viability is usually measured by transfer of stationary-phase cells to fresh medium and determination of the number of cells that can form colonies, the loss of colony-forming ability may reflect a defect in the exit from stationary phase and subsequent resumption of proliferation. Thus, it is important to distinguish between the different mechanisms of viability loss upon nutrient depletion in assessing the role of a given gene product in stationary-phase cells (Werner-Washburne *et al.*, 1993).

10. Conditional mutations that arrest cell proliferation in the G1 phase of the cell cycle

Conditional mutations can arrest cell proliferation in the G1 phase of the cell cycle during incubation at the restrictive temperature. These nonproliferating, unbudded cells

may resemble either mating-pheromone-arrested cells or nutrient-arrested cells. For example, *cdc28* mutant cells incubated at the restrictive temperature arrest cell proliferation in G1, continue to grow (accumulate mass), and undergo morphological changes characteristic of cells arresting by mating pheromones (Johnston et al, 1977a; Hartwell, 1973a). These mutant cells do not acquire characteristics of nutrient-stressed cells. However, other conditional mutations that arrest cell proliferation in the unbudded phase of the cell cycle do confer stationary-phase characteristics on the arrested cells. For example, *cdc25* and *cdc33* mutant cells accumulate storage carbohydrates, become thermotolerant (Iida and Yahara, 1984a, 1984b; Matsumoto *et al.*, 1985), and cease growing (Johnston et al, 1977a; Iida and Yahara, 1984a) when arrested as unbudded cells at a non-permissive temperature. Thus, conditional mutations that arrest cells in G1 at a restrictive temperature can also confer the characteristics of nutrient-stressed cells. Further investigation of the *CDC25* and *CDC33* genes has proved informative with respect to the processes involved in a stationary-phase cessation of cell proliferation.

10 A. The cAMP-dependent protein kinase activation pathway. Conditional mutations in *CDC25* impose a stationary-phase arrest of cell proliferation in G1 during incubation at a restrictive temperature. Cdc25 has been demonstrated to be an important component of a signal-transduction pathway that responds to the presence of nutrients by the production of cAMP (Robinson *et al.*, 1987; Broach and Deschenes, 1990). In the presence of fermentable carbohydrates, the cAMP signaling pathway is activated and adenylate cyclase synthesizes cAMP from ATP. The sole known role for cAMP is the activation of cAMP-dependent protein kinase (A-kinase). Substrates of A-kinase include enzymes responsible for the synthesis and degradation of storage carbohydrates, key enzymes in the glycolytic/gluconeogenic pathway, and ribosomal

proteins (Broach and Deschenes, 1990; Broach, 1991). When intracellular cAMP levels fall as a result of mutations in *CDC25*, cells become unresponsive to the presence of nutrients, and haploid cells acquire stationary-phase characteristics in nutrient-replete medium. Decreasing cAMP levels and decreased A-kinase activity is correlated with glucose depletion in wild-type cells proliferating on glucose-based medium. This decreased A-kinase activity is responsible for the accumulation of reserve carbohydrates in glucose-depleted cells, since the enzymes responsible for the synthesis and degradation of storage carbohydrates are regulated by A-kinase activity (reviewed in Broach and Deschenes, 1990). Thus the maintenance of A-kinase activity is important for continued growth and proliferation, and the absence of this activity permits a regulated arrest of cell proliferation in G1 with the concomitant acquisition of properties of nutrient-stressed cells.

Decreased A-kinase activity is in fact required for the regulated (G1) arrest of proliferation in the absence of nutrients. Mutations in the regulatory subunit of A-kinase (*bcy* mutations) which cause constitutive activation of A-kinase result in an inability to respond normally to starvation conditions (Broek *et al.*, 1985). *bcy* mutant cells fail to accumulate storage carbohydrates, acquire thermotolerance, arrest as unbudded cells, or maintain viability upon nutrient deprivation (Broek *et al.*, 1985). Thus, constitutive activation of A-kinase prevents cells from responding normally to nutrient deprivation. However, mutations in A-kinase which allow a low constitutive level of A-kinase activity restore the ability of cells to respond normally to nutrient deprivation. These mutant cells arrest cell proliferation as unbudded, thermotolerant cells that remain viable in the absence of nutrients (Cameron *et al.*, 1988). Thus, A-kinase activity must be low for acquisition of stationary-phase properties, but not necessarily responsive to decreasing nutrient levels. Apparently, in the absence of A-

kinase activity that is regulated by nutrient levels, other pathways signal the regulated arrest of cell proliferation from G1 in response to nutrient depletion.

The importance of decreased A-kinase activity for acquisition of stationary-phase properties is also seen in the transcriptional regulation of those genes that are highly expressed in stationary-phase cells. Some of these genes show increased expression at the diauxic shift, which coincides with a decrease in intracellular cAMP levels and a corresponding decrease in A-kinase activity. For example, *UBI4* and *SSA3* gene expression increases at the diauxic shift, and the transcription of these genes has been shown to be repressed by A-kinase activity (Tanaka *et al.*, 1988; Boorstein and Craig, 1990a,b). Thus several properties of stationary-phase cells are conferred by the decrease in A-kinase activity that accompanies the depletion of glucose from the growth medium.

As previously described, the depletion of glucose from the growth medium of proliferating cells eventually results in a shift from fermentative to respiratory growth. This metabolic shift is accomplished by the expression of genes required for growth on non-glucose carbon sources and for respiration, which are normally repressed during growth on glucose (reviewed in Johnston and Carlson, 1993). Interestingly, mutations which impair the transition between fermentative and respiratory growth may interfere with the decrease in A-kinase activity that normally occurs at the diauxic shift. The Snf1 protein kinase is required for the transition from fermentative to respiratory growth, and *snf1* mutant cells have a phenotype consistent with an inability to decrease A-kinase activity in response to starvation. *snf1* mutant cells fail to accumulate reserve carbohydrates in response to nutrient deprivation and lose viability in stationary phase (Thompson-Jaeger *et al.*, 1991). These phenotypes of *snf1* mutant cells are alleviated by mutations in the cAMP pathway that reduce levels of intracellular cAMP. Thus, the

pathway that signals the transition between fermentative and respiratory growth may also be required for the decrease in A-kinase activity in response to nutrient limitation.

10B. Defects in protein synthesis impose a stationary-phase-like arrest of proliferation As previously described, decreasing the rate of protein synthesis preferentially lengthens the G1 interval of the cell cycle due to the requirement for the accumulation of labile G1-cyclin proteins during the transition from G1 to S phase. Decreasing the rate of protein synthesis may also play a role in the arrest of cells in stationary phase. Certain conditional mutations that affect the rate of protein synthesis can arrest cell proliferation in the G1 interval of the cell cycle with concomitant indications of nutrient stress. For example, conditional mutations affecting translation initiation, such as *cdc33*, impose arrest of cell proliferation in G1 with acquisition of thermotolerance, cessation of mass accumulation, and accumulation of carbohydrates during incubation at a restrictive temperature (Johnston et al., 1977a; Iida and Yahara, 1984a)). *CDC33* encodes the cap-binding protein (eIF4E) which complexes with the 5' cap of mRNAs in translation initiation (Brenner *et al.*, 1988). Interestingly, although mutations in *CDC33* inhibit translation of most capped mRNAs, the expression of *UBI4 -lacZ* fusion genes, measured as ∫-galactosidase activities, was actually enhanced by mutations in *CDC33* (Brenner *et al.*, 1988). Thus, mutations in *CDC33* may confer a stationary-phase arrest of cell proliferation through differential effects on the production of particular polypeptides. In fact, decreasing the global rate of protein synthesis by treatment with cycloheximide results in a lengthening of the G1 interval but does not result in arrest of cell proliferation in G1 (Unger and Hatwell, 1976; Shilo *et al.*, 1978). Thus, arrest of cell proliferation in G1 by inhibition of translation initiation may involve differential effects on gene expression rather than a global decrease in the rate of protein synthesis.

11. Stationary-phase cells show a lag in resumption of proliferation

For stationary-phase cells, resumption of cell proliferation in the presence of fresh growth medium is characterized by a lag period, consisting of the interval between the transfer of cells to fresh medium and initiation of the cell cycle (as defined by the formation of a bud). The length of this lag period is proportional to the length of time that cells have been in stationary phase (Iida and Yahara, 1984a). Conditional mutations such as *cdc25* and *cdc33*, that arrest cell proliferation from the G1 interval of the cell cycle with indications of nutrient stress, also impose a lag in resumption of proliferation after transfer to the permissive temperature that is proportional to the length of time that cells have been incubated under restrictive conditions (Iida and Yahara, 1984a). This incubation-dependent lag in resumption of proliferation was not observed for cells arrested by mating pheromone or by conditional mutations in *CDC28*, indicating that the lag is specific to G1-arrest conditions that are accompanied by nutrient stress and concomitant cessation of mass accumulation (Iida and Yahara, 1984a).

The lag period preceding resumption of cell proliferation from stationary phase has been suggested to be related to the small size of stationary-phase cells and a requirement for growth (increase in cell size) before resumption of cell proliferation (Johnston *et al.*, 1977a). However, the requirement for small cells to grow to a larger cell size does not explain why cells arrested in stationary-phase for longer times show longer lag periods (Iida and Yahara, 1984a). As previously described, increase in the lag period preceding resumption of proliferation from stationary phase has been observed for *ubc1* mutant cells with defects in ubiquitin-mediated protein turnover (Seufert *et al.*, 1990). Other mutants have been isolated which show increased lag periods for resumption of proliferation from stationary phase, but most of these have

not been well-characterized (Bedard *et al.*, 1982). Further investigation of mutations which increase the lag period preceding resumption of proliferation from stationary phase without affecting the doubling time of actively dividing cells should provide insight into those functions that are specific for the resumption of proliferation from stationary phase.

The lag period preceding resumption of cell proliferation in the presence of fresh medium may involve loss of stationary-phase properties, particularly properties of stationary-phase cells that could be detrimental to resumption of proliferation. This reversal of stationary-phase properties may involve reactivation of processes required for growth, including regeneration of a chromatin structure compatible with increased transcription, and resynthesis of proteins essential for proliferation which may have been degraded during stationary phase. Furthermore, resumption of cell proliferation may require restructuring of the relatively disorganized cytoskeleton of stationary-phase cells (G.C. Johnston, personal communication).

It is also possible that part of the lag in resumption of proliferation is due to events required for resumption of proliferation from stationary phase that are part of a nutrient-signaling pathway that is not well understood. A number of events occur shortly after the addition of glucose to starved cells that may relate to such a nutrient-signaling pathway. The addition of glucose to starved cells results in a sharp peak in cAMP levels and a corresponding increase in A-kinase activity that occurs within seconds after glucose addition (Eraso and Gancedo, 1985). Within minutes of glucose addition to starved cells, the activity of the plasma membrane H^+-ATPase is stimulated, resulting in a transient increase in intracellular pH (Gillies *et al.*, 1981; Serrano *et al.*, 1986). This glucose-induced activation of plasma-membrane ATPase and concomittant proton efflux results in hyperpolarization of the cell membrane and activation of Ca^{2+} channels, resulting in transient Ca^{2+} efflux followed by Ca^{2+} influx in yeast (Eilam

and Otham, 1990; Nakajima-Shimada *et al.*, 1991). The transient rise in intracellular calcium may activate other signaling pathways required for growth (Anraku *et al.*, 1991). Thus, the stimulation of starved cells by nutrients may activate different signaling pathways that function together in the activation of processes (as yet undefined) required for growth and resumption of proliferation from stationary phase.

12. The reentry-mutant criterion for stationary phase

Our understanding of the requirements for resumption of cell proliferation from stationary phase would be greatly aided by a collection of mutants that were conditionally defective for resumption of proliferation from stationary phase but otherwise unimpaired for maintenance of ongoing mitotic cell cycles. In this way, steps that are required for resumption of proliferation might be identified, and dependency relationships among these steps could be established. Several unsuccessful attempts have been made to isolate such mutants (Bedard *et al.*, 1982). Following these unsucessful mutant hunts, one such "reentry" mutant was serendipitously isolated in this laboratory, and has since been shown by several criteria to be conditionally impaired for the resumption of proliferation from stationary phase (Drebot, 1987). As previously desribed, these reentry-mutant cells are cold-sensitive only for the resumption of proliferation from stationary phase. Actively proliferating reentry-mutant cells maintain ongoing proliferation at the temperature that is restrictive for stationary-phase cells. Genetic analysis showed that this phenotype required the interaction of two mutations, *gcs1-1* and *sed1-1* (Drebot et al., 1987). Single-mutant *gcs1-1* cells (growth cold sensitive) were cold-sensitive both for the resumption of proliferation from stationary phase and for maintenance of ongoing proliferation. Single-mutant *sed1-1* cells (suppressor of exponential defect) had no detectable phenotype. However, reentry-

mutant *gcs1-1 sed1-1* cells were cold-sensitive only for the resumption of proliferation from stationary phase.

The defect in resumption of proliferation from stationary phase for *gcs1-1 sed1-1* reentry-mutant cells is independent of the method used to achieve a stationary-phase arrest of cell proliferation. At the permissive temperature, *gcs1-1 sed1-1* mutant cells cease proliferation and arrest in stationary phase after starvation for carbon, nitrogen, or sulphur (Drebot *et al.*, 1990). These stationary-phase *gcs1-1 sed 1-1* mutant cells, produced by starvation for different essential nutrients, are all cold-sensitive for the resumption of cell proliferation in fresh medium. In contrast, arrest of *gcs1-1 sed1-1* mutant cells in the G1 interval of the cell cycle by treatment with mating pheromones, or arrest of *gcs1-1 sed1-1* mutant cells in S phase by treatment with hydroxyurea (Drebot *et al.*, 1990), does not cause cells to become cold-sensitive. Thus the cold-sensitive phenotype of *gcs1-1 sed1-1* mutant cells requires the prior arrest of cell proliferation in stationary phase.

Despite being unable to resume proliferation in fresh medium at the restrictive temperature, stationary-phase *gcs1-1 sed1-1* reentry-mutant cells respond to the presence of fresh growth medium by loss of stationary-phase properties. Mutant cells become thermosensitive, degrade their storage carbohydrates, and increase RNA and protein synthesis (Drebot, 1987; Drebot *et al.*, 1987). Thus *gcs1-1 sed1-1* reentry-mutant cells exit stationary phase in the presence of fresh medium by the criterion of loss of stationary-phase properties, and yet remain unable to resume cell proliferation.

The reentry-mutant phenotype provides a genetic criterion for arrest of cell proliferation in stationary phase that can be used to analyze other mutations that are thought to arrest cells in a stationary-phase state. Two such mutations, *cdc25* and *cdc33*, were analyzed to determine if these mutations arrest cells in stationary phase by the reentry-mutant criterion. The triple-mutant strains *gcs1-1 sed1-1 cdc25* and *gcs1-1*

sed1-1 cdc33 acquired cold sensitivity for resumption of proliferation after prior arrest of cell proliferation at 37ºC, the restrictive temperature for the *cdc25* and *cdc33* mutations (Drebot *et al.*, 1990). Thus, the *cdc25* and *cdc33* mutations do arrest cells in stationary phase by the reentry-mutant criterion, demonstrating that the reentry-mutant criterion is a reliable indicator of stationary-phase status.

Further investigation of the genetic and physiological basis for the reentry-mutant phenotype should explain how stationary-phase cells can be adversely affected for resumption of proliferation without impairing the ability of actively proliferating cells to maintain ongoing cell proliferation. In this thesis, a molecular analysis of the *GCS1* gene has extended our characterization of the reentry-mutant phenotype.

II. Materials and Methods

1. Strains and Plasmids

The genotypes and sources of yeast and bacterial strains used in this work are shown in Table 1. Plasmids used in this work are described in Table 2.

Strains containing disruptions of the *GCS1* gene were constructed during this work, and are listed in Table 1. Strains GWD and GWT, carrying the *gcs1-2* and *gcs1-3* truncation alleles, respectively, were constructed using the disruption plasmids pLI[1-2] and pLI[1-3] (Table 2). These plasmids were constructed as follows: the internal 487-bp *Bgl*II-*Pst*I fragment of the *GCS1* ORF was subcloned into the *URA3* integrating vector YIp352, generating plasmid pLI[1-2]. pLI[1-2] was then digested with *Hin*dIII and religated to make plasmid pLI[1-3]. Recombinogenic *GCS1* DNA ends were created by linearizing pLI[1-2]at a unique *Xba*I site and pLI[1-3] at a unique *Hpa*I site within the *GCS1* insert sequences. These linearized plasmids were used to direct integrative transformation of W303 a/α diploid cells to uracil prototrophy. Sporulation of these transformed diploids yielded asci containing four viable spore clones; all of the Ura$^+$ haploid segregants were cold sensitive when assayed by replica-plating to solid medium and incubation at 15°C. Cold-sensitive Ura$^+$ segregants containing the *gcs1-2* and *gcs1-3* disruption alleles were analyzed by Southern hybridization to confirm disruption of the *GCS1* locus by integrative transformation.

The GWK-8A strain containing the *gcs1-6* null allele was constructed by replacement of the entire ORF of the *GCS1* gene with the *URA3* gene. For this construction, plasmid pBNΔ4 (Table 2), containing a *GCS1* gene-replacement cassete, was constructed in two steps. First, the *Bam*HI-*Nde*I fragment containing the entire *GCS1* gene and flanking sequences was subcloned into the *Sma*I site of pUC19. This

plasmid, pBN19 (Table 2), was then cut with the enzymes *Bsa*AI and *Bst*EII to remove the *GCS1* ORF, and a *Sma*I-*Cla*I fragment containing the *URA3* gene was ligated into the deleted plasmid pBN19 to make plasmid pBNΔ4. Thus the gene-replacement plasmid pBNΔ4 contains a *URA3* gene inserted between flanking *GCS1* DNA sequences. Recombinogenic *GCS1* DNA ends were created by digestion of pBNΔ4 with *Bam*HI and *Eco*RI; and this digested plasmid was used to transform W303 diploid cells to uracil prototrophy. Two independent Ura$^+$ diploids were sporulated; all four segregants from 11 tetrads were viable and all of the Ura$^+$ haploid segregants were cold sensitive when assayed by replica-plating to solid medium and incubation at 15°C. One Ura$^+$ haploid segregant was subjected to Southern analysis to confirm that the *GCS1* sequences were eliminated by gene replacement. Since these experiments showed that the *GCS1* gene was not essential, subsequent strain constructions to put *gcs1* mutant alleles in different strain backgrounds were done with haploid strains. Using the same plasmids and procedures described above, the *gcs1-2*, *gcs1-3* and *gcs1-6* alleles were constructed in the 21R genetic background to make the GRD, GRT and GRK strains respectively (Table 1).

2. Media and Growth conditions

A) Yeast strains

Yeast strains were maintained for long-term storage as frozen stocks in 15% glycerol at -70°C. For routine use, strains were restreaked from frozen stocks monthly and maintained at 23°C on YEPD solid medium containing 2% glucose, 2% bactopeptone, 1% bacto-yeast extract, and 2% agar (Hartwell, 1967). Cells were also grown in YM-1 enriched liquid medium containing 1% succinic acid, 0.6% sodium hydroxide, 1% bactopeptone (Difco Laboratories, Detroit, MI), 0.5% bacto-yeast extract (Difco), and 0.67% bacto-yeast nitrogen base without amino acids (Difco) at pH 5.8 (Hartwell,

Table 1. Yeast strains used in this study

STRAIN	GENOTYPE	SOURCE
W303-1A	*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	J. Friesen
W303-1B	*MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	J. Friesen
MDgcs1-3X	*MATa gcs1-1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	M. Drebot
W303a/α	*MATa/α ade2-1 /ade2-1 his3-11,15 /his3-11,15 leu2-3,112 /leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1*	this work
21R	*MATa ade1 leu2-3,112 ura3-52*	this lab
MD-025-5	*MATα gcs1-1 ade1 his6 leu2-3,112 ura3-52*	M. Drebot
GR2	*MATa his6 ura1*	this lab
MDG02	*MATa gcs1-1 sed1-1 his6 ura1*	M. Drebot
FY56	*MATα his4-9126∂ lys2-12∂ ura3-52*	E. Malone/ F. Winston
111XD	*MATα cdc4-6 his*	this lab
	Strains containing disruptions of the *GCS1* gene in the W303 genetic background	
GWK-8A	*MATa gcs1-6::URA3 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	this work
GWK-2A	*MATα gcs1-6::URA3 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	this work
GWD-12C	*MATa gcs1-2::URA3 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	this work
GWD-12D	*MATα gcs1-2::URA3 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	this work
GWT-3B	*MATa gcs1-3::URA3 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	this work
GWT-3D	*MATα gcs1-3:URA3 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	this work
GWDHIS	*MATa gcs1-2::HIS3 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	X. Wang

Table 1. (cont.)

STRAIN	GENOTYPE	SOURCE
	Strains containing disruptions of the *GCS1* gene in the 21R genetic background	
GRK4-7	*MATa gcs1-6::URA3 ade1 leu2-3,112 ura3-52*	this work
GRD-1	*MATa gcs1-2::URA3 ade1 leu2-3,112 ura3-52*	this work
GRD-2	*MATa gcs1-2::URA3 ade1 leu2-3,112 ura3-52*	this work
GRT-2	*MATa gcs1-3::URA3 ade1 leu2-3,112 ura3-52*	this work
	Yeast strains used for mapping *GCS1*	
LI-8	*MATα gcs1-1 URA3 ::[GCS1] ade1 leu2-3,112 ura3-52*	this work
ARM-4R2	*MATa cdc37 lys4 prt3 trp1 tyr1 ura3-52*	
LARM 4-16B	*MATa cdc65-1 ade1 ade8 arg4 leu2 rna3 trp4 ura3*	
EP4	*MATα cdc36-16 his6 ura1*	
H120-17-4	*MATα cdc13-1 ade1 ade2 leu 2 ura1*	
LIcdc13	*MATα cdc13-1 leu2 ura3-1*	
H89-3-1	*MATα cdc2-2 ade1 ade2 leu ura1*	
EP9	MATα cdc7	
J0217-3a	*MATa HO::LACZ::URA3 ade2 his3 leu2 lys2 trp1 ura3*	
LI-JO217-3a	*MATa HO::LACZ::URA3 ade2 his3 leu2 trp1 ura3*	
	tester strains for complementation of temperature-sensitive mutations	
EP25 (*cdc37* tester)	*MATa cdc37-1 his6*	
SR37 (*cdc37* tester)	*MATα cdc37-1 cyh2 met2? tyr1*	
A298-65C (*rna3* tester)	*MATa rna3 ade8 arg4 leu2-3 met thr trp4*	
X4120-19D (*rna3* tester)	*MATα rna3 ade2 ade5 ade8 arg1 leu1 leu2 lys2 met1 met10 trp3*	
LARM4-9C (*prt3-1* tester)	*MATa prt3-1 lys4 trp1 ura3 his2*	
STX82-3A (*prt3-1* tester)	*MATα prt3 -1 lys4 met2 pha2*	

Table 2. Plasmids used or constructed during this study

PLASMID	DESCRIPTION
p6d-3 (M. Drebot)	6-kbp genomic insert containing the *GCS1* gene flanked by 340 bp of upstream sequence and 4.6 kbp of downstream sequence in a YCp50 vector; from a library containing *Sau*3A genomic fragments inserted into the YCp50 *Bam*HI site.
pE3.3 (L. Veinot)	2.8-kbp genomic insert containing the *GCS1* gene flanked by 340 bp of upstream sequence and 1.4 kbp of downstream sequence in the centromeric vector YCp50. Subcloned from p6d-3 as a *Sau*3A fragment into the *Bam*HI site of YCp50. The *Sau*3A /*Bam*HI junction maintained the YCp50 *Bam*HI site at the 5' end of the *GCS1* gene.
pB23 (L. Veinot)	2.8-kbp genomic insert containing the *GCS1* gene in the high-copy vector YEp24. The insert from pE3.3 was transferred as an *Eco*RI-*Sal*I fragment to YEp24.
YIpL1 (L. Veinot)	6-kbp genomic insert containing the *GCS1* gene in the integrating vector YIp5. The insert from p6d-3 was transferred as an *Eco*RI-*Sal*I fragment to YIp5.
pBN19	The *GCS1* gene with 340 bp of upstream sequence and 450 bp downstream sequence in pUC19. The *Bam*HI-*Nde*I fragment from pE3.3 was blunt-ended by filling the cohesive ends and cloned into the *Sma*I site of pUC19.
pBN316	The *GCS1* gene including 340 bp of upstream sequence and 450 bp of downstream sequence, in the centromeric *URA3* vector pRS316. Constructed by transferring the *Bam*HI-*Kpn*I fragment from pBN19 containing the *GCS1* gene to pRS316.

Table 2. (cont.)

pBNΔ4 and pBNΔ1	Plasmid pBN19 with *URA3* replacing the *GCS1* ORF. The *Bst*EII-*Bsa*AI fragment containing the *GCS1* ORF was deleted from pBN19 and replaced with the *URA3* gene that had been excised as a *Sma*I-*Cla*I fragment from YEp24 and blunt-ended. Plasmid pBNΔ4 contains the *URA3* gene in the same orientation as the *GCS1* gene, and pBNΔ1 contains the *URA3* gene in the opposite orientation to the *GCS1* gene.
pNB314	The *GCS1* gene with 135 bp of upstream sequence and 450 bp of downstream sequence in the centromeric *TRP1* vector pRS314. The *Bst*EII-*Bam*HI fragment from pBN19 containing the *GCS1* gene was blunted-ended and ligated into the *Sma*I site of pRS314. The *Bgl*II and *Hin*dIII sites are unique in this construct.
pL27	N-terminally truncated *GCS1* gene containing an 880 bp deletion from the *Hin*dIII site in the *GCS1* promoter to the *Hin*dIII site in the *GCS1* ORF. Constructed by digestion of pBN316 with *Hind*III and religation of the deleted plasmid.
pRS1-1	The mutant *gcs1-1* gene in the low-copy centromeric *URA3* vector pRS316. Constructed by transformation of cells containing the *gcs1-1* allele with *Hin*dIII-digested pL27 and recovery from transformed yeast cells. pRS1-1 contains genomic *gcs1-1* sequences from the *Hin*dIII site in the *GCS1* promoter to the *Hind*III site in the *GCS1* ORF.
YEp1-1	The mutant *gcs1-1* gene in the high-copy *URA3* vector YEp352. Constructed by transfer of the pRS1-1 insert, as a *Bam*HI-*Kpn*I fragment, to YEp352.
pRS1-1LEU	The mutant *gcs1-1* gene in the low-copy centromeric *LEU2* vector pRS315. Constructed by transfer of the YEp1-1 insert, as a *Sal*I-*Sst*I fragment, to pRS315.

Table 2. (cont.)

pKS1-1	The mutant *gcs1-1* gene in the pBluescript II KS$^+$ vector. Constructed by transfer of the pRS1-1LEU insert, as an *Xho*I-*Sst*I fragment, to KS$^+$.
pRS1-1HIS	The mutant *gcs1-1* gene in the low-copy centromeric *HIS3* vector pRS313. Constructed by transfer of the pRS1-1LEU insert, as a *Xho*I-*Sst*I fragment, to pRS313.
pLI[1-2]	The internal *Bgl*II-*Pst*1 fragment of the *GCS1* ORF cloned into the *URA3* integrating vector YIp352. This plasmid contains a unique *Xba*I site within the *GCS1* sequences, which was used to direct integration and disruption of the *GCS1* gene to construct the *gcs1-2* allele.
pLI[1-3]	The internal *Bgl*II-*Hin*dIII fragment of the *GCS1* ORF cloned into the *URA3* integrating vector YIp352. Constructed by digestion of pLI[1-2] with *Hin*dIII and religation. This plasmid contains a unique *Hpa*I site within the *GCS1* sequences, which was used to direct integration and disruption of the *GCS1* gene to construct the *gcs1-3* allele.
pL23	The N-terminal two thirds of the *GCS1* gene in YIp352 capable of encoding the first 230 amino acids of Gcs1. Constructed by transfer of a *Bam*HI-*Pst*I fragment from pE3.3 toYIp352. This plasmid contains a unique *Xba*I site within the *GCS1* sequences.
CSC2A (M. Parsons)	10-kbp genomic insert containing the *CSS1* gene flanked by 2 kbp of upstream sequence and 6 kbp of downstream sequence in YEp24; from a library containing *Sau*3A genomic fragments inserted into the YEp24 *Bam*HI site.

Table 2. (cont.)

pL45	6-kbp genomic insert containing the *CSS1* gene flanked by 1.7 kbp of upstream sequence and 2.7 kbp of downstream sequence in YIp352. Constructed by transferring a *Hin*dIII-*Sst*I fragment from CSC2A to YIp352.
pL46	6-kbp genomic insert containing the *CSS1* gene flanked by 1.7 kbp of upstream sequence and 2.7 kbp of downstream sequence in YEp352. Constructed by transferring a *Hin*dIII-*Sst*I fragment from pL45 to YEp352.
pL47	3.3-kbp genomic insert containing the *CSS1* gene flanked by 1.65 kbp of upstream sequence containing most of *CDC12* and 0.2 kbp of downstream sequence in YEp352. Constructed by subcloning the 3.3-kbp *Hpa*I fragment from pL45 in the *Sma*I site of YEp352. The *CSS1* gene is cloned in the (-) orientation.
pL48	1.7-kbp genomic insert containing the N-terminally truncated *CSS1* gene lacking the first 48 codons of the *CSS1* ORF. Constructed by digestion of pL47 with *Xba*I and religation of the deleted plasmid.
pL49	2.75-kbp genomic insert containing the C-terminally truncated *CSS1* gene lacking the C-terminal 154 codons of the *CSS1* ORF. Constructed by digestion of pL47 with *Bgl*II and religation of the deleted plasmid.
pSFG2	1.6-kbp genomic insert containing most of *CDC12* . Constructed by transfer of the 1.6-kbp *Bam*HI-*Xba*I fragment from pL47 to YEp352.

1967). YM-1 medium was supplemented with either glucose or galactose to a final concentration of 2%. To maintain selection for nutritional auxotrophies, cells were grown in YNB synthetic defined medium (Johnston *et al.*, 1977a), containing 1% succinic acid, 0.6% sodium hydroxide, 2% glucose, 0.67% bacto-yeast nitrogen base without amino acids or ammonium sulfate, which was supplemented to 40 μg/ml with the appropriate amino acids, 20 μg/ml with the required purines and pyrimidines and 0.1% with ammonium sulfate prior to use. Synthetic complete (SC) medium consisted of YNB solid medium supplemented to 40 μg/ml with each of the following L-amino acids: arginine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, tryptophan, threonine, tyrosine, and valine. The nitrogenous bases adenine and uracil were also added to 20 μg/ml. "Dropout" medium used to follow the segregation of auxotrophic markers consisted of SC medium lacking one of the above amino acids or bases. YEPG solid medium used to identify petite strains were prepared from YM-1 medium containing 2% glycerol and 2% agar. Liquid sporulation medium (SPO) consisted of 1% potassium acetate and 0.1% glucose.

B) *E. coli* strains

E. coli strains were maintained as frozen stocks in 15% glycerol at -70°C. Cells were grown in 2xYT medium (Messing, 1983) containing (per liter) 16 g bactotryptone, 10 g bacto-yeast and 5 g NaCl at 37°C. Solid 2xYT medium was prepared by the addition of 20 g Bacto-agar (Difco) per liter of 2xYT medium. 2xYT medium containing ampicillin (100 μg/ml) was used to select for cells containing plasmids that conferred resistance to ampicillin.

3. Measurement of cell concentration

Cell concentrations were determined as follows (Johnston *et al.*, 1979). 0.5-ml samples of cell culture were fixed by the addition of 4.5 ml of fixing solution (0.15 M NaCl

containing 3.7% formaldehyde); fixed cells were sonicated to disrupt cell clumps for 5 sec at 50% power (Hartwell *et al.*, 1970) using a Model W140 Sonifier Cell Disrupter (Heat Systems Ultrasonics Inc., Long Island, NY). Fixed, sonicated cells were diluted 10 or 100-fold in Isotonic Saline Solution (Fisher-Scientific, Nepean, Ont.) and counted using a Coulter Particle Counter (Model ZM, Coulter Electronics, Hialeah, Fl).

4. Determination of budding index

Fixed and sonicated cells were collected by centrifugation and resuspended in 0.1 ml of fixing solution. Since stationary-phase cells in some strains tended to clump, these cells were then sonicated again for 5 sec at 50% power as before, and proportions of cells without buds were determined visually using a phase-contrast microscope (Carl Zeiss, Oberkochen, West Germany). One hundred to two hundred cells were scored for each determination.

5. Viable cell counts

The number of viable cells in a culture sample was determined by making 10-fold serial dilutions in phosphate buffered saline (PBS) (per liter: 8 g NaCl, 0.2 g KCl, 2.8 g Na_2HPO_4, and 2.8 g KH_2PO_4). YEPD solid medium was then spread with 0.1 ml of diluted cells and incubated for three days at 29°C before colonies were counted. Determination of viable cell number was made from at least two different dilutions of the same culture sample.

6. Measurement of thermotolerance

The number of thermotolerant cells in a population was determined by a modification of the procedure of Walton *et al.* (1979). 0.1-ml samples of cell culture were transferred to 12-mm x 100-mm tubes and incubated in a heat block (Canlab, Mississauga, Ont.) at

55°C for 5 min. Heat-shocked cells were then diluted in PBS at 23°C, and 0.1 ml of diluted samples were spread on YEPD solid medium and incubated for three days at 29°C. The proportion of thermotolerant cells in the population was determined by comparing the number of cells that survived the heat treatment with the total number of viable cells in samples taken from the same culture.

7. Yeast genetic techniques

A) Diploid construction

Haploid strains of opposite mating types were mated by mixing cells on YEPD solid medium and incubating for 5-12 h (Sherman *et al.*, 1979). Diploid cells in the mating mixture were then selected by transferring samples of the mating mixture to media or temperatures in which the haploid cells from the mating mixture could not proliferate (Pomper and Burkholder, 1949). Alternatively, in the absence of selectable markers, cells from the mating mixture were streaked on an agar slab and zygotes, identified by their typical "dumbell" shape (Sherman *et al.*, 1979), were collected by micromanipulation using a Singer MK. III Micromanipulator (Singer Instrument Co., Ltd, Watchet, England) and a phase-contrast microscope (Carl Zeiss). Confirmation of the diploid status of cells derived from the collected zygotes was obtained by the halo assay described by Fink and Styles (1972). Diploid cells were patched onto a lawn of mating-factor super-sensitive *sst2-1* cells of each mating type. For this, a large colony of *sst2-1* tester cells was resuspended in 5 ml of YM-1 medium. Top agar, consisting of YM-1 medium plus 0.7% agar, was melted and cooled to 45°C; 10 ml of melted top agar was inoculated with 125 µl of resuspended *sst2-1* tester cells and overlayed on YEPD solid medium. Colonies to be tested were patched onto the surface of the overlay and incubated for 1-3 days. Haploid cells secrete either a-factor or α-factor, which inhibits the growth of *sst2-1* cells of the opposite mating type. When haploid

cells are patched onto the surface of the overlay of *sst2-1* cells of the opposite mating type, a clear zone of growth inhibition develops around the haploid-cell colony due to the secretion of mating factor by the haploid cells. Since diploid cells do not produce mating factor, a zone of inhibition does not develop around diploid-cell colonies patched onto the surface of the overlay of mating-factor super-sensitive *sst2-1* cells of either mating type.

B) Sporulation of diploid cells and tetrad analysis

Diploid cells were sporulated by washing and resuspending 4 ml of a late-log culture (approx. 2 x 10^7cells/ml) in 3 ml of sporulation medium. These cells were then incubated at 23°C on a roller drum (New Brunswick Scientific Co., New Brunswick, NJ) for 4-6 days, and checked for ascus formation by phase-contrast microscopy. For tetrad dissection, cells from 1 ml of a sporulated culture were washed and resuspended in 1 ml PBS, and ascus walls were digested by addition of 60 µl of β-gluronidase (Sigma Chemical Co., St. Louis, MO) and incubation for 1-2 hrs at 30°C. Digested cultures were then washed and resuspended in PBS, and approximately 10 µl of digested cells were spread on an agar slab. The spores from individual asci were then separated and deposited at 4-mm intervals on the agar surface using a Singer MK. III Micromanipulator. These isolated spores were incubated on the agar slab at 23°C for 2-3 days until the spore clones grew into visible colonies. For convenience of replica-plating, the haploid colonies were patched in an ordered array to YEPD solid medium and grown up a second time. The genotypes of these haploid segregants were determined by replica-plating to the approprate selective conditions. Recombination frequencies between markers were determined by standard tetrad analysis (Mortimer and Hawthorne, 1969) according to the equation of Perkins (1949): distance in cM= [(6NPD+TT)x100]/2(PD + NPD +TT).

C) Complementation analysis.

When two recessive mutant alleles with the same phenotype were present in the same strain, the segregation of each mutant allele could not be scored directly due to the presence of a second mutant allele conferring the same phenotype. In this case, the segregation of mutant alleles in haploid segregants was analyzed by construction of diploids with tester strains carrying previously characterized mutations in those mutant alleles. For this analysis, the segregants were replica-plated to solid medium and each segregant to be tested was mated to the tester strain by "cross-stamping" with the tester strain using wooden tongue depressors. These cells on solid medium were incubated for 24 h under permissive conditions to allow diploid formation. The cross-stamped streaks of cells were then replica-plated and incubated under restrictive conditions for the haploid cells and the growth of the resultant diploids was analyzed. Non-complementation, or lack of growth of the resultant diploid under restrictive conditions, indicated that the haploid segregant and the tester strain contained a mutant allele of the same locus. Complementation, or growth of the resultant diploid, indicated that the haploid segregant and the tester strain contained mutant alleles at different loci.

D) Mapping the *GCS1* gene

The *GCS1* gene was initially localized to chromosome IV by hybridizing radiolabelled DNA from plasmid p6d-3 (Table 2) to a Southern blot of whole yeast chromosomes separated by contour-clamped homogeneous electric field electrophoresis (Chu *et al.*, 1986) as prepared by L. C. Schalkwyk (Department of Biochemistry, Dalhousie University). Further attempts to localize the *GCS1* gene concentrated on determining genetic linkage to other genetic loci on the left arm of chromosome IV. These crosses utilized the integrant strain LI-8 containing at the *gcs1-1* locus an integrated wild-type *GCS1* gene along with associated plasmid sequences, including the vector marker gene

URA3. Linkage to the *gcs1* locus was therefore conveniently scored by analyzing linkage to the *URA3* gene in genetic crosses between LI-8 and strains carrying mutations in genetic loci previously mapped to chromosome IV. No linkage was found between the *URA3* gene at the *gcs1-1* locus and the *trp1*, *lys4*, *cdc37*, *trp4*, and *ade8* loci distributed along the left arm of chromosome IV (see Table 4).

Attempts to localize the *GCS1* gene with respect to genetic loci on the right arm of chromosome IV began with the cold-sensitive *gcs1-1* mutant strain MDG02. However, genetic crosses of MDG02 to strains H120, EP9, H89, and EP4, containing temperature-sensitive alleles of genes previously mapped to the right arm of chromosome IV, indicated that temperature sensitivity and cold sensitivity were not showing the expected 2:2 segregation; in these crosses only half of the of the tetrads analyzed showed 2:2 segregation for both cold sensitivity and temperature sensitivity. Linkage was, however, suggested between *gcs1-1* and *cdc13* , because the five tetrads in the cross of MDG02 to H120 that showed 2:2 segregation for both cold sensitivity and temperature sensitivity were parental ditypes.

Strains were then constructed to quantify the linkage between *cdc13* and *gcs1* and to alleviate genetic background effects. The GWD-12C strain contains the *gcs1-2* truncation allele marked with the *URA3* gene at the *GCS1* locus. A *cdc13 ura3* mutant strain was constructed by crossing cells of strain H120 (*Matα ade1 ade2 ura1 cdc13-1*) to cells of strain GWT-3b (*Mata gcs1-3::URA3 ura3-1 leu2 ade2 his3 trp1-1*) and selecting a resultant tetrad in which cold sensitivity segregated 2:2 and all 4 spores were ura$^-$. The two cold-sensitive ura$^-$ spores were not temperature-sensitive and therefore had the genotype *gcs1-3::URA3 ura1 CDC13*; thus the two temperature-sensitive ura$^-$ spores had the desired *cdc13-1ura3-1* genotype. A cross of the GWD-12C strain, containing the *gcs1-2* truncation allele marked with the *URA3* gene, to the *cdc13-1 ura3-1* strain LI-13 showed tight linkage between the *URA3* gene at the *GCS1*

locus and the *cdc13* locus (Table 4). Since the *cdc13* locus is only 7 cM from the *HO* locus, the linkage between *GCS1* and *HO* was determined. The GWD-12C strain was crossed to an *HO* strain containing a *URA3* gene at the *HO* locus. In 15 tetrads analyzed all 4 spores were Ura^+, indicating no recombination between the *URA3* markers at the *GCS1* and *HO* loci. The cross was repeated, this time with a strain carrying the *gcs1-2* truncation allele marked with *HIS3* . Tight linkage was again found (Table 4).

8. Yeast cell transformations

A) Lithium Acetate: long method

Yeast cells were transformed by the method of Ito *et al.* (1983) using the lithium acetate transformation protocol essentially as described in Current Protocols in Molecular Biology (Ausubel *et al.*, 1989). Yeast cells were grown up in 50 ml of YM-1 + glucose medium to cell densities of 1 - 2 x 10^7 per ml, cells were collected by centrifugation (IEC Universal model UV, International Equipment Co., Needham Hts., Mass) at 2000 rpm in 50-ml sterile Falcon tubes for 3 min, washed first with 50 ml dH_2O and then with 10 ml of lithium acetate solution (0.1 M lithium acetate, 10 mM Tris, pH8.0, 1 mM EDTA), and finally resuspended in 0.5 ml of lithium acetate solution, transferred to 10-ml sterile tubes and incubated on a roller drum at either 23° or 30°C for 30 min. Cells were then distributed, 100 µl per sterile microfuge tube. 4 µl of carrier DNA (10 µg/µl in dH_2O, salmon sperm, sheared and sonicated) and transforming DNA (0.1-10 µg) was added in a total volume of less than 15 µl, and microfuge tubes were incubated for 30 min at 30°C. 0.7 ml of PEG solution (40% PEG 3000, 0.1 M lithium acetate, 10 mM Tris.Cl, pH 8.0, 1 mM EDTA) was then added and cells were incubated for 45 min at 30°C. Finally, cells were heat-shocked by incubation at 42°C for 5-7 min and plated on

appropriate selective media. Transformant colonies were visible after incubation for 2-3 days at 30°C.

B.) Lithium Acetate : short method

For routine introduction of plasmids into yeast cells another transformation protocol which used stationary-phase cells was employed. This procedure is essentially as described in Chen *et al.* (1992) with some modification. One ml of fresh stationary-phase cell culture (per transformation) was transferred to a sterile microfuge tube and cells were pelleted by centrifugation. The cell pellet was washed in 1.5 ml of lithium acetate solution and resuspended in 5 µl of the transforming DNA plus 5 µl of carrier DNA (salmon sperm, 10 mg/ml). 100 µl of PEG solution (36% PEG 3500, 0.2 M lithium acetate in TE, 0.1 M DTT) was added to the cells-plus-DNA mixture and the mixture was incubated at 45°C for 30 min in a heat block. To select for transformants, the cells were plated on selective medium and incubated at 30°C.

9. Isolation of plasmid DNAs from yeast

Plasmid DNA was isolated from yeast essentially as described in Current Protocols in Molecular Biology (Ausubel *et al.*, 1989). Briefly, 2 ml of selective medium was innoculated with a single fresh yeast colony from selective medium and grown to stationary phase at 23°C on a roller drum. Cells were transferred to microfuge tubes, pelleted in a microcentrifuge, and resuspended in 200 µl of breakage buffer (2% Triton-X-100, 1% SDS). Sterile acid-washed glass beads were added to just below the meniscus, 200 µl of TE-buffered (pH 7.5) phenol:chloroform (1:1), was added, and the mixture was vigorously mixed at highest speed on a multi-vortex mixer (Vortex Genie 2, Fisher-Scientific) for 2 min. Organic and aqueous phases were separated by centrifugation, and the aqueous phase was transferred to a fresh microfuge tube, diluted

3-fold with dH_2O, and used directly for transformation of *E. coli* cells by electroporation.

10. Isolation of genomic DNA from yeast

Genomic DNA was isolated from yeast as described in Current Protocols in Molecular Biology (Ausubel *et al.*, 1989), with the following modifications. Cells from 5 ml of fresh stationary-phase cultures were collected by centrifugation (IEC Universal) at 2500 rpm for 3 min, resuspended in 0.5 ml of sorbitol solution (0.9 M Sorbitol, 0.1 M Tris, pH 8, 0.1 M EDTA) and transferred to microfuge tubes. 10 mg (the amount remaining on the end of a flat toothpick) of lyticase (Sigma) was dissolved in 0.5 ml of sorbitol solution in a separate tube, and 50 µl of this lyticase solution was added to the cell suspension, which was then incubated at 37°C for periods from 30 min to 2 h until the cell walls were digested and the cells had formed spheroplasts. Spheroplasting was verified by mixing 2 µl of treated cells with 20 µl of dH_2O and 2 µl of 10% SDS on a microscope slide, and looking for spheroplast lysis under a phase-contrast microscope. Spheroplasted cells were pelleted at 6,000 rpm in a Picofuge (Stratagene, La Jolla, CA), resuspended in 50 mM Tris, pH 8.0, 20 mM EDTA, and lysed by the addition of 50 µl of 10% SDS followed by incubation for 20 min at 65°C. To the lysed spheroplasts, 200 µl of 5 M potassium acetate (pH 6.0) was added and the mixture was incubated on ice for 30 min. Precipitated cellular debris was removed by centrifugation at 12,000 rpm in a microcentrifuge (Micro Centaur, Johns Scientific Inc, Canada) for 3 min. Supernatants were transferred to fresh microfuge tubes; the tubes were filled with 95% ethanol and left on ice for 10 min. The precipitated nucleic acid was collected by centrifugation at 12,000 rpm in a microcentrifuge for 10 min at 4°C, resuspended in 100 µl of dH_2O and redissolved by incubation at 37°C with 4 µl of a 10-mg/ml boiled solution of RNase A (Worthington) for 30 min. Genomic DNA was then precipitated

by the addition of 150 µl of dH_20 and 500 µl of isopropanol. The microfuge tubes were mixed individually by slow inversion (not vortexing) for 60 sec, during which the DNA fibers aggregated into one large clump. This clump of DNA was transferred on the end of a pipette tip to a fresh microfuge tube and resuspended in 100 µl dH_20. Approximately 15 µl was then used for restriction analysis and preparation of Southern blots.

11. Isolation of plasmid DNA from *E.coli.*

Plasmid DNA was routinely extracted and purified from E. coli by the method of Birnboim and Doly (1979) as described in the alkali lysis protocol in Maniatis *et al.* (1982). This protocol was adapted for 1-ml overnight cultures of *E. coli* DH5αF' cells using 1.5-ml microfuge tubes and centrifugation in a microcentrifuge at 12,000 rpm for all centrifugation steps. Cells were transferred to microfuge tubes and pelleted by centrifugation for 2 min. Cell pellets were either processed immediately or stored at -20°C. For processing, cell pellets were resuspended in 100 µl of solution 1 (50 mM glucose, 10 mM EDTA, 25mM Tris.Cl, pH 8) and lysed by the addition of 2 vol of freshly prepared 0.2 M NaOH, 1% SDS. Chromosomal DNA and associated proteins were precipitated by the addition of 150 µl of ice-cold 3 M potassium acetate, pH 4.8, followed by mixing and incubation on ice for 5 min. The precipitate was removed by centrifugation at 4°C for 5 min, and the supernatant was transferred to fresh microfuge tubes. Remaining nucleic acids (plasmid DNA and RNA) were precipitated by the addition of 2 vol of 95% ethanol. Precipitated nucleic acids were collected by centrifugation for 5 min at room temperature and redissolved in 100 µl dH_2O containing 4 µl of a 10 -mg/ml boiled solution of RNase A (Worthington). Mixtures were incubated for 30 min at 37°C, followed by precipitation of the DNA with 1/2 vol of 7.5 M ammonium acetate and 2 vol of 95% ethanol. After centrifugation to collect

the precipitated plasmid DNA, the plasmid DNA was resuspended in 50 µl dH_2O, and 5 µl was used routinely for restriction digests.

Large-scale isolation of plasmid DNA was performed essentially as above. For 50-ml overnight cultures of *E. coli* DH5αF' cells, cells were transferred to 50-ml sterile centrifuge tubes (Falcon 2070) and all centrifugation was done in a refrigerated IEC (Clinicool, Damon/IEC refrigerated centrifuge) at maximum speed (7,000 rpm). Additional purification steps included two TE-buffered phenol:chloroform (1:1) extractions after the incubation with RNase, and two ethanol precipitations. The yield from a large-scale isolation was typically 200-300 µg of plasmid DNA, and the yield from a small-scale isolation was 5-10 µg of plasmid DNA.

12. Restriction analysis and purification of DNA fragments

Restriction endonucleases were purchased from Bethesda Research Laboratories (Gaithersburg, Md; BRL) and New England Biolabs (Beverly, MA; NEB), and used with the buffers supplied according to the supplier's recommendations. Typically 1 µg of plasmid DNA was digested with 10 u of enzyme in a volume of 20 µl for 1 h at 37°C. Approximately 0.1 µg of digested plasmid DNA was analyzed by aga. :e gel electrophoresis using either TAE (40 mM Tris-acetate, 1 mM EDTA) or TBE (.089 M Tris-borate, .089 M boric acid, 2 mM EDTA) buffers and molecular biology grade agarose (BRL) or SeaPlaque (FMC BioProducts, Rockland, ME) as described by Maniatis *et al.* (1982). Ethidium bromide (0.5 µg/ml) was added to the molten gel before casting. Concentrated loading buffers (6x) were prepared essentially as described by Maniatis *et al.* (1982) and contained 25% Ficoll, 0.01% bromophenol blue and 0.01% xylene cyanol. Lambda *Hin*dIII digests (BRL) were resolved for use as size markers and for approximate quantitation of samples. Size-fractionated DNAs were

purified from agarose gel slices using a Geneclean kit (BIO 101, La Jolla, CA) according to the manufacturer's instructions.

13. DNA ligations for plasmid contructions

Ligations were performed using T4 DNA ligase (BRL) in the ligation buffer supplied by the manufacturer as described by King and Blakesley (1986). DNA ligase was inactivated by heating at 65°C for 10 min prior to transformation of *E. coli* with the ligated mixture. Ligations in low-melt agarose (SeaPlaque) were performed as described by Struhl (1985).

14. Transformation of *E. coli* by $CaCl_2$ treatment.

E. coli cells competent for transformation were prepared as described in Maniatis *et al.* (1982), and were stored at -70°C. 400 ml of 2xYT were innoculated with 200 µl of DH5αF' cells from a fresh overnight culture. *E. coli* cells were grown for 2-6 h in a 37°C shaking water bath to an A_{650} of 0.5-1.0, then transferred to 50-ml centrifuge tubes and chilled for 15-20 min on ice. All subsequent steps were on ice, and centrifugations were at 2500 rpm in a refrigerated IEC centrifuge at 4°C. Chilled cells were pelleted by centrifugation for 10 min, resuspended in 20 ml of ice-cold 0.1 M $CaCl_2$, and pelleted again. Cell pellets in each 50-ml centrifuge tube were resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$; resuspended cells were combined and incubated on ice at 4°C for 24 h (Dagert and Ehrlich, 1979). Finally, cells were mixed with 1/2 volume of ice-cold 50% glycerol, and 300 µl was aliquoted per microfuge tube and stored at -70°C.

For use in transformations with M13 DNA, one microfuge tube of frozen competent cells was thawed on ice and divided into three 100-µl aliquots. 0.1 ng of M13 RF DNA was added to each tube and the cell mixtures were incubated for 40 min

on ice. Cells were heat-shocked for 2.5-3 min in a 42°C water bath and allowed to stand at room temperature for 5 min. 3 ml of top agar (YT + 0.7% agar) at 52°C was added to the cells; the mixture was mixed by vortexing and poured onto YT solid medium and incubated at 37°C until plaques were visible. Smaller amounts (50 µl) of these frozen competent *E. coli* cells were used for plasmid transformations; 1 ml of 2xYT was added to heat-shocked cells, followed by incubation at 37°C for 1 hr. 100 µl of the cell mixture was spread directly on YT+ Amp solid medium and incubated at 37°C until transformed colonies were visible.

15. Transformation of *E. coli* by electroporation

Electroporation-competent *E. coli* cells were prepared essentially as described in Current Protocols in Molecular Biology (Ausubel *et al.*, 1989) and stored at -70°C. Briefly, *E. coli* DH5αF' cells were grown up to an A_{650} of 0.5-0.7 in 400 ml of 2xYT medium. Cells were transferred to 50-ml sterile centrifuge tubes and chilled in ice-water for 10 -15 min. All subsequent steps were on ice, with centrifugation at maximum speed (7000 rpm) at 4°C in a refrigerated centrifuge (IEC Clinicool). Cells were pelleted by centrifugation for 10 min, resuspended and washed 3x with 50 ml dH_2O. Cell pellets were suspended in ice-cold 10% glycerol, combined in one 50-ml tube and mixed well. Cells were pelleted again and resuspended in 1 ml of 10% glycerol. Cells were distributed (80 µl) to prechilled microfuge tubes and stored at -70°C. For use, frozen cells were thawed and 40 µl of cells was used per transformation. Usually 0.5-5 pg of DNA, in 1 µl, was added to the cells, and cells plus DNA were transferred to electroporation cuvettes (Bio-Rad Laboratories, Mississauga, Ont.). The cells were then electroporated using a BioRad Gene Pulser (1.5 V at 200 ohms) with a time constant of between 4.5-4.8 msec. One ml of 2xYT was added to

cells immediately after electroporation. To select for transformants, 100 µl of cells were spread on YT+Amp solid medium and incubated overnight at 37°C.

16. DNA sequence analysis

DNA sequence was obtained by Sanger dideoxy chain-termination reactions, using modified T7 polymerase as provided in the Sequenase Version 2.0 kit (United States Biochemical Company, Cleveland, Ohio; USB) or Taq DNA polymerase as provided in the Taquence kit (USB). Sequencing artifacts due to regions of strong secondary structure in the DNA template were resolved by using combinations of Sequenase or Taq DNA polymerase and dGTP or 7-deaza-dGTP (USB) in four parallel sequencing reactions. Sequencing reactions were resolved on 6% polyacrylamide denaturing buffer-gradient gels (Biggin *et al.*, 1983).

Both strands of the *GCS1* gene were sequenced from the *Bgl*II site, 150 bp upstream of the presumed translation start site, to the *Bsa*AI site 100 bp downstream of the stop codon. A combination of directed subcloning, and ordered M13 deletions using the rapid deletion subcloning method of Dale and Arrow (1987), as described in the IBI Cyclone system (IBI cat. #77200), was used to generate 95% of the sequence on both strands. M13 subclones used in sequencing are described in Table 3. The remaining gaps in the double-strand sequence were filled using specifically designed oligonucleotides as primers in sequencing reactions on single-stranded or double-stranded templates.

Sequencing from double-stranded templates was performed on 2-3 µg of plasmid DNA using the Sequenase Version 2.0 kit with the following modifications. The plasmid DNA in 5 µl was denatured by incubation with 1 µl primer (20 ng) and 1 µl 1 M NaOH at 85°C in a heat block for 5 min. The denatured DNA was neutralized by the addition of 1 µl 1 M HCl and 2 µl of sequencing buffer (5x). After incubation of this DNA

Table 3. M13 subclones of *GCS1* and *CSS1* used in sequencing and probe construction

SUBCLONE	DESCRIPTION
BaP21	The N-terminal two thirds of the *GCS1* gene in the M13um21 vector, encompassing the first 230 codons of *GCS1*. Constructed by transfer of a *Bam*HI-*Pst*I fragment from pE3.3 to M13um21. The *GCS1* gene is in the (-) orientation
BaP20	The N-terminal two thirds of the *GCS1* gene in the M13um20 vector encompassing the first 230 codons of *GCS1*. Constructed by transfer of a *Bam*H1-*Pst*1 fragment from pE3.3 to M13um20. The *GCS1* gene is in the (+) orientation
HH21[3-6]	The N-terminal 880-bp *Hin*dIII fragment of *GCS1* encompassing the first 190 codons of *GCS1*, in M13um21. The *GCS1* gene is in the (-) orientation.
Bg21[4-1]	The N-terminal 427-bp *Bgl*II fragment of *GCS1*, from pE3.3, encompassing the first 63 codons of *GCS1*, in M13um21. Tl. *GCS1* gene fragment is in the (+) orientation.
BgS21[B5]	The C-terminal 2.5-kbp *Bgl*II-*Sph*I fragment from of *GCS1* from pE3.3, encompassing the C-terminal 289 codons of the *GCS1* gene, in M13um21. The *GCS1* gene fragment is in the (-) orientation.
PsS21[P6]	The C-terminal 2-kbp *Pst*I-*SphI* fragment from pE3.3, encompassing the C-terminal 122 codons of *GCS1*, in M13um21. The *GCS1* gene fragment is in the (-) orientation.
XbS21[C1]	The C-terminal 2.1-kbp *Xba*I-*Sph*I fragment from pE3.3, encompassing the C-terminal 151 codons of *GCS1*, in M13um21. The *GCS1* gene fragment is in the (-) orientation.

Table 3 (cont.)

SX20	The C-terminal 2.1-kbp *Xba*I-*Sph*I fragment from pE3.3, encompassing the C-terminal 151 codons of *GCS1*, in M13um20 . The *GCS1* gene fragment is in the (+) orientation.
XbΔS	A collection of deleted subclones of mL16. mL16 was subjected to successive deletions from the *Xba*I end by the Cyclone method (IBI).
	M13 subclones of *CSS1*
HX20	1.8-kbp *Hin*dIII-*Xba*I fragment of pL45, encompassing most of *CDC12* and the first 48 codons of *CSS1*, cloned in the (+) orientation in M13um20.
XH21	1.8-kbp *Hin*dIII-*Xba*I fragment of pL45 encompassing most of *CDC12* and the first 48 codons of *CSS1* cloned in the (-) orientation in M13um21.
EH21	4.2-kbp *Hin*dIII-*Eco*RI fragment of pL45, encompassing most of *CDC12*, all of *CSS1*, and 214 codons of ORF2 cloned in the (-) orientation in M13um21.
XS20	2.6-kbp XbaI-SstI fragment of pL45, encompassing 98% of ORF2 and all of ORF3, cloned in the (+) orientation in M13um20.
SX21	2.6-kbp *Xba*I-*Sst*I fragment of pL45, encompassing 98% of ORF2 and all of ORF3, cloned in the (-) orientation in M13um21.
XX20(+)	1.7-kb *Xba*I fragment of pL45, encompassing 90% of *CSS1* and the N-terminal 10 codons of ORF2, cloned in the (+) orientation in M13um20
EE21(-)	1.5-kb *Eco*RI fragment of pL45, encompassing the C-terminal 294 codons of ORF2 and the N-terminal 138 codons of ORF3, cloned in the (-) orientation in M13um21.

mixture at 37°C for 10 min, the annealed DNA was transferred to 23°C and sequenced according to the standard Sequenase Version 2.0 protocol.

For analysis of the sequencing autoradiograms, sequence data were typed directly into the SeqSpeak program for the Apple Macintosh computer (developed by Keith Conover, described in Ausubel *et al.*, 1989). The DNA sequence data, read from the sequencing gels and entered into the SeqSpeak program was verified using the program's audio-feedback. Assembling sequence data from multiple subclones into one large overlapped sequence was done on mainframe computers using the GelAssemble program from the University of Wisconsin Genetics Computer Group (GCG) Sequence Analysis Software for Vax/VMS computers (Devereux *et al.*, 1984). Analysis of ORFs and restriction enzyme sites was performed with DNA Strider software for Macintosh computers (Marck, 1988).

17. Southern Analysis

Southern transfer was performed by the method of Southern (1975) with the following modifications (Wan Lam,personal communciation). Genomic DNA that had been digested with restriction enzymes was resolved by agarose-gel (0.7-1%) electrophoresis in buffer containing ethidium bromide (0.5µg/ml). After photographing the stained gel, the electrophoretically separated DNA fragments were depurinated by immersion of the gel in 0.25 M HCl for 20 min on a platform shaker (New Brunswick Scientific). The gel was then washed in dH_2O, and the depurinated DNA was transferred in 0.4 M NaOH to GeneScreen nylon membranes (NEN Research Products, Boston, MA) or Amersham nylon membranes (Amersham Canada Ltd, Oakville, Ont.). After transfer the membrane was washed in 2 x SSC (1 x SSC=0.15 M NaCl, 0.015 M sodium citrate) and the DNA was cross-linked to the membrane by UV irradiation using a model 2400 UV crosslinker (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

18. RNA isolation and Northern blots

Cells from 50-ml culture samples were pelleted, resuspended in 300 µl of LETS buffer without SDS (0.1 M $LiCl_2$, 10 mM EDTA, 10 mM Tris.Cl, pH 7.0), transferred to microfuge tubes and rapidly frozen in liquid nitrogen. RNA was isolated from thawed cell pellets as described by Penn *et al.* (1984). Separation of RNAs on formaldehyde agarose gels and transfer to nylon membranes (Amersham) was performed as described by Maniatis *et al.* (1982).

19. [α^{32}P]dATP labelled probes for Southern and Northern Hybrizations

Probe preparation and hybridization conditions were identical for Southern and Northern hybridizations. Restriction fragments of DNA to be labelled were resolved by agarose gel electrophoresis, purified from gel slices with the GeneClean kit (Bio101), and labelled to high specific activity by the random-primer method of Feinberg and Vogelstein (1983) with a random-primed DNA-labelling kit (Boehringer Mannheim, GmbH, Mannheim, West Germany) according to the supplier's instructions. Unincorporated [α^{32}P]dATP (ICN) was separated from the DNA using NICK columns supplied by Pharmacia (Baie d'Urfe, Quebec).

Strand-specific single-stranded probes were prepared from M13 templates using the M13 "-40" primer. Labelling conditions were the same as for the random- primer method except for the substitution of a single-stranded template instead of double-stranded template and a single primer instead of random hexamers. Since the random-primer kit contains hexamers in the primer-extension buffer, a separate 10x primer-extension buffer (0.5 M Tris.Cl, pH 7.6, 0.1 M $MgCl_2$, 10 mM DTT, 2 mg BSA/ml) was used for labelling single-stranded templates. The use of the M13 "-40" primer and large amounts of M13 template (100 ng) ensured that the amount of labelled dATP in

the reaction was only sufficient to extend the primer a short distance such that most of the labelled dATP was concentrated near the primer, and not distributed over the entire M13 template thus resulting in strand-specific high-specific-activity probes.

Hybridization conditions were as recommended by the manufacturers of GeneScreen. DNA or RNA blots were incubated in hybridization buffer (5 x SSC, 0.05 M $Na_2H_2PO_4$, pH 6.5, 1 x Denhardt's solution [50 x Denhardt's consists of 5 g Ficoll, 5 g polyvinylpyrrolidone, and 5 g bovine serum albumin per liter], 50% formamide, 0.2 µg salmon sperm DNA/ml, 1% SDS) for 3-6 hrs before adding 1-2 x 10^6 cpm of labelled probe DNA per ml of hybridization buffer and incubation overnight at 42°C. Blots were washed twice in 0.1 x SSC, 0.5% SDS at 50-55°C for 20-min periods on a platform shaker. Autoradiograms were prepared from hybridized blots by exposure at -70°C to Kodak X-OMAT AR or Amersham film using Dupont Cronex Lightning-Plus intensifying screens (Dupont Co., Wilmington, DE).

20. Primer-extension reactions

Primer-extension reactions were performed on 40 µg of total RNA by modification of the method described by Fouser and Friesen (1986). The primer-extension oligonuceotide was labelled with [γ^{32}P]ATP (ICN) to high specific activity with T4 polynucleotide kinase. The kinase reaction contained in 20 µl: 10 pmol of oligonucleotide, 26 pmol of [γ^{32}P]ATP (4500 Ci/mmol) and 10 u of T4 kinase (BRL) in BRL reaction buffer. The labelled oligonucleotide was purified from unincorporated ATP using the Mermaid kit (Bio 101) according to the manufacturer's instructions. Briefly, to the kinase reaction in 20 µl, 60 µl of high-salt solution and 2 µl of "glass fog" were added and the mixture was mixed continuously on the vortexer (Vortex Genie 2) for 15 min. The glass-fog was pelleted by centrifugation in a picofuge (20 sec) and washed with 300 µl of 95% ethanol. Most of the ethanol was removed with a

micropipette, the last drops of ethanol were removed by a second spin in the picofuge and the ethanol wash was repeated. To elute the oligonucleotide from the glass fog, 20 µl of dH_2O was added and the mixture was incubated at 53°C for 5 min. The glass fog was pelleted by centrifugation in the picofuge and the supernatant containing the labelled oligonucleotide was transferred to a fresh microfuge tube. The elution was repeated with a second 20-µl volume of dH_2O and the two supernatants were combined. 6 µl of this purified labelled oligonucleotide primer was subsequently used in each extension reaction.

The primer-extension reaction was performed using 40 µg of total RNA and 200 u of M-MLV reverse transcriptase (BRL) in a volume of 30 µl with the buffer provided by the supplier. The extension reaction was incubated at 42°C in 10 mM DTT, 1 mM dNTPs, 50 ng actinomycin D/ml, with 1 pmol of [γ^{32}P]ATP labelled oligonucleotide (6µl) for 2 h. Primer-extended products and RNA were precipitated by the addition of 5.5 µl of 2.1 M sodium acetate, pH 7.0, and 100 µl of cold 95% ethanol. The precipitate was pelleted by centrifugation in a microfuge at 12,000 rpm for 10 min at room temperature and washed with 300 µl of 70% ethanol. The last drops of ethanol were pelleted again and removed. The pellet was resuspended in 5 µl TE buffer containing 200 ng of RNase A/ml and incubated at 37°C for 30 min. After the addition of 5 µl of gel-loading buffer (Sequenase kit version 2.0, USB), the primer-extended products were resolved by electrophoresis through an 8% polyacrylamide, 6 M urea sequencing gel.

III. RESULTS

1. Identification of the wild-type *GCS1* gene

Prior to the initiation of the present study, two plasmids, each from a different yeast genomic library, had been shown to restore a cold-resistance phenotype to cells carrying the *gcs1-1* mutation. One of the plasmids, p6d-3 (Table 2), was isolated from a low-copy YCp50-based yeast genomic library while the other plasmid, CSC-2A (Table 2), was isolated from a high-copy YEp24-based yeast genomic library.

Initially I set out to determine if p6d-3 and CSC-2A contained the same genomic insert sequences. For this analysis, restriction maps were constructed of the p6d-3 and CSC-2A insert sequences (Fig. 1). Comparison of the restriction maps from the two plasmids revealed that p6d-3 contains a 5-kbp insert and CSC-2A contains a 9-kbp insert, and that the two insert sequences appear to be unrelated. Since two different plasmids had been isolated from wild-type genomic libraries by reversal of the cold sensitivity of *gcs1* mutant cells, at least one of these two plasmids had to contain a suppressor.

To compare the relative abilities of the inserts from plasmids p6d-3 and CSC-2A to reverse the cold sensitivity of cells carrying the *gcs1-1* mutation, *gcs1-1* mutant cells were transformed with the original high-copy plasmid CSC-2A and with a derivative of p6d-3, plasmid pE3.3 (Fig. 1; Table 2). pE3.3 contained a 2.8-kbp insert that could still reverse the cold sensitivity of cells carrying the *gcs1-1* mutation. The relative abilities of the plasmid insert sequences from pE3.3 and CSC-2A to reverse the cold sensitivity of *gcs1-1* mutant cells were analyzed for cells growing in liquid medium. Transformants were allowed to enter stationary phase at 29°C and then were tested for cell proliferation in fresh medium at the restrictive temperature of 14°C (Fig. 2A).

Figure 1. Restriction maps of plasmids CSC-2A, p6d-3, pE3.3 and pd2.4.

The restriction maps of the 9-kbp insert from plasmid CSC-2A and the 5-kbp insert from plasmid p6d-3 are shown with the following restriction sites: *Bam*HI (B), *Bgl*II (G), *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), *Hpa*I (A), *Kpn*I (K), *Nde*I (N), *Pst*I (P), *Pvu*II (V), *Sst*I (S), *Xba*I (X). Derivatives of p6d-3, plasmids pE3.3 and pd2.4, were also restriction mapped and tested for reversal of the cold-sensitive phenotype of *gcs1-1* mutant cells. Plasmids pE3.3 and pd2.4, containing the *URA3* gene as a selectable marker, were used to transform cells carrying the *gcs1-1* and *ura3-52* mutations (strain MD-025-5) to uracil prototropy. Ura^+ transformants were replica-plated to solid medium and incubated at 14°C. + = complementation (colony formation at 14°C), − = no complementation (failure to form colonies at 14°C).

Figure 1

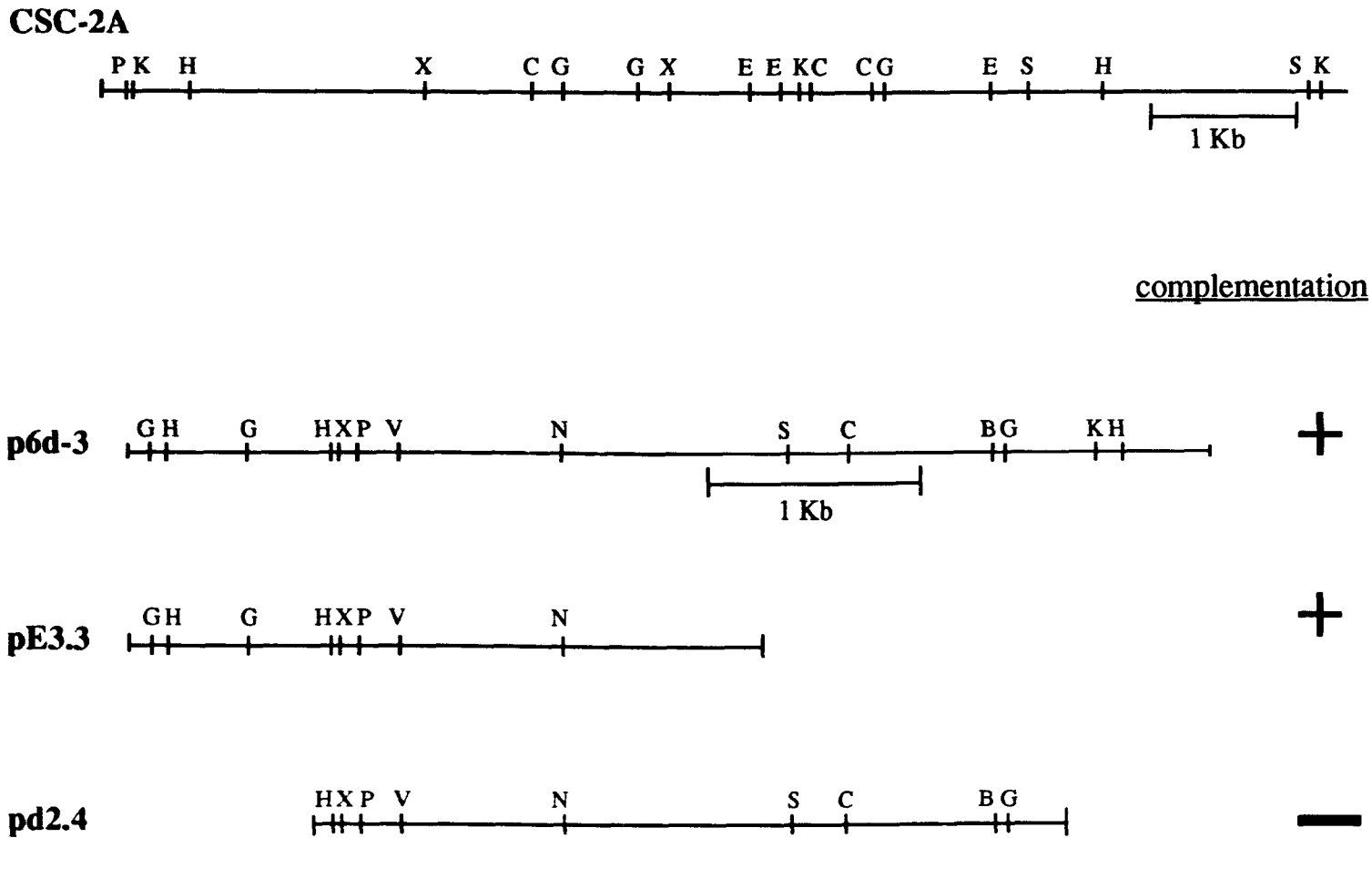

CSC-2A
P K H X C G G X E E K C C G E S H S K
1 Kb
complementation
p6d-3
G H G H X P V N S C B G K H
1 Kb
+
pE3.3
G H G H X P V N
+
pd2.4
H X P V N S C B G
−

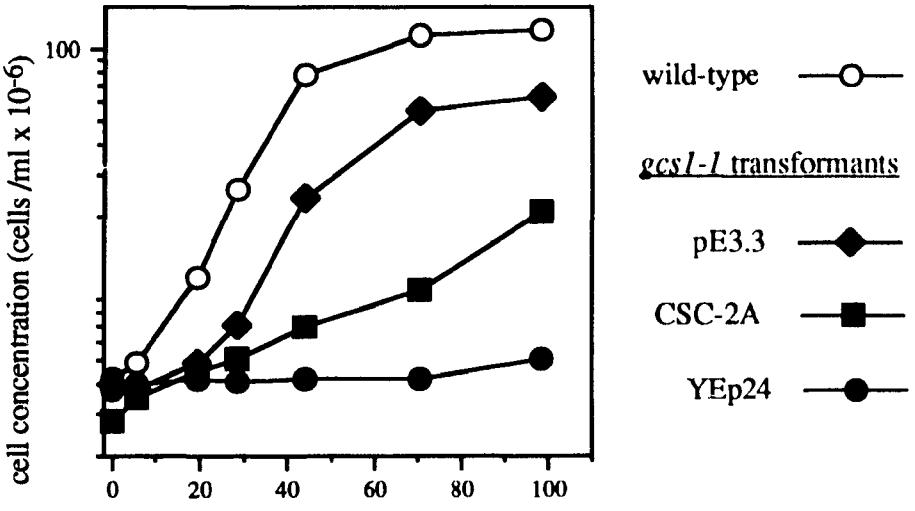
Figure 2. Reversal of the cold sensitivity of *gcs1-1* mutant cells by transformation with plasmids CSC-2A or pE3.3.

(A) Stationary-phase wild-type cells of strain 21R (open circles), or stationary-phase *gcs1-1* mutant cells (filled symbols) were diluted into fresh YEPD medium and incubated at 14°C. At intervals, samples were removed for determination of cell concentration. *gcs1-1* mutant cells (strain MD-025-5) were transformed with YEp24 vector (filled circles), with plasmid pE3.3 (filled diamonds), or with plasmid CSC-2A (filled squares).

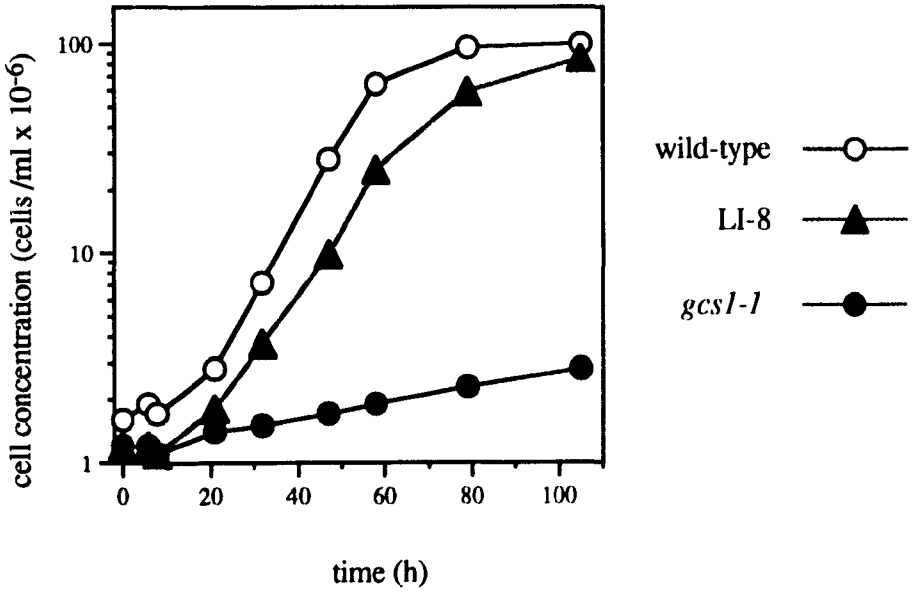
(B) Stationary-phase wild-type cells of strain 21R (open circles), stationary-phase *gcs1-1* mutant cells (filled circles), or stationary-phase cells of strain LI-8 (filled triangles) were diluted into fresh YEPD medium and incubated at 14°C. At intervals samples were removed for determination of cell concentration. The integrant strain LI-8 was produced by directing integration of p6d-3 insert sequences to their homologous chromosomal locus in *gcs1-1* mutant cells (as described in text).

Figure 2

A


cell concentration (cells /ml x 10-6)
100
0
20
40
60
80
100
wild-type
gcs1-1 transformants
pE3.3
CSC-2A
YEp24

B


cell concentration (cells /ml x 10-6)
100
10
1
0
20
40
60
80
100
time (h)
wild-type
LI-8
gcs1-1

gcs1-1 mutant cells separately transformed with each of these plasmids grew with different rates of cell proliferation. The cells transformed with the p6d-3 subclone proliferated more rapidly than the cells transformed with the high-copy CSC-2A plasmid. In particular, the low-copy p6d-3 derivative pE3.3 restored wild-type rates of proliferation at the restrictive temperature to cells carrying the *gcs1-1* mutation. Since the p6d-3 subclone reversed the cold sensitivity of *gcs1-1* mutant cells when present in only one to two copies per cell, it was likely that this p6d-3 subclone contained the *GCS1* gene (rather than an unlinked suppressor gene which suppressed the cold-sensitivity phenotype indirectly when present in multiple copies). Therefore the p6d-3 insert was subjected to a more complete genetic analysis to verify that this insert contained the wild-type *GCS1* gene.

Plasmid YIp5L1 (Table 2), containing the genomic insert from p6d-3 in the integrating vector YIp5, was used to direct integration of p6d-3 sequences to their homologous chromosomal locus in cells carrying the *gcs1-1* mutation. For directing integration, recombinogenic DNA ends (Orr-Weaver *et al.*, 1981; 1983) were created by linearizing the plasmid at a restriction site within the insert sequences, in this case at a unique *Kpn*I site within the genomic insert. This linearized DNA of plasmid YIp5L1, which also contained the *URA3* gene as a selectable marker, was used to transform cells carrying the *gcs1-1* and *ura3-52* mutations to uracil prototrophy. Stationary-phase cells of a resultant integrant strain, LI-8, were tested for resumption of cell proliferation at the restrictive temperature of 14°C as described above. The integrant strain LI-8 showed wild-type rates of proliferation at the restrictive temperature (Fig. 2B). Therefore the integrated p6d-3 insert sequences suppressed the cold-sensitive phenotype of *gcs1-1* mutant cells.

The Ura$^+$ integrant strain LI-8, containing integrated p6d-3 sequences, was then subjected to genetic analysis to determine if the plasmid had integrated at the

homologous *gcs1-1* locus, and therefore contained the wild-type *GCS1* gene. Plasmid integration at the *gcs1-1* locus would position both the plasmid sequences conferring cold resistance and the *gcs1-1* allele at the same locus (Fig. 3). All the haploid segregants of a cross of this integrant strain to a wild-type strain would then be cold-resistant, since the *gcs1-1* mutant allele would not readily segregate away from the tightly linked sequences conferring cold resistance. The absence of cold-sensitive segregants in crosses between the integrant strain and strains containing a wild-type *GCS1* gene would therefore indicate that the p6d-3 sequences had been directed by sequence homology to integrate at the *gcs1-1* locus, and thus would contain the wild-type *GCS1* gene On the other hand, if plasmid p6d-3 did not carry the wild-type *GCS1* gene but instead an unlinked suppressor gene, then the integrated suppressor gene and associated plasmid sequences would segregate independently of *gcs1-1* in subsequent crosses to wild-type cells, resulting in the production of cold-sensitive segregants.

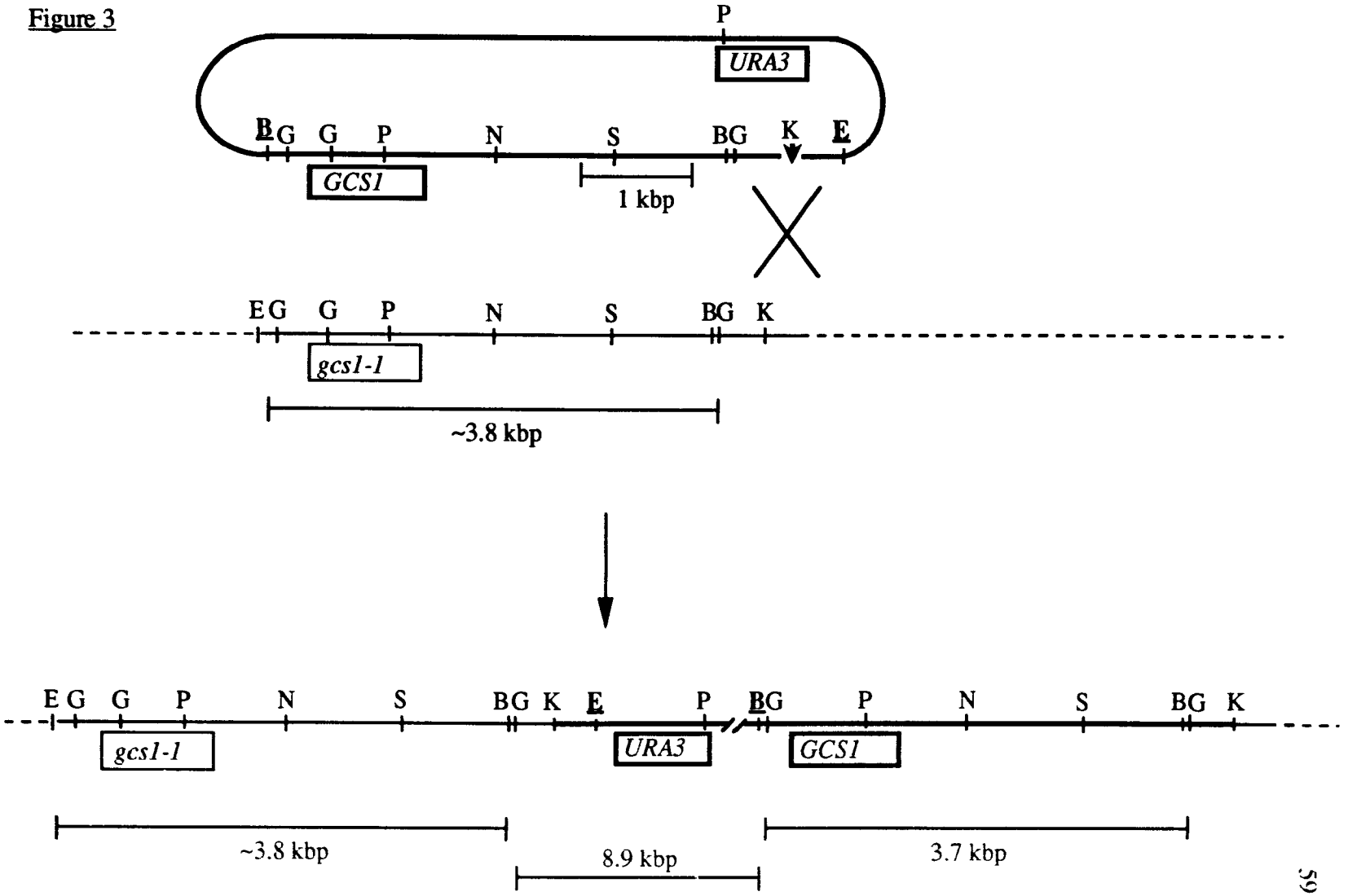
For this test of genetic linkage, the integrant strain LI-8 carrying integrated p6d-3 sequences was mated to strain ARM4R2 carrying a wild-type *GCS1* gene, and the resulting diploid strain was sporulated. In 33 tetrads analyzed from the cross all haploid segregants were cold-resistant, indicating that the sequences carried by plasmid p6d-3 were tightly linked (< 2 cM) to the *gcs1-1* gene. Together with the finding that one to two copies per cell of the p6d-3 insert sequences suppress the cold sensitivity of cells carrying the mutant *gcs1-1* allele (Fig. 2A), these data indicate that p6d-3 contains the wild-type *GCS1* gene.

(It should be noted that cryptic cold-sensitive mutations were revealed during crosses of cold-resistant wild-type strains. For example, crosses of the cold-resistant integrant strain LI-8 to the cold-resistant strain LARM 4-16B produced both cold-sensitive and cold-resistant haploid segregants. However, complementation tests showed that these cold-sensitive segregants were not due to the *gcs1-1* mutation. When

Figure 3. Construction of strain LI-8.

Integration of plasmid YIp5L1 (containing p6d-3 insert sequences) was directed by linearization at the unique *KpnI* site within the plasmid insert sequences. The insert sequences of plasmid YIp5L1 are directed to their homologous chromosomal locus. If this chromosomal locus is the *gcs1-1* genomic locus, then the resulting integration event will generate a *gcs1-1/GCS1* genomic locus in cells of the integrant strain LI-8. This integration event is illustrated using the following restriction sites: *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Kpn*I (K), *Nde*I (N), *Pst*I (P), *Sst*I (S). The *Bam*HI (B) and *Eco*RI (E) restriction sites flanking the plasmid insert sequences are derived from the vector. The position of the genomic *Eco*RI site is approximate and was deduced from the size of the genomic *Bam*HI-*Eco*RI fragment hybridizing to a radiolabelled p6d-3 derivative (data not shown).

Figure 3


P
URA3
B G G P N S BG K E
GCS1
1 kbp
E G G P N S BG K
gcs1-1
~3.8 kbp
E G G P N S BG K E P BG P N S BG K
gcs1-1
URA3
GCS1
~3.8 kbp
8.9 kbp
3.7 kbp

diploids were constructed between these cold-sensitive segregants and cells carrying the *gcs1-1* mutation, the diploids were all cold-resistant. Thus these cold-sensitive mutations were unrelated to the *gcs1-1* mutation. These unrelated cold-sensitive mutations hampered previous attempts to identify the wild-type *GCS1* gene).

2. Sequence of the *GCS1* gene

Several smaller subclones of p6d-3 were available from previous work. These subclones had been prepared by partial *Sau*3A digestion of the entire 5-kbp insert of p6d-3 and religation of the resultant 2- to 3-kbp size-fractionated DNA fragments into the *Bam*HI site of the same low-copy vector, YCp50. These smaller subclones of p6d-3 were restriction mapped and tested for complementation of the cold-sensitivity phenotype conferred by the *gcs1-1* mutation (Fig. 1). Complementation analysis of subclones of p6d-3 (Fig. 1) indicated that the *GCS1* gene sequences were located at the left end of the insert in pE3.3. Therefore, the left end of the pE3.3 insert was subjected to nucleotide sequence analysis; the insert from pE3.3 was subcloned into the related vectors M13 um20 and um21 and sequenced from the *Bam*HI site to the *Nde*I site at position 1914, as diagrammed in Fig. 4A. This sequence revealed one continuous ORF of 1056 bp capable of encoding a 352-amino-acid polypeptide (Fig. 4B).

To confirm that the 1056-bp ORF defines the *GCS1* gene, a 1.9-kbp *Bam*HI-*Nde*I fragment containing the ORF was subcloned into a low-copy vector to generate plasmid pBN316 (Table 2), which was then tested for complementation of the cold-sensitivity phenotype conferred by the *gcs1-1* mutation. Mutant cells transformed with plasmid pBN316 or with the vector alone were replica-plated to YNB solid medium and incubated at 15°C or at 29°C. Transformants carrying plasmid pBN316 were cold-resistant and proliferated at 15°C, while *gcs1-1* mutant cells transformed with vector

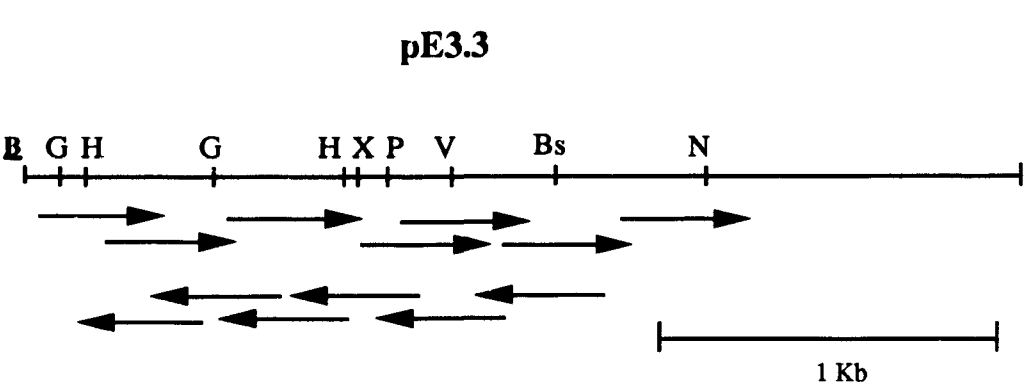
Figure 4. Strategy for sequencing the *GCS1* gene

(A) Sequencing strategy for *GCS1*. The restriction map of the 2.8-kbp complementing subclone, pE3.3, is illustrated using the following enzyme: *Bam*HI (B), *Bgl*II (G), *Bsa*AI(Bs), *Hin*dIII (H), *Nde*I (N), *Pst*I (P), *Pvu*II (V), *Sst*I (S), *Xba*I (X). The underlined *Bam*HI site is derived from the multiple cloning site of the YCp50 vector. The pE3.3 insert was sequenced on both strands from the 5' *Bgl*II site through the *Bsa*AI site. Directed subcloning in M13 and ordered deletions in M13 generated 95% of the sequence (as described in the Materials and Methods). The remaining gaps in the sequence were filled using specifically designed oligonucleotide primers in sequencing reactions on single-stranded or double-stranded templates. Arrows correspond to sequence obtained either from individual M13 subclones or from specifically designed primers.

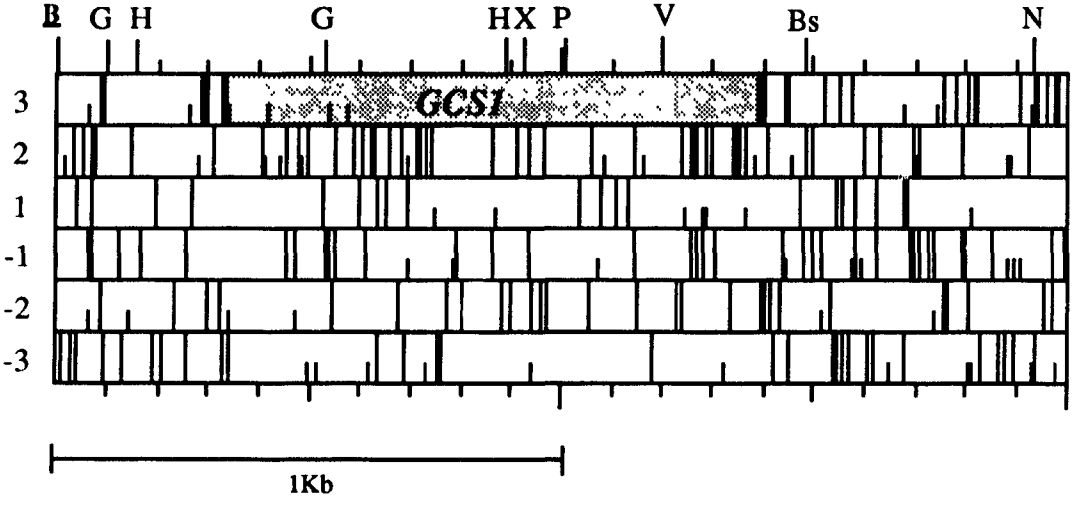
(B) ORF map depicting the *GCS1* ORF was generated using DNA Strider software (Marck, 1988). The restriction sites within the sequenced region of pE3.3 are superimposed upon the ORF map. The six possible reading frames are numbered from +3 to -3 and the ORF corresponding to the *GCS1* gene is shaded in grey. Positions of methionine codons are marked with short bars, and positions of stop codons are marked with full length bars.

Figure 4

A



B



alone remained cold-sensitive in this assay (data not shown). Therefore the ORF diagrammed in Fig. 4B encodes the wild-type Gcs1 protein.

The entire sequence of the *GCS1* gene together with the derived sequence of the Gcs1 polypeptide is shown in Fig. 5, and an annotated Gcs1 peptide sequence is shown in Fig. 6A. As indicated in Fig. 6A, the most suggestive feature of the 39-kD Gcs1 protein is a C-x2-C-x16-C-x2-C putative metal-binding motif encompassing amino acids 26 to 49 at the Gcs1 N-terminus. Cysteines can serve as ligands to a number of different metals, most commonly Fe^{2+} and Zn^{2+} (Richardson and Richardson, 1989). Typically four cysteines cluster together to form a group around a metal ion, producing a finger-like structure from the peptide sequence between the cysteines (Struhl, 1989). Zinc-binding motifs of the C2-C2 type have been shown to function in the stabilization of protein subdomains (Coleman, 1992); frequently, those subdomains function as DNA-binding domains of eukaryotic transcription factors (Coleman, 1992; Klug and Rhodes, 1987; Struhl, 1989). The particular C2-C2 motif found in the Gcs1 protein shows some resemblance to the C2-C2 motifs found in the GATA family of DNA-binding proteins (Orkin, 1992; Martin and Orkin, 1990). This similarity is shown here by highlighting those conserved positions within the consensus GATA C2-C2 motif, C-x2-C-x5-**P**-x-**W**-x5-**G**-x3-**C**-x2-**C**, that can be aligned with the Gcs1 C2-C2 motif C-x2-C-x4-**P**-x-**W**-x5-**G**-x3-**C**-x2-**C** (see Discussion).

The N-terminus of the Gcs1 protein also contains a putative nuclear localization signal, R-R-R-x10-K-K (Fig. 6A), which is similar (in inverted form) to *bona fide* nuclear localization signals in *Saccharomyces cerevisiae* (Dingwall and Laskey, 1991). Also shown in Figure 6A are putative phosphorylation sites that correspond to consensus sites for protein kinase C, casein kinase II, cAMP-dependent protein kinase, and Cdc28 kinase (Kenelly and Krebs, 1991; Bairoch, 1990). One of the putative

Figure 5. The *GCS1* gene and flanking sequences.

The nucleotide sequence of the *GCS1* gene is numbered from the presumptive ATG initiation codon of the *GCS1* ORF. The predicted amino-acid sequence is also numbered from the initiator methionine with amino-acid numbering indicated on the right. Nucleotide numbering is shown on the left beginning with the *Sau*3A site at position -337. The dot scale above the nucleotide sequence places a dot at every 10th nucleotide. Restriction sites described in the text are underlined. The Genbank accession number for the *GCS1* nucleotide sequence is L24125.

Figure 5

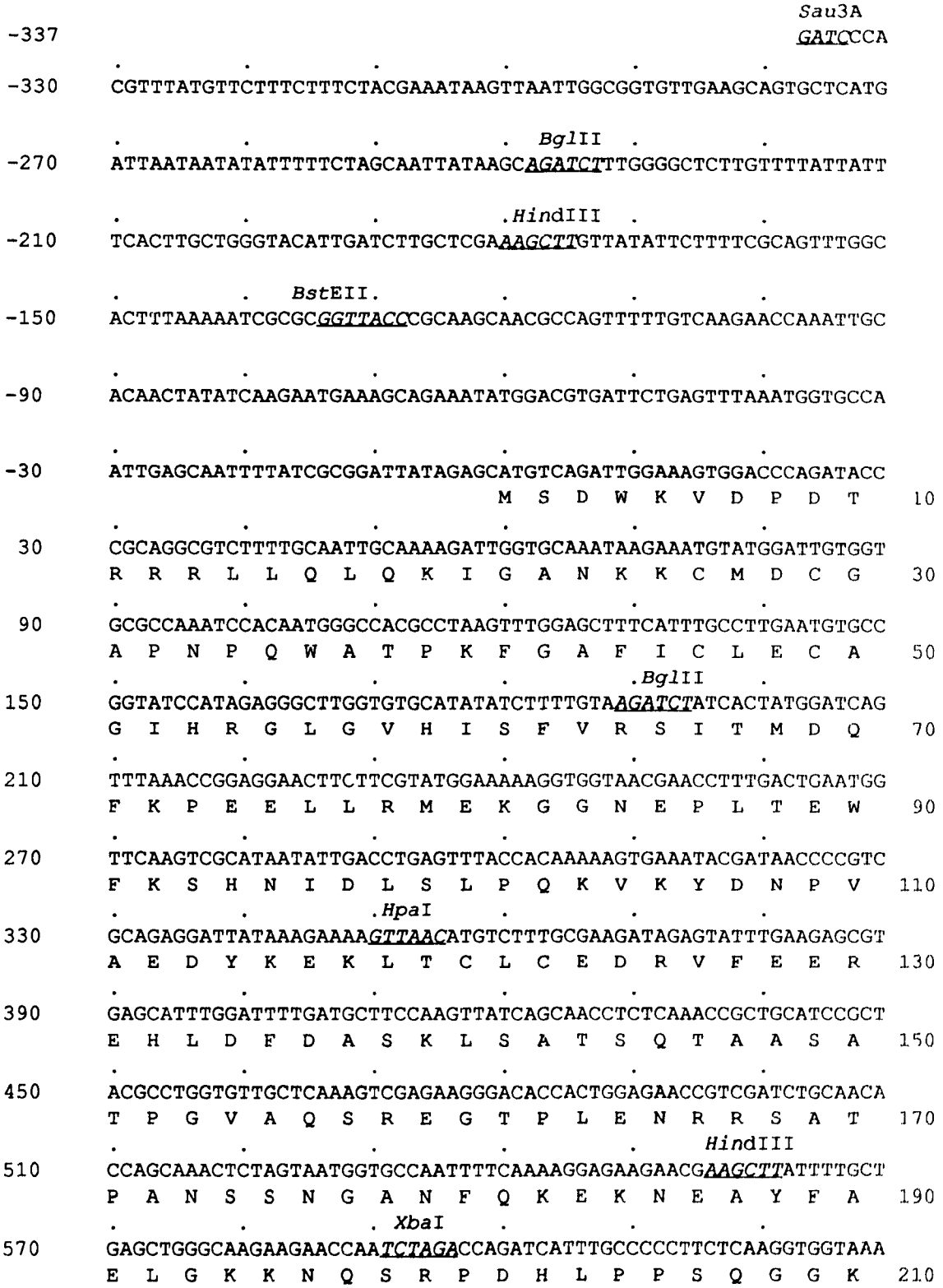

Sau3A
GATCCCA
-337
-330 CGTTTATGTTCTTTCTTTCTACGAAATAAGTTAATTGGCGGTGTTGAAGCAGTGCTCATG
BglII
-270 ATTAATAATATATTTTTCTAGCAATTATAAGCAGATCTTTGGGGCTCTTGTTTTATTATT
HindIII
-210 TCACTTGCTGGGTACATTGATCTTGCTCGAAAGCTTGTTATATTCTTTTCGCAGTTTGGC
BstEII
-150 ACTTTAAAAATCGCGCGGTTACCCGCAAGCAACGCCAGTTTTTGTCAAGAACCAAATTGC
-90 ACAACTATATCAAGAATGAAAGCAGAAATATGGACGTGATTCTGAGTTTAAATGGTGCCA
-30 ATTGAGCAATTTTATCGCGGATTATAGAGCATGTCAGATTGGAAAGTGGACCCAGATACC
M S D W K V D P D T 10
30 CGCAGGCGTCTTTTGCAATTGCAAAAGATTGGTGCAAATAAGAAATGTATGGATTGTGGT
R R R L L Q L Q K I G A N K K C M D C G 30
90 GCGCCAAATCCACAATGGGCCACGCCTAAGTTTGGAGCTTTCATTTGCCTTGAATGTGCC
A P N P Q W A T P K F G A F I C L E C A 50
BglII
150 GGTATCCATAGAGGGCTTGGTGTGCATATATCTTTTGTAAGATCTATCACTATGGATCAG
G I H R G L G V H I S F V R S I T M D Q 70
210 TTTAAACCGGAGGAACTTCTTCGTATGGAAAAAGGTGGTAACGAACCTTTGACTGAATGG
F K P E E L L R M E K G G N E P L T E W 90
270 TTCAAGTCGCATAATATTGACCTGAGTTTACCACAAAAAGTGAAATACGATAACCCCGTC
F K S H N I D L S L P Q K V K Y D N P V 110
HpaI
330 GCAGAGGATTATAAAGAAAAGTTAACATGTCTTTGCGAAGATAGAGTATTTGAAGAGCGT
A E D Y K E K L T C L C E D R V F E E R 130
390 GAGCATTTGGATTTTGATGCTTCCAAGTTATCAGCAACCTCTCAAACCGCTGCATCCGCT
E H L D F D A S K L S A T S Q T A A S A 150
450 ACGCCTGGTGTTGCTCAAAGTCGAGAAGGGACACCACTGGAGAACCGTCGATCTGCAACA
T P G V A Q S R E G T P L E N R R S A T 170
HindIII
510 CCAGCAAACTCTAGTAATGGTGCCAATTTTCAAAAGGAGAAGAACGAAGCTTATTTTGCT
P A N S S N G A N F Q K E K N E A Y F A 190
XbaI
570 GAGCTGGGCAAGAAGAACCAATCTAGACCAGATCATTTGCCCCCTTCTCAAGGTGGTAAA
E L G K K N Q S R P D H L P P S Q G G K 210

Figure 5 (cont.)

```
                                                    PstI.
630   TATCAGGGCTTTGGAAGTACACCCGCAAAACCTCCACAAGAACGGTCTGCAGGGTCCAGC
      Y  Q  G  F  G  S  T  P  A  K  P  P  Q  E  R  S  A  G  S  S   230
690   AATACTTTGAGCCTGGAAAATTTTCAAGCTGATCCTTTGGGAACATTGAGCAGAGGATGG
      N  T  L  S  L  E  N  F  Q  A  D  P  L  G  T  L  S  R  G  W   250
750   GGCCTCTTCTCCAGCGCTGTAACCAAATCGTTCGAGGACGTAAACGAAACGGTTATTAAG
      G  L  F  S  S  A  V  T  K  S  F  E  D  V  N  E  T  V  I  K   270
                                                          PvuII
810   CCTCACGTCCAGCAATGGCAATCCGGAGAGTTGTCAGAAGAAACGAAGAGAGCAGCTGCC
      P  H  V  Q  Q  W  Q  S  G  E  L  S  E  E  T  K  R  A  A  A   290
870   CAGTTTGGCCAAAAGTTTCAAGAAACGAGTAGTTATGGATTCCAAGCATTTAGTAACTTC
      Q  F  G  Q  K  F  Q  E  T  S  S  Y  G  F  Q  A  F  S  N  F   310
930   ACTAAAAATTTCAATGGTAATGCAGAGGATAGCTCTACAGCAGGGAATACAACCCATACA
      T  K  N  F  N  G  N  A  E  D  S  S  T  A  G  N  T  T  H  T   330
990   GAATATCAAAAGATTGATAATAACGATAAGAAGAATGAACAGGACGAGGACAAATGGGAC
      E  Y  Q  K  I  D  N  N  D  K  K  N  E  Q  D  E  D  K  W  D   350
1050  GATTTCTAATTTTAATATAGTTTTTCTCAACATTCAAGGAAAGTTCAATTCACATTGTAG
      D  F  *                                                      352
                                    BsaA1
1110  CTATAATGGAGAAGTGGTTCTGTAAAGTTTACACGTAATACTTCTGTCTCTAAATTTGTA

1170  TTTTTCATATCTTGACAAGAAGAGGTTTACTCTCTAGGATTATAAATAAGTCTACACCAG

1230  TATTCATAAATAGTTACCACAAGGCCATATCGTAAATTATTATACAACCTATTAATTACA

1290  AATAGAATTGCAATCCTTTCCCATCGGCGTTGTTGTACTGTTGCAAGGCTAATGACGGCT

1350  ATTATTAAAAAATGTTTTAGACCTGGGACAATCGTTATCCTTTTCTTCATTATTTGGATG

1410  TTTGCGCCTTAAAATTATCCTGGGCACGAGTGAAACAAAGCTAAAACCTTTATTTAGCAT

1470  GGCCATTGAATGTAACAATTATATATATCGCAAGCACAAAAAATCAAGGAGAGAGAACTA

1530  CCACTTTGTTCATGTGTACAATGTTCATTATCTCCATAAGCAAAAAAAAAAAATAGAAAA

      NdeI
1590  CATATGCTATAAGGTTGATATTCTCACGAGTAAGCGGCACTTACTTATTGACATTGAGGA
```

Figure 6. Gcs1 protein sequence.

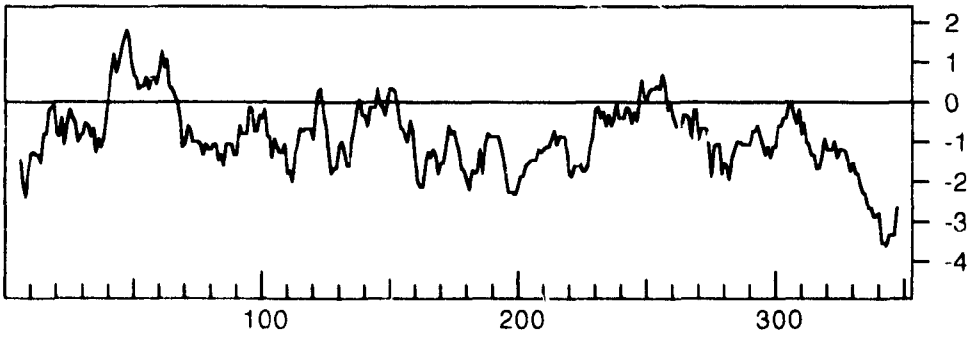
(A) Annotated peptide sequence of Gcs1. The C2-C2 motif (enclosed within the large oval) encompasses amino-acids 26-49; the four cysteine residues are indicated by large type. The putative nuclear localization signal sequences (Dingwall and Laskey, 1991; Boulikas, 1993) are indicated by the shaded boxes. Consensus phosphorylation sites for protein kinase C, Cdc28 kinase, casein kinase II, and cAMP-dependent protein kinase are described by Kenelly and Krebs, 1991. Consensus phosphorylation sites for protein kinase C (R/K_{1-3} -X_{2-0} -**S/T** -X_{2-0} -R/K_{1-3}) > (**S/T** -X_{2-0} -R/K_{1-3}) ≥ (R/K_{1-3} -X_{2-0} -**S/T**) are indicated by filled circles above threonine residues (bold type). Open circles above threonine residues (bold type) indicate consensus phosphorylation sites for Cdc28 kinase (**S/T** -P -X -R/K). The filled square above the serine residue (bold type) indicates a consensus phosphorylation site for casein kinase II (**S/T** -D/E_{1-3}). Consensus phosphorylation sites for cAMP-dependent kinase (R -R/K -X -**S/T**) > (R -X_{1-2} -**S/T**) are indicated by open squares above threonine residues (bold type). The charged nature of the C-terminus of the Gcs1 protein is indicated.

(B) Hydropathy plot for the Gcs1 protein. Kyte-Doolittle hydropathy plot was generated by DNA Strider software (Marck, 1988) for the Macintosh computer.

A Figure 6

```
1    MSDWKVDPDTRRRLLQLQKIGANKK   25
26   CMDCGAPNPQWATPKFGAFICLEC   49
50   AGIHRGLGVHISFVRSITMDQFKPEELL   77
78   RMFKGGNFPITEWFKSHNIDLSLPQK   103
104  VKYDNPVAFDYKEKLTCLCEDRVFEE   130
131  REHLDFDASKLSATSQTAASATPGVA   156
157  QSREGTPLENRRSATPANSSNGANLQ   182
183  KEKNEAYFAELGKKNQSRPDHLPPSQ   208
209  GGKYQGFGSTPAKPPQERSAGSSNTL   234
235  SLENFQADPLGTLSRGWGIFSSAVTK   259
260  SFEDVNETVIKPHVQQWQSGELSEET   285
286  KRAAAQFGQKFQETSSYGFQAFSNFT   311
312  KNFNGNAEDSSTAGNTTHTFYQKID   336
337  NNDKKNEQDEDKWDDF   352
     -++ - - --+ - -
```

B



protein kinase C sites is found with the C2-C2 motif of the Gcs1 protein, suggesting that C2-C2 domain interactions could be modulated by phosphorylation.

The amino-acid composition of the Gcs1 polypeptide is compared with the composition of an average yeast protein (Sharp and Cowe, 1991) in Fig. 7B. The Gcs1 polypeptide sequence has fewer of the hydrophobic amino-acid residues isoleucine, leucine, methionine and valine and slightly elevated proportions of polar and charged amino-acid residues compared to the average yeast protein. The hydropathy plot shown in Fig. 6B indicates that the highest density of hydrophobic amino-acid residues occurs within the C2-C2 motif of the Gcs1 protein, while the C-terminus of Gcs1 shows a high density of charged amino-acid residues. Two hydrophobic residues are included within this charged C-terminal region (Fig. 6A). These features of the Gcs1 protein; charged C-terminus with hydrophobic residues, potential phosphoprotein, potential nuclear localization signal and C2-C2 motif, are common in proteins involved in the regulation of gene expression (Struhl, 1989; Leuther *et al.*, 1993).

3. Expression of the *GCS1* gene

The codon usage bias in the *GCS1 ORF* is shown in Fig. 7A. The codon bias index (CBI) (Bennetzen and Hall, 1982) was calculated to be 0.09 (on a scale of 0 to 1). This value predicts that *GCS1* is expressed at a relatively low level. By comparison the codon bias index of *SSB1*, a member of the stress protein Hsp70 gene family (Slater and Craig, 1989; Craig, 1993), is 0.91 (Sharp and Cowe, 1991). To characterize the expression of the *GCS1* gene, the transcript abundance of the *GCS1* gene was determined in wild-type cells of strain GR2 proliferating at 23°C or 14°C, and in stationary-phase wild-type GR2 cells Total RNA isolated from stationary-phase cells, and after dilution of stationary-phase cells in fresh medium, was resolved on agarose gels and transferred to nylon membranes. Blots of total RNA were probed with

Figure 7. Codon usage and predicted amino-acid composition of the *GCS1* gene.

(A) Codon usage table shows the frequency (freq.) of occurrence of each codon in the *GCS1* gene. Boxed codons correspond to codons over represented in highly expressed genes in *S. cerevisiae* (Sharp and Cowe, 1991).

(B) Amino acid composition of the Gcs1 predicted protein sequence showing the number of occurrences (n) of each amino acid, and the percent (n%) of each amino acid relative to the average yeast protein (av %).

A

Figure 7

codon	freq.	codon	freq.	codon	freq.	codon	freq.
UUU phe	12	UCU ser	9	UAU tyr	5	UGU cys	4
UUC phe	8	UCC ser	5	UAC tyr	1	UGC cys	2
UUA leu	3	UCA ser	3	UAA OCH	-	UGA OPA	-
UUG leu	9	UCG ser	2	UAG AMB	-	UGG trp	6
CUU leu	6	CCU pro	7	CAU his	6	CGU arg	4
CUC leu	1	CCC pro	3	CAC his	1	CGC arg	1
CUA leu	-	CCA pro	8	CAA gln	17	CGA arg	2
CUG leu	4	CCG pro	1	CAG gln	5	CGG arg	1
AUU ile	5	ACU thr	4	AAU asn	13	AGU ser	7
AUC ile	2	ACC thr	5	AAC asn	9	AGC ser	5
AUA ile	1	ACA thr	8	AAA lys	12	AGA arg	6
AUG met	4	ACG thr	5	AAG lys	16	AGG arg	1
GUU val	2	GCU ala	10	GAU asp	15	GGU gly	11
GUC val	2	GCC ala	4	GAC asp	6	GGC gly	4
GUA val	4	GCA ala	12	GAA glu	18	GGA gly	6
GUG val	3	GCG ala	1	GAG glu	11	GGG gly	4

B

amino acid		n	n(%)	av %
A ala	alanine	27	7.7	6.4
C cys	cysteine	6	1.7	1.2
D asp	aspartic acid	21	6.0	6.0
E glu	glutamic acid	29	8.2	6.6
F phe	phenylalanine	20	5.7	4.3
G gly	glycine	25	7.1	5.6
H his	histidine	7	2.0	2.1
I ile	isoleucine	8	2.3	6.3
K lys	lysine	28	8.0	7.4
L leu	leucine	23	6.5	9.2
M met	methionine	4	1.1	2.1
N asn	asparagine	22	6.2	5.8
P pro	proline	19	5.4	4.4
Q gln	glutamine	22	6.2	5.8
R arg	arginine	15	4.3	4.4
S ser	serine	31	8.8	8.2
U thr	threonine	22	6.2	5.8
V val	valine	11	3.1	6.0
W trp	tryptophan	6	1.7	1.0
Y tyr	tyrosine	6	1.7	3.3

radiolabelled pE3.3 plasmid, which contains both the *GCS1* and *URA3* genes (Fig. 8A). The *GCS1* transcript was found to be of relatively low abundance in comparison to the *URA3* transcript, but increased in abundance (as did the *URA3* mRNA) upon incubation of stationary-phase wild-type cells in fresh medium at 23° (Fig. 8A). To more readily visualize the low-abundance *GCS1* transcript, a higher-specific-activity probe was prepared using subclones of *GCS1* in M13 to produce strand-specific radiolabelled *GCS1* fragments (see Materials & Methods). Reprobing the same blot with this probe indicated that some *GCS1* transcript is also present in stationary-phase cells (Fig. 8B; 23°C). The *GCS1* transcript increases in abundance upon incubation of stationary-phase wild-type cells in fresh medium at either 23°C or 14°C (Fig. 8B). The relatively low-abundance *GCS1* transcript is therefore present in stationary-phase cells, is transcribed at a low level during the resumption of proliferation from stationary phase, and increases in abundance in exponentially proliferating cells.

4. Mapping the *GCS1* gene

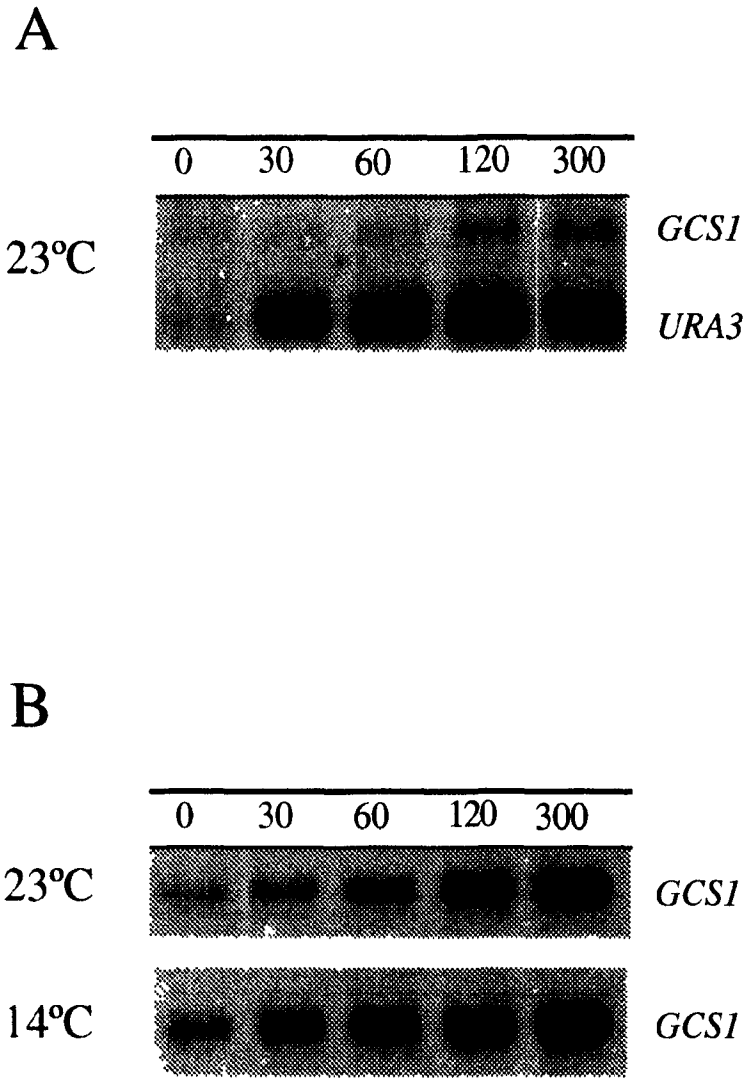
To determine if the *GCS1* gene is a novel gene or if it had been previously identified by mutation and positioned on the yeast genetic map, the *GCS1* locus was mapped by a combination of standard genetic techniques and molecular hybridization analysis. As a first step to map the *GCS1* locus, a radiolabelled *GCS1* fragment from plasmid 6d-3 was used to probe a Southern blot of separated yeast chromosomes (see Materials &Methods). This analysis localized *GCS1* to chromosome IV (data not shown). To further localize the *GCS1* gene with respect to other genetic loci on chromosome IV, several genetic crosses were performed using the integrant strain LI-8. This integrant strain contains at the *gcs1-1* locus an integrated wild-type *GCS1* gene along with associated plasmid sequences, including the vector marker gene *URA3* (Fig. 3). Linkage between genetic loci on chromosome IV and the *GCS1* locus was therefore

Figure 8. Relative abundance of the *GCS1* transcript in wild-type cells.

(A) RNA was isolated from stationary-phase cells wild-type cells (strain GR2) at time 0 and after dilution of stationary-phase cells in fresh YEPD medium and incubation for 30, 60, 120 and 300 min at 23°C. RNA was resolved by formaldehyde-agarose gel electrophoresis and transferred to nylon membranes (as described in Materials and Methods). The RNA blot was probed with radiolabelled pE3.3 plasmid as described in the text. The *GCS1* and *URA3* transcripts are indicated.

(B) The RNA blot described in (A) was reprobed with a higher-specific-activity probe specific for the *GCS1* transcript. This higher-specific-activity probe was prepared using a subclone of pE3.3 in M13 (BaP20; Table 3) as described in the Materials and Methods. In addition, a second RNA blot was prepared from stationary-phase wild-type cells (strain GR2) before (0) and after transfer to fresh medium and incubation at 14°C for 30, 60, 120 and 300 min. This RNA blot was also probed with the higher-specific-activity probe specific for the *GCS1* transcript as described above.

Figure 8



conveniently scored by analyzing linkage to the *URA3* gene integrated at the *gcs1-1* locus. Genetic crosses did not show any linkage between this *URA3* gene integrated at the *GCS1* locus and markers distributed along the right arm of chromosome IV, but did show linkage to markers on the left arm of chromosome IV (Table 4). Subsequent genetic crosses positioned the *GCS1* gene between the *HO* and *CDC13* genes on the left arm of chromosome IV, within 1 cM of the *HO* locus (Fig. 9A).

The tight genetic linkage observed between the *GCS1* and *HO* genes suggested that the physical relationship between these two genes could also be clarified. From the observed genetic linkage data, the restriction maps of *GCS1* and *HO* flanking sequences could be expected to overlap, since 1 cM of genetic distance corresponds to an average of 3 kbp (Mortimer *et al.*, 1989). The restriction maps of the regions flanking the *HO* gene (Jensen *et al.*, 1983; Nasmyth, 1985) were therefore examined for patterns characteristic of the restriction map of p6d-3, the plasmid containing the *GCS1* gene within the largest complementing insert. As shown in Fig. 9B, significant overlap was found between the restriction map of the p6d-3 genomic insert and the restriction pattern in the region upstream of the *HO* gene. This physical correspondence places the 3' end of the *GCS1* gene 3 kbp upstream of the 5' end of the *HO* ORF. Since the *HO* upstream activating region extends 1.8 kb upstream of the *HO* gene (Tebb *et al.*, 1993), there may be at least one other gene positioned between *HO* and *GCS1*.

Another gene, *SSB1*, has also been mapped to the approximate location of *GCS1* (Mortimer *et al.*, 1992). The restriction map of *SSB1* did not show any overlap with that of *GCS1*; consistent with this observation, *SSB1* has been physically localized, by sequence comparison, to within 300 bp of the 3' end of the *HO* gene (Mortimer *et al.*, 1992). Thus, the order of the genetic loci in this region is *GCS1-HO-SSB1*. Genetic and physical mapping therefore show that *GCS1* is indeed a novel gene that has not been previously characterized by genetic studies.

Table 4. Genetic linkage between *gcs1* and chromosome IV markers

		Ascus Type			Map distance
Diploid	Interval	PD[a]	NPD[b]	TT[c]	cM[d]
LI-8 x LARM 4-16B	*gcs1-1* - *rna3*	2	5	24	> 90 cM
	gcs1-1 - *ade8*	4	6	20	> 93 cM
	gcs1-1 - *trp4*	9	5	16	> 77 cM
LI-8 x ARM-4R2	*gcs1-1* - *lys4*	2	2	4	> 100 cM
	gcs1-1 - *cdc37*	2	1	5	> 50 cM
	gcs1-1 - *trp1*	0	1	7	> 81 cM
GWD12C x LIcdc13	*gcs1-2* - *cdc13-1*	30	0	2	3.1 cM
GWDHIS x LI-J0217-3a	*gcs1-2* - *HO::URA3*	44	0	1	1.1 cM

(a) PD, parental ditype

(b) NPD, non-parental ditype

(c) TT, tetratype

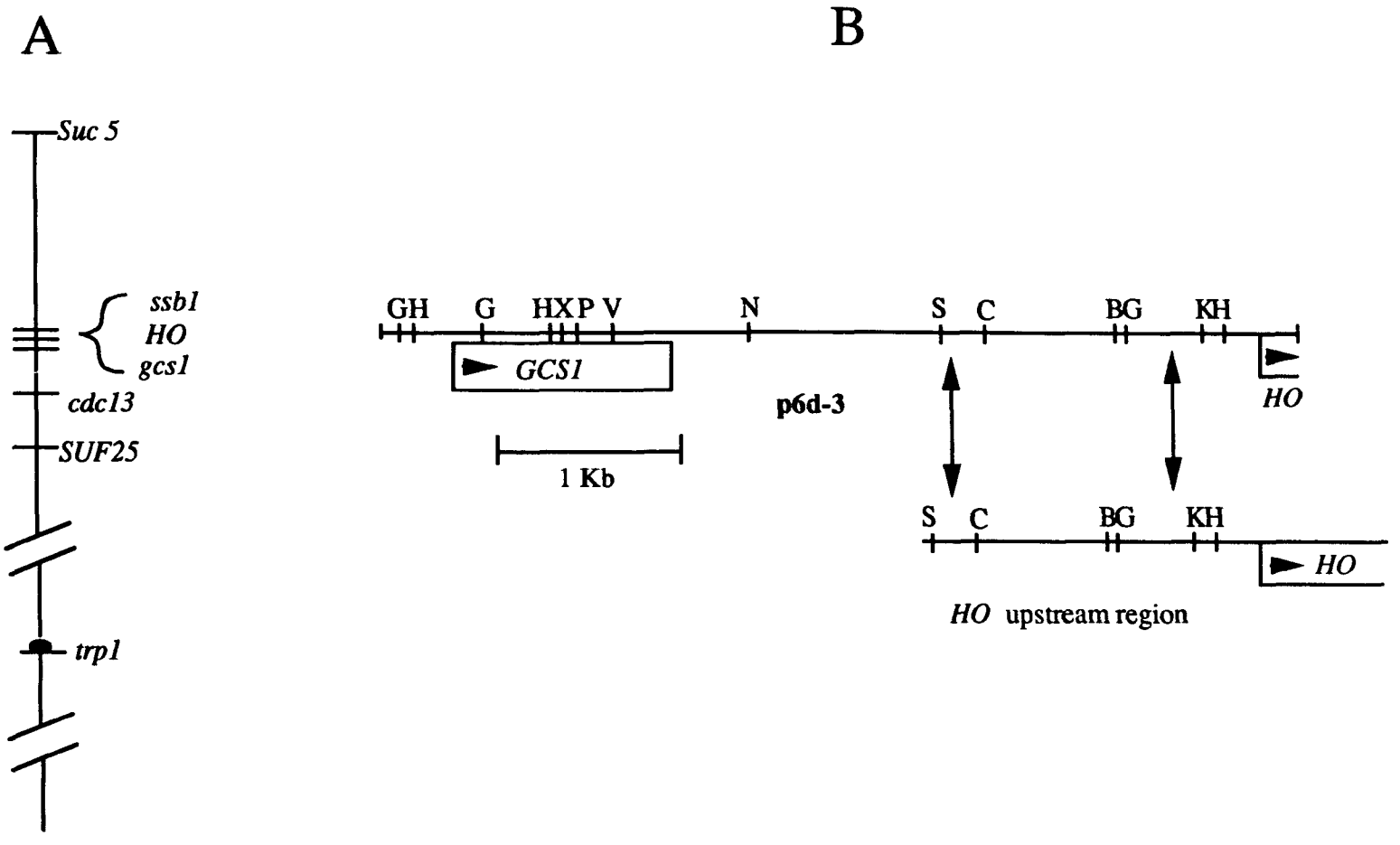
(d) cM, Distance in centimorgans (cM) was determined using the equation of Perkins (1949); distance in cM=[(TT + 6NPD) x 100] / 2 (PD + NPD + TT).

Figure 9. Mapping the *GCS1* gene

(A) Genetic mapping of the *gcs1-2* mutation. The *gcs1-2* mutation was positioned relative to *cdc13* and *HO*genomic loci by crossing *gcs1-2* mutant strains with mapping strains and analyzing the resulting tetrads (listed in Table 4). The left arm of chromosome IV is diagrammed with the *gcs1* locus positioned between the *HO* and *cdc13* genes. However, another interpretation of this mapping data would position the *HO* locus between the *gcs1* and *cdc13* genes. Thus, the gene order could also be *gcs1-HO-ssb1-cdc13*.

(B) Physical mapping of the *GCS1* gene. The tight genetic linkage between the *GCS1* and *HO* genes suggested that the restriction maps of the *GCS1* and *HO* flanking sequences may overlap. The restriction maps of the insert of p6d-3 and the *HO* upstream region (Jensen et al., 1983; Nasmyth, 1985) were therefore compared. The overlap is shown using the following restriction sites: *Bam*HI (B), *Bgl*II (G), *Cla*I (C), *Hin*dIII (H), *Kpn*I (K), *Nde*I (N), *Pst*I (P), *Pvu*II (V), *Sst*I (S), *Xba*I (X).

Figure 9



Chromosome IV

5. Cloning the *gcs1-1* mutant allele

To identify the mutation in the *gcs1-1* mutant allele, the *gcs1-1* mutant allele was cloned using the technique of gap-repair (Orr-Weaver and Szostak, 1983). Gap-repair allows the cloning of a mutant gene by transformation of the mutant cell with a plasmid containing only the flanking regions of the wild-type gene. This plasmid is constructed so that when it is linearized by cleavage with the appropriate restriction enzymes, recombinogenic DNA ends containing sequences homologous to DNA flanking the mutant chromosomal locus are produced. Upon transformation into mutant yeast cells, these recombinogenic ends can then direct the plasmid sequences to the DNA flanking the mutant chromosomal locus. The sequences within the mutant chromosomal locus can then be copied from the chromosomal allele, to fill in the "gap" of missing sequences in plasmid DNA, by a process thought to be similar to gene conversion. Reisolation of the plasmid from the mutant yeast cells is the final step in cloning the mutant allele.

The selection of the homologous flanking sequences in the gap-repair plasmid determines the sequence information that can be obtained from the chromosomal allele. If the approximate position of the mutation in a mutant allele is known, then a gap-repair plasmid can be designed to recover only those sequences which contain the mutation.

5A. Localization of the mutation in the *gcs1-1* mutant allele. To simplify the gap-repair procedure, the *gcs1-1* mutation was first localized within the gene. The *gcs1-1* mutation was initially localized to the 5' half of the ORF as follows. First, a *Bam*HI-*Pst*I fragment containing the entire *GCS1* promoter and two thirds of the *GCS1* ORF was subcloned into the *URA3* integrating vector YIp352 to make plasmid pL23. Integration of this plasmid was directed to the *gcs1-1* chromosomal locus by linearizing

the plasmid at a unique *Xba*I site within the *GCS1* ORF (Fig. 10A). This integration event results in two versions of the *gcs1* gene at the *gcs1-1* locus. One version contains the 5' sequences from the plasmid-borne wild-type *GCS1* gene up to the *Xba*I site coupled to the remaining 3' sequences from the *gcs1-1* chromosomal locus, resulting in a chimeric *GCS1/gcs1* gene, while the other version is a C-terminal truncation of *gcs1-1*. Since the phenotype of an identically truncated *GCS1* gene has been shown to be cold sensitivity (see section 6A below), the phenotype of this integrant will be cold resistance only if the chimeric *GCS1/gcs1* gene can confer wild-type *GCS1* gene function.

Cells carrying the *gcs1-1* and *ura3-1* mutations were transformed to uracil prototrophy with plasmid pL23, and the phenotype of the Ura^+ transformants was determined by replica-plating to solid medium and incubation at 15°C. All of the Ura^+ transformants were cold-resistant; thus the chimeric gene conferred wild-type *GCS1* gene function. Therefore the wild-type sequences comprising the 5' half of the *GCS1* chimeric gene must replace the sequences containing the mutation in the *gcs1-1* chromosomal locus. In this way, the mutation was localized to the region of the *GCS1* gene corresponding to the first 200 amino acids of the 352-amino-acid Gcs1 protein.

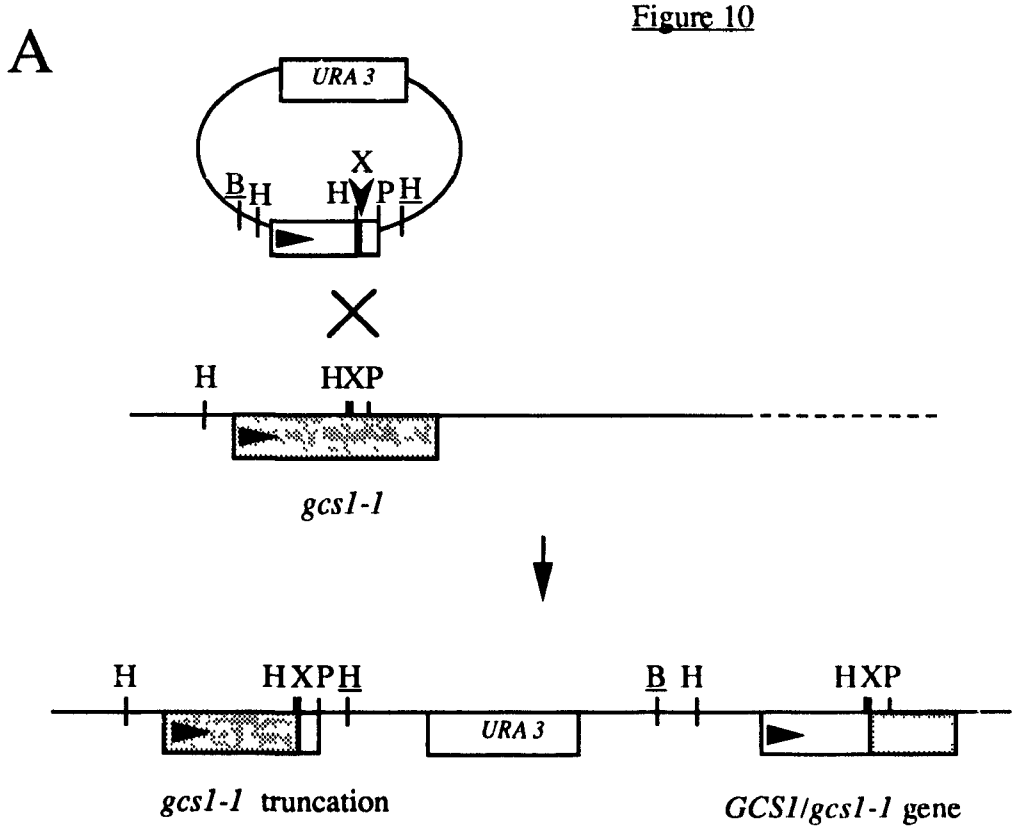
Confirmation that the 3' half of the *gcs1-1* mutant ORF did not contain any mutations was obtained by using the polymerase chain reaction to amplify the region of the *gcs1-1* mutant allele between the *Hin*dIII site located in the middle of the *GCS1* ORF and the 3' end of the ORF (Fig. 10A; M. Drebot, personal communication). This amplified product was subcloned into M13 vectors and two clones were sequenced. The sequence of the mutant *gcs1-1* gene between the internal *Hin*dIII site and the 3' end of the mutant gene was found to be wild-type; thus the mutation was localized to the 5' half of the *GCS1* gene. In this way the *gcs1-1* mutation was localized to the region

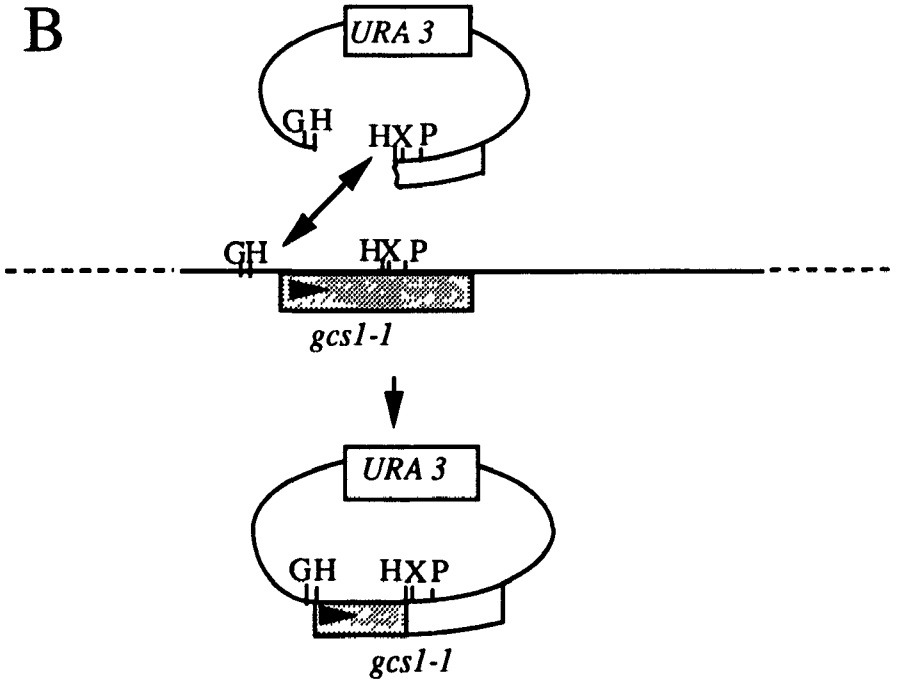
Figure 10. Cloning the *gcs1-1* mutation

(A) Localization of the *gcs1-1* mutation. Plasmid L23, containing 5' sequences from the *GCS1* gene (the 5' end of the *GCS1* gene is indicated by the solid arrow), was linearized with *Xba*I to direct integration to the *gcs1-1* locus. Integration of this construct at the *gcs1-1* locus creates a hybrid *GCS1/gcs1-1* gene as well as a truncated *gcs1-1* gene. The hybrid *GCS1/gcs1-1* gene will be a functional wild-type gene if the *gcs1-1* mutation is located in the 5' half of the *gcs1-1* gene.

(B) Gap repair of the *gcs1-1* locus. The gap-repair plasmid, pL27, was linearized at the unique *Hin*dIII site (created by the deletion of the 880-bp *Hin*dIII fragment of the cloned *GCS1* gene). Transformation of *gcs1-1* mutant cells (strain MDgcs1-3X) with the linearized gapped plasmid was followed by the recovery of a plasmid (from *gcs1-1* transformants) that contained the 880-bp *Hin*dIII fragment copied from the *gcs1-1* locus.

Figure 10


A
URA 3
X
BH
HXPH
H
HXP
gcs1-1
H
HXPH
B H
HXP
URA 3
gcs1-1 truncation
GCS1/gcs1-1 gene


B
URA 3
GH
HXP
GH
HXP
gcs1-1
URA 3
GH
HXP
gcs1-1

corresponding to the first 190 codons of the *GCS1* gene, between the *Hin*dIII site in the *gcs1-1* promoter and the *Hin*dIII site within the *gcs1-1* ORF.

5B. Gap-repair of the *gcs1-1* mutant allele. Localization of the *gcs1-1* mutation to the interval between the *Hin*dIII site in the *gcs1-1* promoter and the *Hin*dIII site within the *ORF* of the *gcs1-1* gene simplified the cloning the *gcs1-1* mutant allele: it was sufficient to gap-repair only the region of the *gcs1-1* gene between these two *Hin*dIII sites. Accordingly, a gap-repair plasmid was constructed by deletion of the 880-bp *Hin*dIII fragment from the cloned wild-type *GCS1* gene in plasmid pBN316. This gap-repair plasmid, pL27 (Table 2), was then digested with *Hin*dIII to generate recombinogenic *GCS1* DNA ends and used to transform cells containing the *gcs1-1* and *ura3-1* mutant alleles to uracil prototrophy. Since plasmids must be circular (or integrated) to be propagated *in vivo*, the *URA3* plasmids carried by the Ura^+ transformants were either simply recircularized by religation *in vivo*, and thus lacked the 880-bp *Hin*dIII fragment, or the "missing" *Hin*dIII fragment had been replaced by a process similar to gene conversion using the chromosomal *gcs1-1* gene as template (Fig. 10B). Plasmids were therefore recovered from transformants that had acquired uracil prototrophy and screened for the presence of the 880-bp *Hin*dIII fragment. One such plasmid, designated pRS1-1, was found to contain this *Hin*dIII fragment, copied from the *gcs1-1* mutant allele.

Nucleotide sequence analysis of the *Hin*dIII fragment copied from the *gcs1-1* mutant allele by gap-repair revealed two alterations in the *gcs1-1* DNA sequence that resulted in changes to the amino-acid sequence of the Gcs1 protein. Both of these alterations were G -> A substitutions. The *gcs1-1* mutant allele resulted from EMS mutagenesis (Drebot, 1987), and both of these alterations are consistent with the expected G-C to A-T transition mutations produced by EMS mutagenesis (Kohalmi *et*

al., 1988). These two sequence alterations were both located in the C2-C2 motif at the N terminus of the Gcs1 protein (Fig. 11A). One alteration causes the conservative substitution of an isoleucine residue for a methionine residue in the amino acid sequence C-M-x-C-x_{16}-C-x_2-C of theC2-C2 motif. The other alteration causes a nonconservative change of the second cysteine in the four-cysteine C2-C2 motif to a tyrosine.

Additional experiments resolved which of the two sequence alterations is responsible for the cold sensitivity conferred by the *gcs1-1* mutant allele. Since the *gcs1-1* mutation was produced by mutagenesis of cells of strain IIIXD (Drebot, 1987), yeast genomic DNA was isolated from this strain, and the *Hin*dIII fragment of the *GCS1* gene from IIIXD was amplified by the polymerase chain reaction and sequenced directly. The sequence of this *Hin*dIII fragment did not contain the cysteine-codon alteration, but did contain the same base-pair change encoding the same conservative substitution of isoleucine for methionine as found in the *gcs1-1* mutant allele (Fig. 11B; M. Drebot, personal communication). Since the wild-type allele from strain IIIXD encodes this conservative substitution of isoleucine for methionine, this polymorphism is unlikely to contribute to the cold sensitivity of cells carrying the *gcs1-1* allele. Therefore the missense mutation responsible for the cold sensitivity conferred by the *gcs1-1* mutant allele is the substitution of tyrosine for the second cysteine in the C2-C2 motif of the Gcs1 protein (Fig. 11C). Clearly the C2-C2 motif of the Gcs1 protein is essential for Gcs1 function.

6. Disruption of the *GCS1* gene

To gain insight into the role of the *GCS1* gene, the phenotype of cells lacking wild-type *GCS1* gene function was determined. Three different gene-disruption alleles of *GCS1* were constructed by integrative transformation of diploid cells. (Diploid cells were

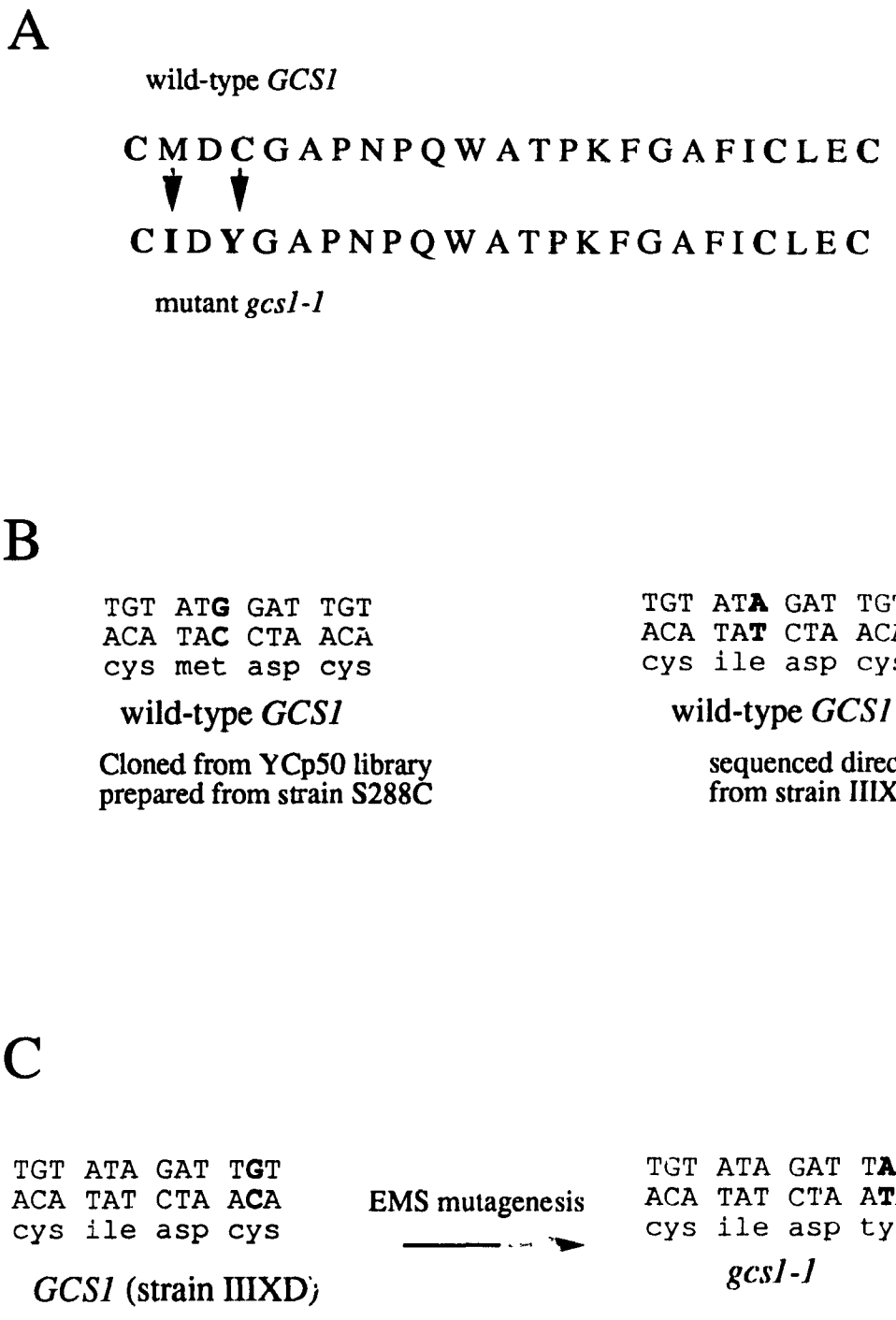
Figure 11. Sequencing of the *gcs1-1* mutation.

(A) Two changes in the derived amino-acid sequence of the *GCS1* gene (indicated by arrows) were revealed by nucleotide sequence analysis of the *Hin*dIII fragment of the cloned *gcs1-1* mutant allele. Both sequence alterations are located within the C2-C2 motif of the Gcs1 protein. One change causes a substitution of isoleucine for methionine (first arrow) and the other change causes a substitution of tyrosine for cysteine (second arrow) in the second cysteine of the four cysteine C2-C2 motif.

(B) Comparison of the sequence of two wild-type alleles of *GCS1*. The derived protein sequence of the wild-type *GCS1* gene isolated during this work (Fig. 5) predicts a methionine residue at amino-acid position 27 of the Gcs1 protein. However, the sequence of the wild-type *GCS1* gene obtained directly from strain IIIXD (see text) predicts an isoleucine residue at position 27 of the Gcs1 protein. Therefore, the G-C to A-T transition mutation (bold type) that results in the substitution of isoleucine for methionine represents a polymorphism of the wild-type *GCS1* gene.

(C) Diagrammatic representation of the production of the *gcs1-1* mutant allele through EMS mutagenesis of the wild-type *GCS1* gene in cells of strain IIIXD. The G-C to A-T transition mutation produced by EMS mutagenesis is illustrated (bold type).

Figure 11


A
wild-type GCS1
C M D C G A P N P Q W A T P K F G A F I C L E C
C I D Y G A P N P Q W A T P K F G A F I C L E C
mutant gcs1-1
B
TGT ATG GAT TGT
ACA TAC CTA ACA
cys met asp cys
wild-type GCS1
Cloned from YCp50 library
prepared from strain S288C
TGT ATA GAT TGT
ACA TAT CTA ACA
cys ile asp cys
wild-type GCS1
sequenced directly
from strain IIIXD
C
TGT ATA GAT TGT
ACA TAT CTA ACA
cys ile asp cys
GCS1 (strain IIIXD)
EMS mutagenesis
TGT ATA GAT TAT
ACA TAT CTA ATA
cys ile asp tyr
gcs1-1

used so that if the *GCS1* gene proved to be essential, the intact homolog of the *GCS1* gene remaining in the transformed diploid could still supply function.) Two mutant alleles, *gcs1-2* and *gcs1-3*, were constructed by integration of an internal fragment of the *GCS1* ORF into the *GCS1* chromosomal locus, and resulted in mutant alleles encoding two different truncations of the Gcs1 polypeptide. In addition to these truncation alleles, a null allele, *gcs1-6*, was constructed by replacing the entire ORF and part of the *GCS1* promoter with the *URA3* gene.

6A. The *gcs1-2* and *gcs1-3* truncation alleles. The *gcs1-2* truncation allele was constructed by integration of an internal fragment of the *GCS1* ORF into the *GCS1* chromosomal locus (see Materials and Methods), thus disrupting the chromosomal locus and creating both an N-terminal truncation (lacking N-terminal coding sequences) and a C-terminal truncation (lacking C-terminal coding sequences) of the *GCS1* gene. Since the N-terminal truncation lacks a promoter, only the C-terminal truncation should be capable of supplying any *GCS1* gene function (Fig. 12A).

The *gcs1-2* allele was constructed in wild-type W303 diploid cells by truncation of one homolog of the *GCS1* gene, allowing the other homolog to supply *GCS1* gene function. The *gcs1-2* disruption plasmid, containing an internal *Bgl*II-*Pst*I fragment of the *GCS1* ORF in the *URA3* integrating vector YIp352, was directed to the *GCS1* chromosomal locus in wild-type diploid cells homozygous for the *ura3-1* mutation. Diploid transformants that acquired uracil prototrophy by integration of the *URA3* vector marker were then sporulated; the tetrads dissected from three of these *URA3* diploids all yielded four viable spores, suggesting that the wild-type Gcs1 protein is not required for exponential growth. Disruption of the *GCS1* chromosomal locus in Ura$^+$ spore clones was confirmed by Southern analysis (Fig. 12B). The *gcs1-3* truncation allele was constructed exactly as described for *gcs1-2* but using a different disruption

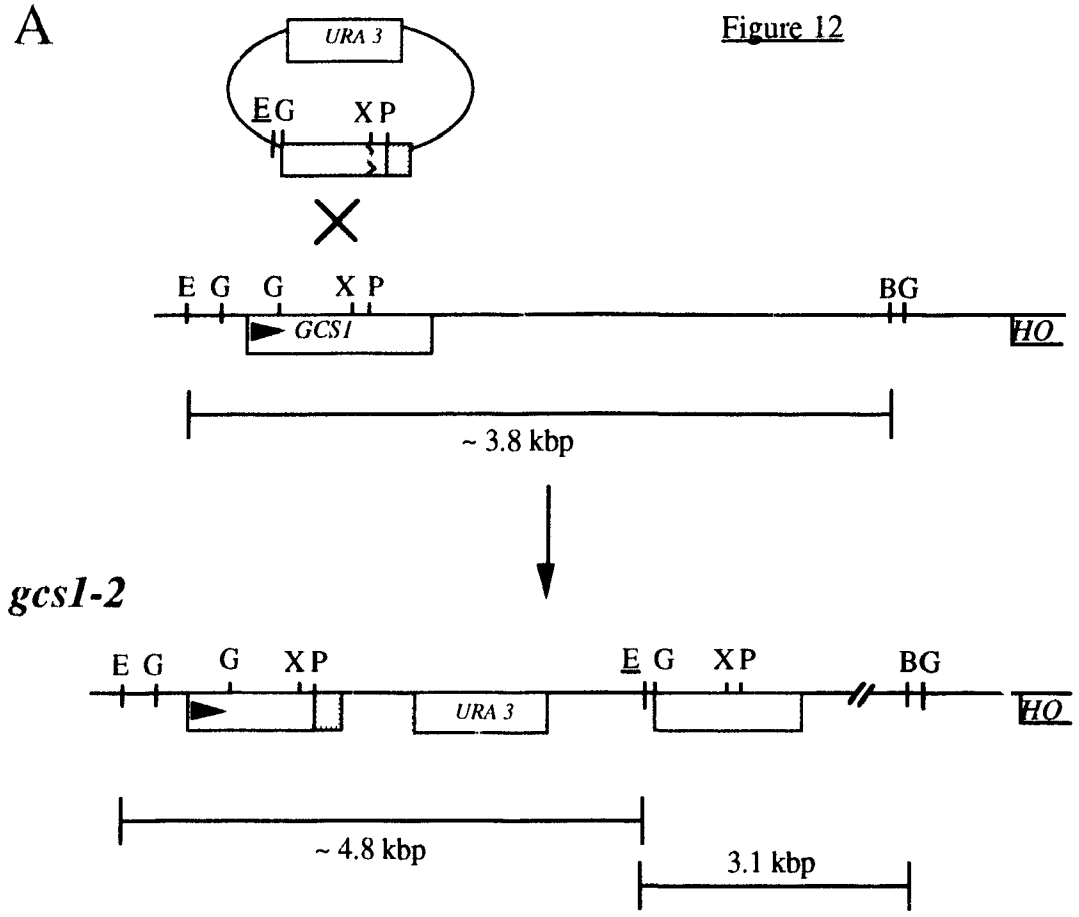
Figure 12. The *gcs1-2* truncation allele.

(A) Construction of the *gcs1-2* truncation allele. The *gcs1-2* disruption plasmid, containing an internal *Bgl*II-*Pst*I fragment of the *GCS1* ORF, was linearized with *Xba*I to direct integration to the *GCS1* locus. Integration at the *GCS1* locus creates both an N-terminal truncation (contained within a 3.1 kbp genomic *Eco*RI-*Bam*HI fragment) and a C-terminal truncation (contained with a ~4.8 kbp *Eco*RI-*Eco*RI fragment). The predicted fusion protein from the C-terminal truncation is encoded by the 5' 228 codons of *GCS1* sequences (indicated by light shading) fused to 52 codons of frame-shifted *lacZ* sequences (indicated by heavy shading). Selected restriction sites are indicated: *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Pst*I (P), *Xba*I (X). The underlined *Eco*RI site corresponds to the site in the vector multiple cloning site that is introduced into the *gcs1-2* genomic locus. The position of the genomic *Eco*RI site upstream of the *GCS1* ORF is approximate and was deduced from the size of the genomic *Bam*HI-*Eco*RI fragment containing the *GCS1* gene (data not shown).

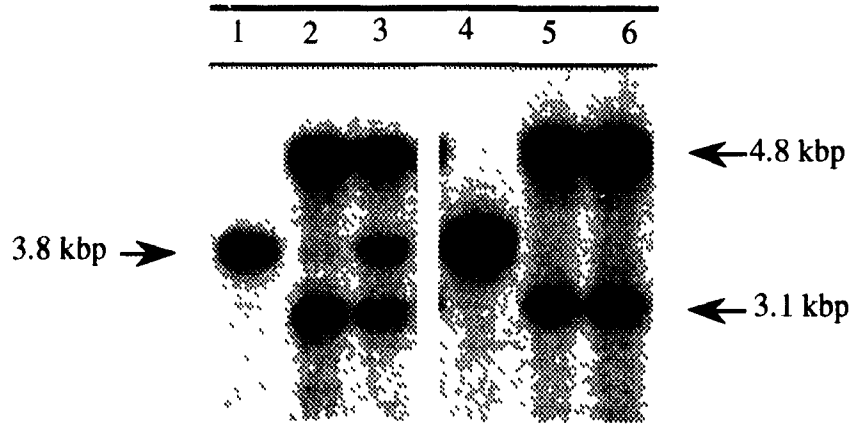
(B) Confirmation of the *gcs1-2* allele by Southern analysis. Genomic DNA was isolated from wild-type and *gcs1-2* mutant cells and digested with *Bam*HI and *Eco*RI restriction enzymes. The *Bam*HI-*Eco*RI restricted DNA was electrophoretically resolved on an agarose gel and transferred to a nylon membrane (see Materials and Methods). The DNA blot was probed with the radiolabelled 725-bp *Bst*EII-*Xba*I fragment of the *GCS1* gene (purified as an ~725-bp *Xba*I-fragment from pNB314 [Table 3]). Lanes 1 and 4 contain DNA from wild-type cells of strain W303 and 21R respectively. Lanes 2 and 3 contain DNA from *gcs1-2* mutant cells with the W303 genetic background. For this, DNA was isolated from cells of strain GWD-12C (lane 2) and GWD-12C-2 (lane 3). Lanes 5 and 6 contain DNA from *gcs1-2* mutant cells with the 21R genetic background. For this, DNA was isolated from cells of strain GRD-1 (lane 5) and GRD-2 (lane 6).

Figure 12

A


URA 3
E G
X P
E G G X P
BG
GCS1
HO
~ 3.8 kbp
gcs1-2
E G G X P
E G X P
BG
URA 3
HO
~ 4.8 kbp
3.1 kbp

B


1 2 3 4 5 6
3.8 kbp
4.8 kbp
3.1 kbp

plasmid, so that the N-terminal truncation of the *GCS1* gene was the same as for *gcs1-2* but the C-terminal truncation extended to the internal *Hin*dIII site of the *GCS1* ORF, thus removing an additional 40 amino acids (compared to the *gcs1-2* polypeptide) from the C-terminus of the protein, as diagrammed in Fig. 13A. As found for *gcs1-2*, the Ura$^+$ haploid spores carrying the *gcs1-3* mutant allele were viable, and disruption of the *GCS1* chromosomal locus in Ura$^+$ spore clones was confirmed by Southern analysis (Fig. 13B).

Although the Ura$^+$ *gcs1-2* and *gcs1-3* segregants could form colonies on rich medium at 23°C and at 29°C, these segregants were found to be cold-sensitive upon transfer by replica-plating to solid medium and incubation at 15°C (Fig. 14). Thus, truncation of the *GCS1* gene results in the phenotype of cold sensitivity. To investigate further, the growth kinetics of *gcs1-2* and *gcs1-3* mutants were determined in liquid medium. Stationary-phase *gcs1-2* and *gcs1-3* cells were clearly impaired for resumption of proliferation when transferred to fresh medium and incubated at the restrictive temperature of 15°C (Fig. 15; A and C). However, actively proliferating *gcs1-2* and *gcs1-3* mutant cells continued to proliferate with exponential kinetics after transfer to the restrictive temperature (Fig. 15; B and D). Thus truncation of the *GCS1* gene results in a reentry-mutant phenotype. That is, stationary-phase *gcs1* mutant cells are unable to resume proliferation at the restrictive temperature of 15°C, while proliferating *gcs1* mutant cells can continue to proliferate after transfer to 15°C.

Although stationary-phase *gcs1-2* and *gcs1-3* cells are clearly impaired for resumption of proliferation from stationary phase at the restrictive temperature, proliferation is observed after extended incubations at 15°C. This cell proliferation may reflect heterogeneity in the starting stationary-phase cell culture. The presence of low numbers of cells in the starting population that are no longer cold-sensitive from stationary phase may account for the observed cell proliferation during the extended

Figure 13. The *gcs1-3* truncation allele.

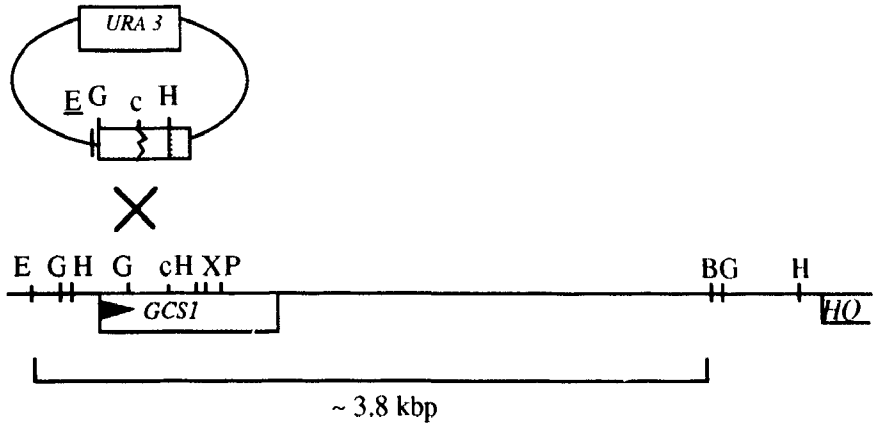
(A) Construction of the *gcs1-3* truncation allele. The *gcs1-3* disruption plasmid, containing an internal *Bgl*II-*Hin*dIII fragment of the *GCS1* ORF, was linearized with *Hin*cII to direct integration to the *GCS1* locus. Integration at the *GCS1* locus creates both an N-terminal truncation (contained within a 3.1 kbp genomic *Eco*RI-*Bam*HI fragment) and a C-terminal truncation (contained with a ~4.7 kbp *Eco*RI-*Eco*RI fragment). The predicted fusion protein from the C-terminal truncation is encoded by the 5' 187 codons of *GCS1* sequences (indicated by light shading) fused to 49 codons of frame-shifted lacZ sequences (indicated by heavy shading). Selected restriction sites are indicated: *Bam*HI (B), *Bgl*II (G), *Hin*cII (c), *Hin*dIII (H), *Eco*RI (E), *Pst*I (P), *Xba*I (X). The underlined *Eco*RI site corresponds to a site in the vector that is introduced into the *gcs1-3* genomic locus. The position of the genomic *Eco*RI site upstream of the *GCS1* ORF is approximate and was deduced from the size of the genomic*Bam*HI-*Eco*RI fragment containing the *GCS1* gene (data not shown).

(B) Disruption of the *GCS1* gene was confirmed by Southern analysis. Genomic DNA was isolated from wild-type and *gcs1-3* mutant cells and digested with *Bam*HI and *Eco*RI restriction enzymes. The *Bam*HI-*Eco*RI restricted DNA was electrophoretically resolved by agarose gel electorphoresis and transferred to nylon membranes (see Materials and Methods). The DNA blot was probed with the radiolabelled 725-bp *Bst*EII-*Xba*I fragment of the *GCS1* gene (purified as an ~725-bp *Xba*I-fragment from pNB314 [Table 3]).

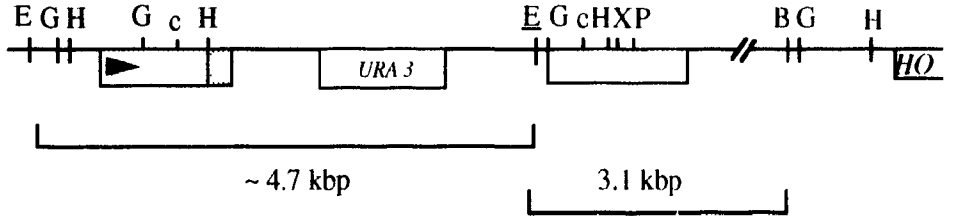
Lanes 1 and 4 contain DNA from wild-type cells of strain W303 and 21R respectively. Lanes 2 and 3 contain DNA from *gcs1-3* mutant cells with the W303 genetic background. For this, DNA was isolated from cells of strain GWT-3b (lane 2) and GWT-3d (lane 3). Lanes 5 contains DNA from *gcs1-3* mutant cells with the 21R genetic background (strain GRT-2).

Figure 13

A



gcs1-3



B

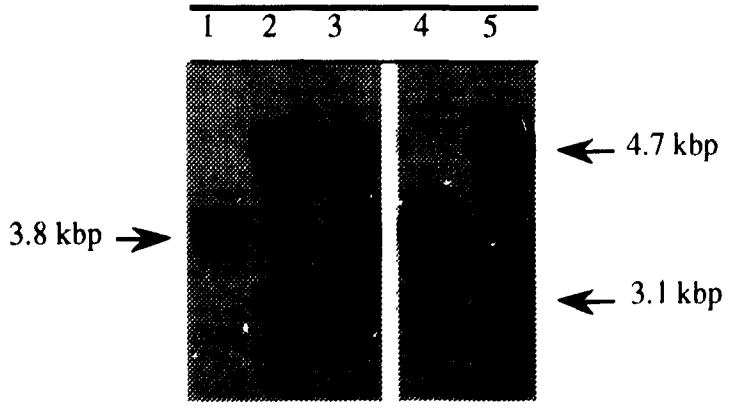


Figure 14. Cold sensitivity of *gcs1-3* segregants.

The *gcs1-3* disruption plasmid, containing an internal *Bgl*II-*Hin*dIII fragment of the *GCS1* ORF in the *URA3* integrating vector YIp352 (Fig. 13), was integrated at the *GCS1* chromosomal locus in wild-type diploid cells homozygous for the *ura3-1* mutation. Diploid transformants that acquired uracil prototrophy by integration of the *URA3* vector marker were then sporulated, and 7 tetrads were dissected from one of these sporulated *URA3* diploid strains. All four isolated spores from each tetrad were viable and formed colonies at 23°C. The four colonies from each tetrad were patched in an ordered array to YEPD solid medium and grown up a second time at 29°C. These patches, which all grew at 29°C, were then replica-plated to YEPD solid medium and incubated at 15°C for 9 days (tetrads 1-7). Cold sensitivity segregated 2:2, and all cold-sensitive segregants were also Ura^+. Only Ura^- haploid segregants could proliferate at 15°C. The Ura^+ cold-sensitive segregants corresponded to *gcs1-3* mutant cells (confirmed by Southern analysis, Fig. 13). However, isolated papillations of cold-resistant *gcs1-3* cells can also be detected.

Figure 14

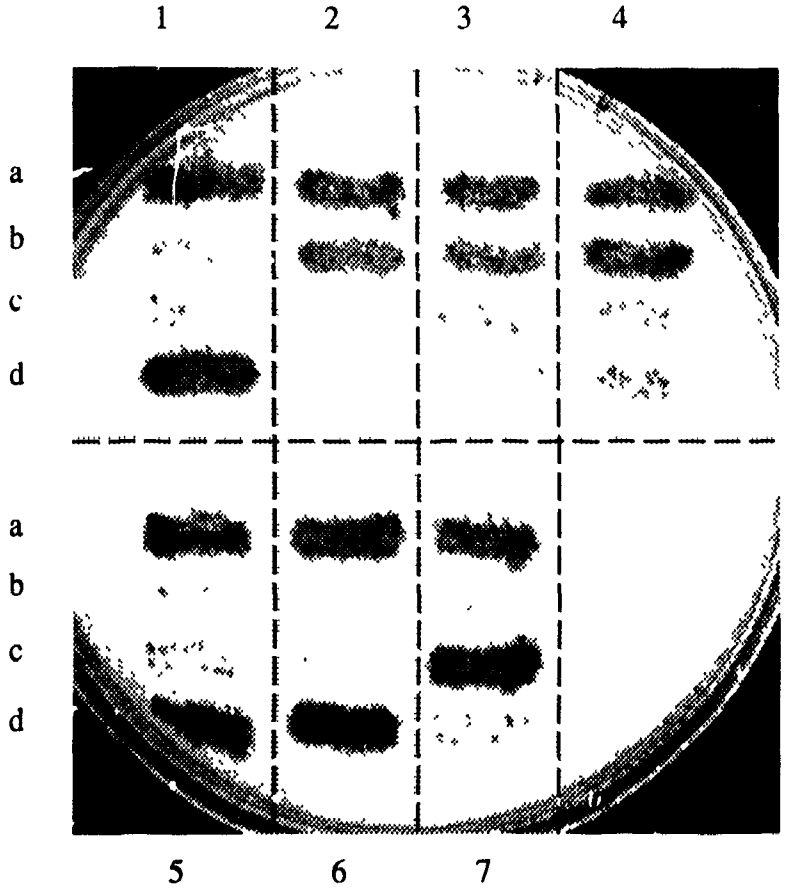
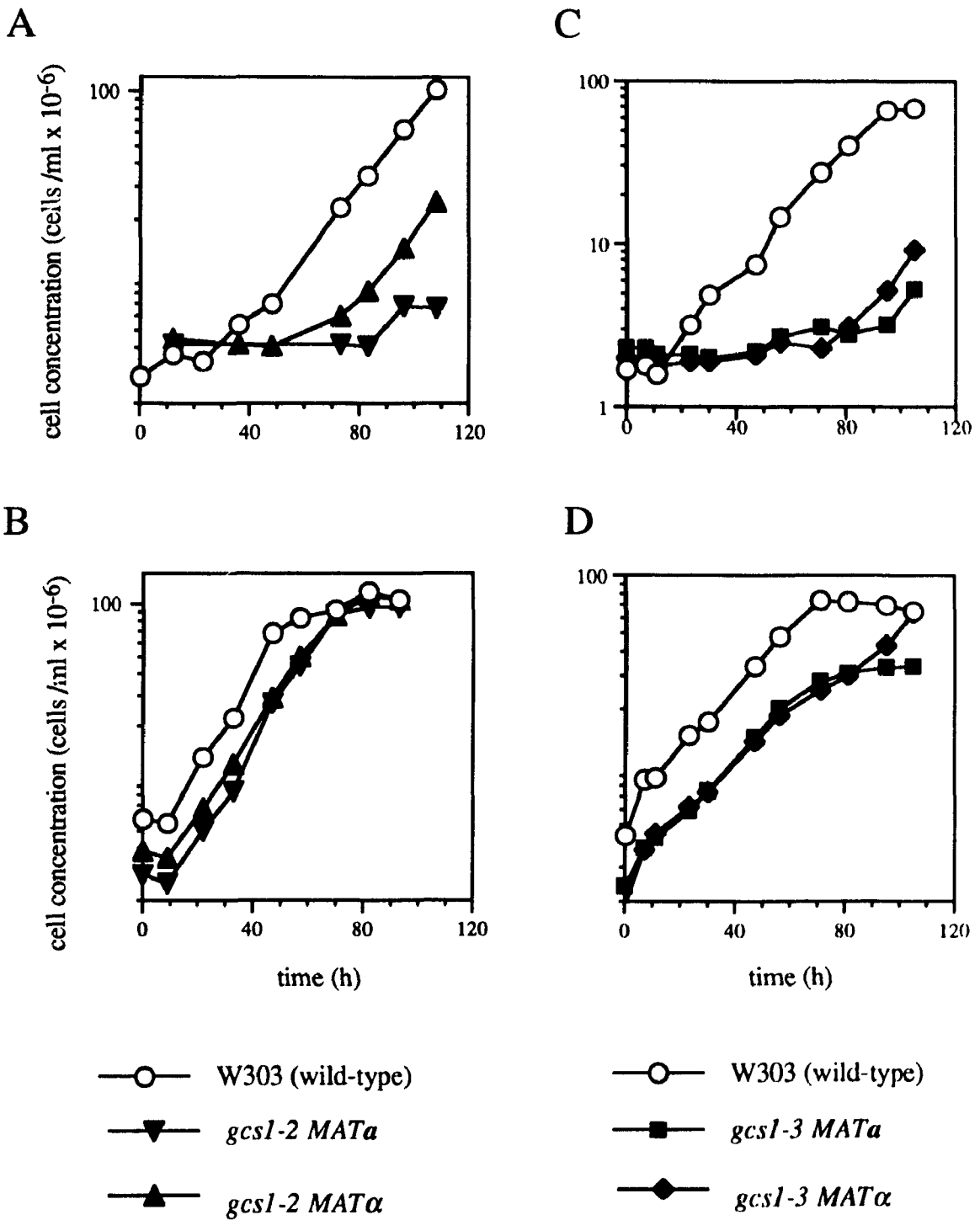


Figure 15. Growth kinetics of cells containing the *gcs1-2* or *gcs1-3* truncation alleles.

(A and C) Stationary-phase wild-type (open symbols) and *gcs1* mutant cells (filled symbols) were diluted into fresh YEPD medium and incubated at 15°C. At intervals samples were removed for determination of cell concentration. In panel A, wild-type cells (open circles), *gcs1-2 MATa* cells (downward triangles), and *gcs1-2 MAT*α cells (upward triangles) were assayed for the ability to resume proliferation from stationary phase at 15°C. In panel C, wild-type cells (open circles), *gcs1-3 MATa* cells (squares) and *gcs1-3 MAT*α cells (diamonds) were assayed for the ability to resume proliferation from stationary phase at 15°C.

(B and D) Actively proliferating wild-type (open symbols) and *gcs1* mutant cells (filled symbols) were transferred from 29°C to 15°C at time 0 and then incubated further at 15°C. At intervals samples were removed for determination of cell concentration. In panel B, wild-type cells (open circles), *gcs1-2 MATa* cells (downward triangles), and *gcs1-2 MAT*α cells (upward triangles) were assayed for the ability to maintain ongoing proliferation at 15°C. In panel C, wild-type cells (open circles), *gcs1-3 MATa* cells (squares), and *gcs1-3 MAT*α cells (diamonds) were assayed for the ability to maintain ongoing cell proliferation during incubation at 15°C.

Figure 15


A
B
C
D
cell concentration (cells /ml x 10-6)
100
10
1
0
40
80
120
time (h)
W303 (wild-type)
gcs1-2 MATa
gcs1-2 MATα
gcs1-3 MATa
gcs1-3 MATα

incubation periods at 15°C. To investigate this hypothesis, the number of cold-resistant cells present in a cell culture from *gcs1-3* mutant cells after 57 h of incubation at the restrictive temperature was measured. Samples of cell culture were diluted in PBS, spread on YEPD solid medium and incubated at 29°C. Colonies that formed were then replica-plated to YEPD solid medium and incubated at the restrictive temperature. By this assay, 5% of the cells sampled at 57 h were cold-resistant in the *gcs1-3* cell cultures. These cold-resistant cells within the starting stationary-phase populations of *gcs1-2* and *gcs1-3* mutant cells may be responsible for the observed cell proliferation in liquid culture after long periods of incubation at the restrictive temperature.

Cold-resistant cells within the starting stationary-phase population of *gcs1-2* and *gcs1-3* mutant cells were also found when stationary-phase cells were replica-plated to solid medium and incubated for 9-11 days at 15°C; these cold-resistant cells formed isolated colonies within the background of cold-sensitive cells (Fig. 14). Some of these cold-resistant cells may arise from recombination at the mutant locus (reversal of the integration event shown in Fig. 12A) to regenerate an intact *GCS1* gene. In fact, Southern analysis of genomic DNA from *gcs1-2* and *gcs1-3* mutant cells shows a faint band corresponding to the size of the intact *GCS1* gene in some preparations of genomic DNA (Fig. 12B, lane 3; Fig. 13B, lane 3).

The reentry-mutant phenotype of the *gcs1-2* and *gcs1-3* truncation alleles indicates that the C terminus of the Gcs1 protein is important for Gcs1 function. The truncation alleles actually encode somewhat larger proteins than would be predicted from the *GCS1* sequences alone. The *gcs1-2* truncation allele contains 228 codons from the *GCS1* gene fused to 52 codons of frame-shifted *lacZ* sequences (Fig. 12A), and the *gcs1-3* truncation allele contains 187 codons of *GCS1* sequence fused to 49 codons of frame-shifted *lacZ* sequences (Fig. 13A). The additional amino acids

encoded by frame-shifted *lacZ* sequences do not restore function to the truncated *gcs1* mutant proteins.

Although this analysis of the phenotype of the *gcs1-2* and *gcs1-3* truncation alleles was sufficient to determine that these alleles confer a reentry-mutant phenotype, the effect of completely eliminating Gcs1 had not been established. Another disruption allele, *gcs1-6*, was therefore constructed to assess the effect of the complete absence of the Gcs1 protein.

6B. The *gcs1-6* null allele

The *gcs1-6* mutant allele was constructed by replacing the entire *GCS1* ORF with the *URA3* gene. To construct this *gcs1-6* null mutation, the *Bam*HI-*Nde*I fragment containing the *GCS1* ORF and flanking regions was first subcloned into pUC19, to make plasmid pBN19. From pBN19 the *Bsa*AI-*Bst*EII fragment containing the entire *GCS1* ORF was removed and replaced with the *URA3* gene, to make plasmid pBNΔ4 (Table 2). This "gene-replacement plasmid" contains the *URA3* gene enclosed within 200 bp of *GCS1* upstream flanking sequences and 450 bp of *GCS1* downstream flanking sequences. To direct integration of this *URA3* gene at the *GCS1* locus, two recombinogenic DNA ends were exposed by digesting pBNΔ4 with *Bam*HI and *Eco*RI. These cleavages release the entire plasmid insert and expose *GCS1* flanking sequences, which can direct homologous recombination at the *GCS1* locus. With this fragment, cells can be transformed to uracil prototrophy by undergoing a double crossover, resulting in the replacement of the wild-type *GCS1* gene with the *URA3* gene at the *GCS1* chromosomal locus (Fig. 16A).

The replacement of the *GCS1* gene was done in diploid cells by replacement of one homolog of the *GCS1* gene, allowing the other homolog to supply *GCS1* gene function. The pBNΔ4 plasmid DNA, digested with *Bam*HI and *Eco*RI, was used to

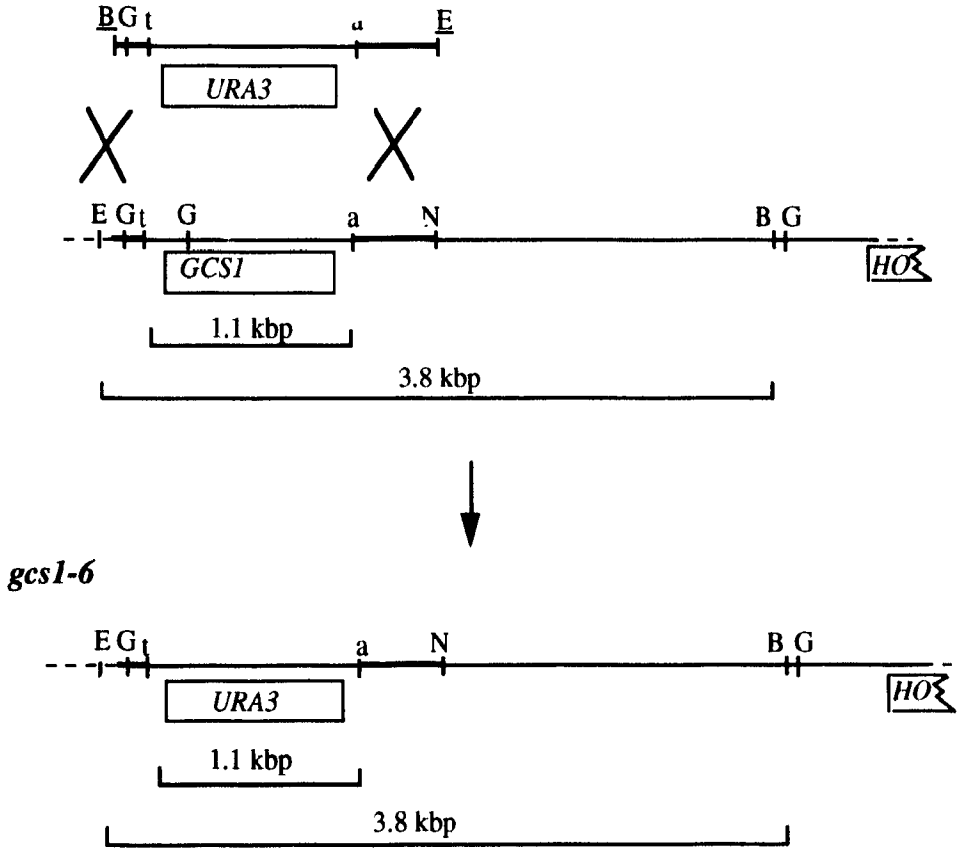
Figure 16. The *gcs1-6* null allele.

(A) Construction of the *gcs1-6* null allele. The *gcs1-6* disruption plasmid, pBNΔ4 (Table 3), was digested with *Bam*HI and *Eco*RI to release a restriction fragment containing the *URA3* gene flanked by 200 bp of *GCS1* promoter sequence and 450 bp of sequence downstream of the *GCS1* ORF (heavy lines). Wild-type cells were transformed to uracil prototrophy with this fragment resulting in the replacement of the wild-type *GCS1* gene by the *URA3* gene at the *gcs1-6* chromosomal locus. Selected restriction sites are indicated: *Bam*HI (B), *Bgl*II (G), *Eco*RI (E), *Nde*I (N), *Bst*EII (t), *Bsa*AI (a), *Xba*I (X). Underlined restriction sites correspond to vector cloning sites.

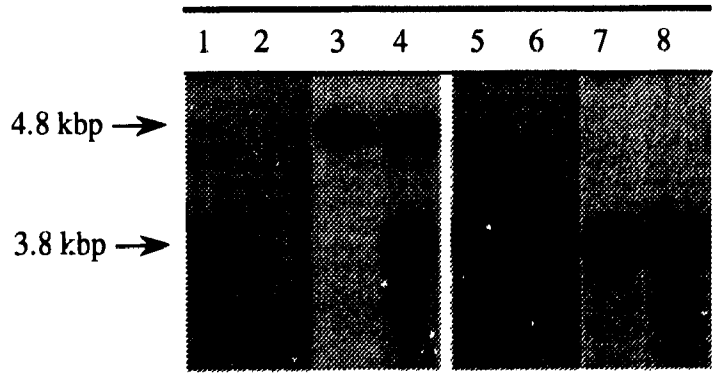
(B) The replacement of the *GCS1* gene in *gcs1-6* null cells was confirmed by Southern analysis. Genomic DNA, isolated from wild-type (lanes 1, 3, 5 , 7) or *gcs1-6* null cells (lanes 2, 4, 6, 8), was digested with *Bam*HI and *Eco*RI, resolved on agarose gels, and transferred to nylon membranes. The DNA blot was probed with the radiolabelled 725-bp *Bst*EII-*Xba*I fragment of the *GCS1* gene (lanes 1, 2, 5, 6). This probe will not hybridize to DNA from *gcs1-6* null cells since *GCS1* sequences 3' to the *Bst*EII site were removed by the construction of the *gcs1-6* allele (Fig. 16A). The DNA blot was stripped and reprobed with the radiolabelled 0.9-kbp *Bam*HI-*Pst*I fragment of the *URA3* gene (lanes 3, 4, 7, 8) isolated from plasmid Ydp-U (provided by L. Murray). Lanes 1 and 3 contain DNA from wild-type cells of strain W303. Lanes 5 and 7 contain DNA from wild-type cells of strain 21R. Lanes 2 and 4 contain DNA from *gcs1-6* mutant cells with the W303 genetic background (strain GWK-8A). Lanes 6 and 8 contain DNA from *gcs1-6* mutant cells with the 21R genetic background (strain GRKΔ4-7).

Figure 16

A


B G t
a
E
URA3
E G t
G
a
N
B G
GCS1
HO
1.1 kbp
3.8 kbp
gcs1-6
E G t
a
N
B G
URA3
HO
1.1 kbp
3.8 kbp

B


1 2 3 4 5 6 7 8
4.8 kbp
3.8 kbp

transform *ura3-1* homozygous diploid W303 cells to uracil prototrophy. Two independent diploid transformants that acquired uracil prototrophy were then sporulated. All of the tetrads dissected from the two independent Ura^+ diploids yielded four viable spores. Gene replacement at the *GCS1* chromosomal locus in Ura^+ spore clones was confirmed by Southern analysis (Fig. 16B). Colony formation by these Ura^+ *gcs1-6* null-mutant segregants indicates that the Gcs1 protein is not essential for cell proliferation on rich medium at 29°C.

As found previously for cells carrying the *gcs1-2* and *gcs1-3* truncation alleles, the haploid *gcs1-6* null segregants were found to be cold-sensitive upon transfer by replica-plating to solid medium and incubation at 15°C. To investigate further, the growth kinetics of haploid cells of strain GWK-8A, carrying the *gcs1-6* null mutation, were determined in liquid medium. Like the *gcs1-2* and *gcs1-3* mutants, *gcs1-6* mutant cells were found to have a reentry-mutant phenotype. Stationary-phase *gcs1-6* mutant cells were clearly impaired in resumption of proliferation when transferred to fresh medium and incubated at the restrictive temperature of 15°C (Fig. 17A). However, actively proliferating *gcs1-6* mutant cells continued to proliferate with exponential kinetics after transfer to the restrictive temperature (Fig. 17B). Therefore, Gcs1 is only essential at 15°C for resumption of cell proliferation from stationary phase.

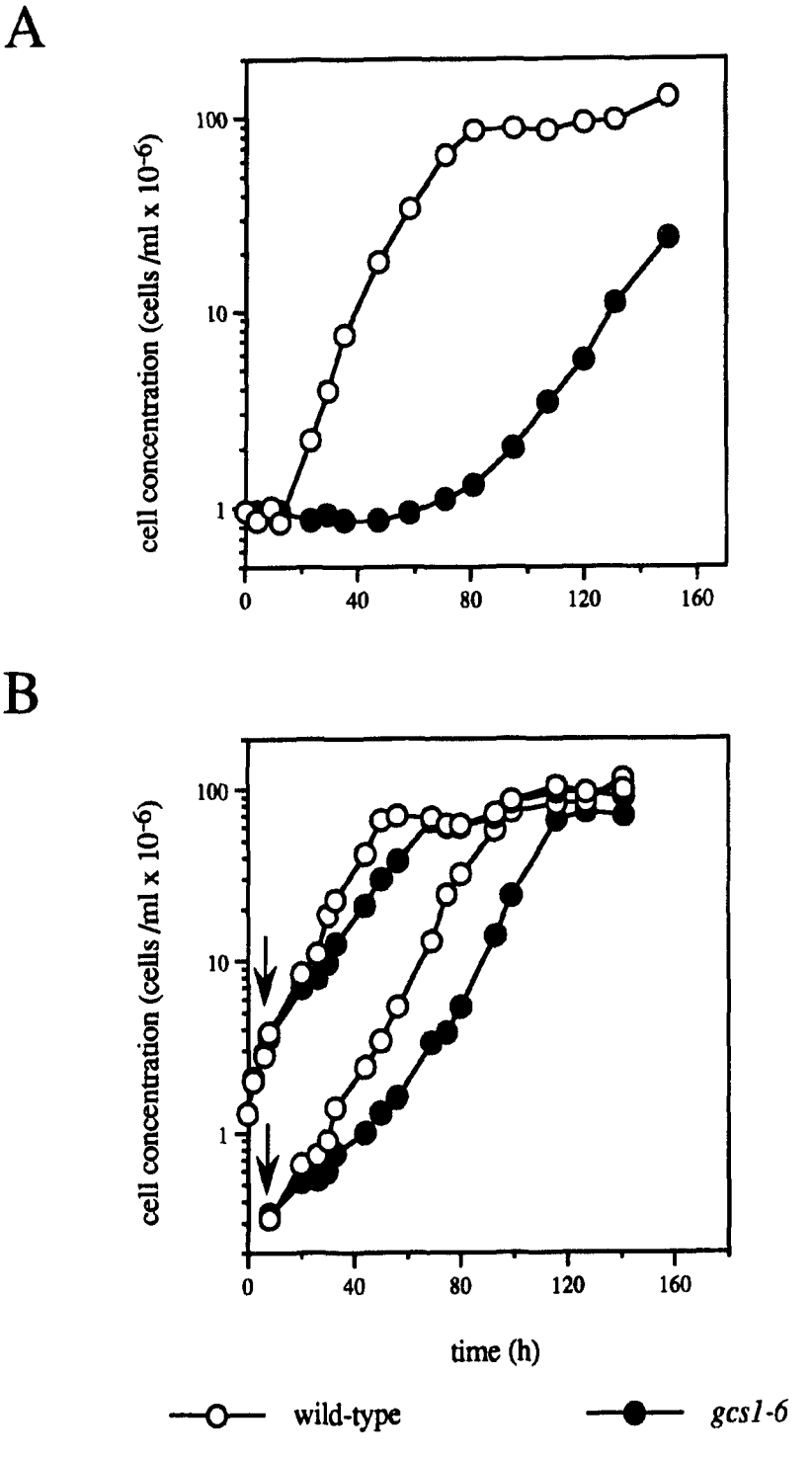
After incubation of stationary-phase *gcs1-6* cells for 80 h at 15°C (equivalent to 7 generation-times of wild-type cells with a generation time of 11 h) proliferation was observed in the cell population (Fig. 17A). In these *gcs1-6* mutant cell cultures, revertant cells that were cold-resistant for resumption of proliferation from stationary phase could not arise by homologous recombination, since *GCS1* sequences had been eliminated from the genome. However, cold-resistant cells could arise from genetic alterations that result in suppression of the cold-sensitivity phenotype. These *gcs1-6* cell cultures were therefore tested for the presence of cells that were cold-resistant for

Figure 17. Growth kinetics of *gcs1-6* mutant cells at 15°C.

(A) Stationary-phase wild-type (open circles) and *gcs1-6* null cells (filled circles) were diluted into fresh YEPD medium and incubated at 15°C. At intervals, samples were removed to determine cell concentration.

(B) Actively proliferating wild-type (open circles) and *gcs1-6* null cells (filled circles) were transferred from 29°C to 15°C and then incubated at 15°C. After 8 h of incubation at 15°C (arrows) a portion of each cell culture was diluted 10-fold with YEPD medium prechilled to 15°C. At intervals, samples were removed from all four cell cultures to determine cell concentration.

Figure 17


A
B
cell concentration (cells /ml x 10-6)
100
10
1
0
40
80
120
160
time (h)
wild-type
gcs1-6

resumption of proliferation from stationary phase. Samples of cell culture were removed after 81, 107 and 131 h of incubation, diluted in PBS, and spread on YEPD solid medium (as described above) and incubated at 29°C. Cells that formed colonies at 29°C were replica-plated to YEPD solid medium and incubated at the restrictive temperature. Since most cells in a colony on solid medium are in stationary phase, this assay tests the ability of stationary-phase cells to resume proliferation at 15°C. By this assay, > 99% of cells within the *gcs1-6* cell population remained cold-sensitive for resumption of proliferation from stationary phase for at least 131 h of incubation at the restrictive temperature. Therefore the increase in cell number observed after 80 h of incubation of *gcs1-6* cells under restrictive conditions was not due to the proliferation of genetically suppressed cold-resistant cells with the population, but instead was from the proliferation of cold-sensitive *gcs1-6* mutant cells. Thus *gcs1-6* mutant cells (with the W303 genetic background) may be capable of resuming proliferation after an extended lag equivalent to 7 generation times of wild-type cells.

An alternative explanation for the observed proliferation of *gcs1-6* mutant cells after extended incubation at the restrictive temperature is that not all of the cells were originally arrested in stationary phase by our standard protocol. A subpopulation of cells that were not in stationary phase could eventually result in the observed proliferation of cells that were still cold-sensitive for the resumption of proliferation from stationary phase. Other procedures, such as time-lapse photography of stationary-phase *gcs1-6* mutant cells spread on solid medium and incubated at the restrictive temperature, would be needed to distinguish between these two possibilities.

In other experiments, the population of proliferating cells observed after extended periods of incubation of *gcs1-6* mutant cells under restrictive conditions has been shown to contain a proportion of cells that are no longer cold-sensitive for resumption of proliferation from stationary phase (data not shown). Thus, genetically

suppressed cells can also be present in *gcs1-6* cell populations. The presence of these cold-resistant cells in a starting stationary-phase culture appears to be correlated with the length of time the cells had remained on solid medium at 23°C before being used to inoculate a liquid culture. This instability of the cold-sensitivity phenotype due to acquisition of suppressor mutations was previously observed for *gcs1-1 sed1-1* mutant cells (M. Drebot, personal communication).

For the budding yeast *Saccharomyces cerevisiae*, reentry into the mitotic cell cycle is coincident with the initiation of a bud on the mother cell (Johnston *et al.*, 1980). *gcs1-6* mutant cells remained greater than 90% unbudded for 60 h at the restrictive temperature (Fig. 18A), indicating that these cells are blocked for reentry into the mitotic cell cycle. In contrast, wild-type cells initiated buds after 9 h of incubation at 15°C, with half of the population budded by 12 h of incubation at 15°C (Fig. 18B).

For wild-type cells, resumption of proliferation from stationary phase coincides with loss of stationary-phase properties, including the ability to survive exposure to high temperatures (thermotolerance). Loss of thermotolerance by mutant cells would indicate that *gcs1-6* mutant cells respond to the presence of fresh medium despite being blocked for reentry into the mitotic cell cycle. Thermotolerance was therefore measured for stationary-phase *gcs1-6* mutant cells and wild-type cells after transfer to fresh medium and incubation at the restrictive temperature, by measuring cell viability before and after exposure to heat shock (Fig. 19). Loss of thermotolerance coincided with resumption of proliferation in wild-type cells (Fig. 19B). Stationary-phase mutant cells were thermotolerant; however, after 35 h of incubation at the restrictive temperature, 97% of mutant cells could no longer survive a 5-min heat shock at 52°C. Thus *gcs1-6* mutant cells respond to transfer to fresh medium by loss of thermotolerance, indicating that these cells lose stationary-phase properties despite being blocked in resumption of proliferation.

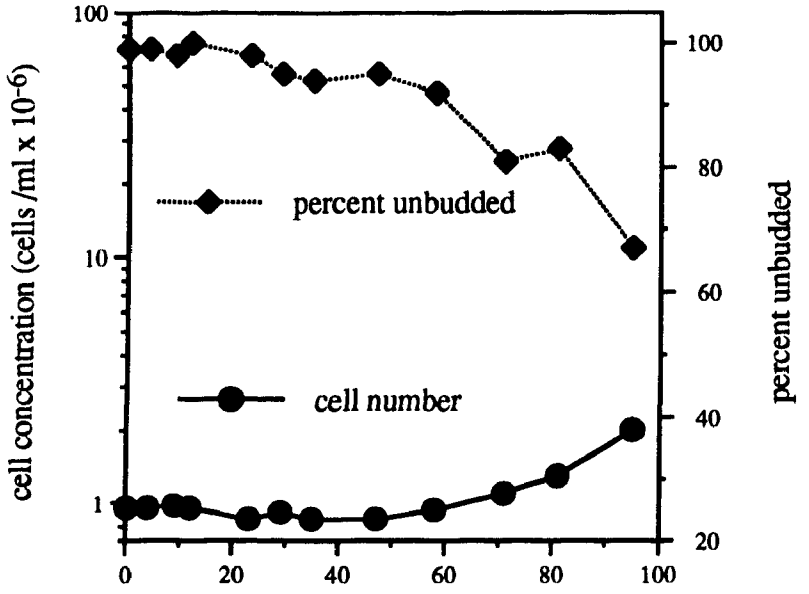
Figure 18. *gcs1-6* mutant cells are impaired for reentry into the cell cycle.

(A) Stationary-phase *gcs1-6* null cell cultures described in Fig. 17A were monitored for both cell concentration (filled circles) and percentage of unbudded cells (filled diamonds). At time 0, stationary-phase cells were diluted in fresh YEPD medium and incubated at 15°C. At intervals, samples were removed to determine cell concentration and percentage of unbudded cells. The cell concentration data is reproduced from Fig. 17A.

(B) Wild-type cell cultures described in Fig. 17A were monitored both for cell concentration (open circles) and percentage of unbudded cells (open diamonds). Stationary-phase cells were diluted in fresh YEPD medium at time 0 and incubated at 15°C. At intervals, samples were removed to determine cell concentration and percentage of unbudded cells. The cell concentration data (open circles) is reproduced from Fig. 17A.

Figure 18

A


100
10
1
cell concentration (cells /ml x 10-6)
percent unbudded
cell number
100
80
60
40
20
percent unbudded
0
20
40
60
80
100

B

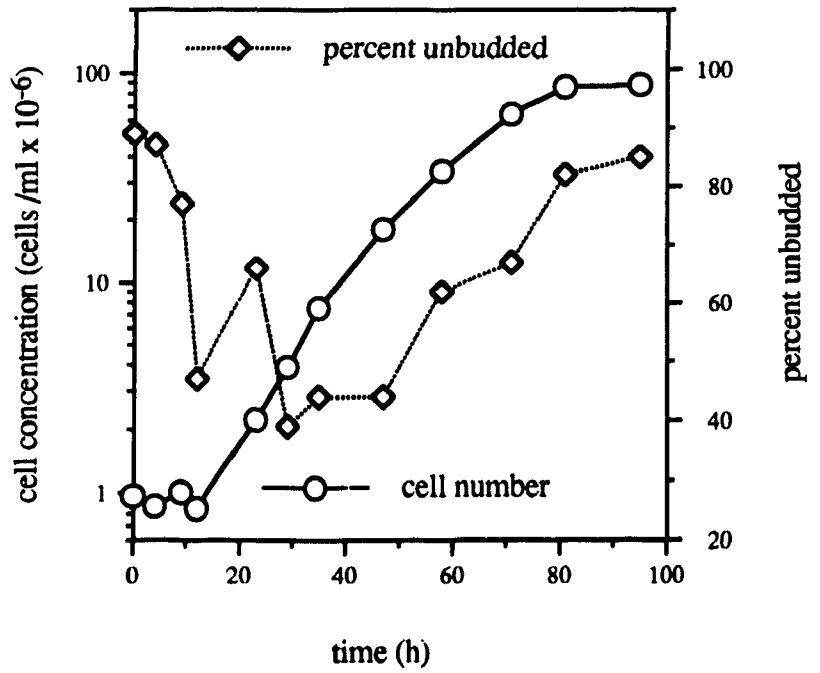

percent unbudded
100
10
1
cell concentration (cells /ml x 10-6)
cell number
100
80
60
40
20
percent unbudded
0
20
40
60
80
100
time (h)

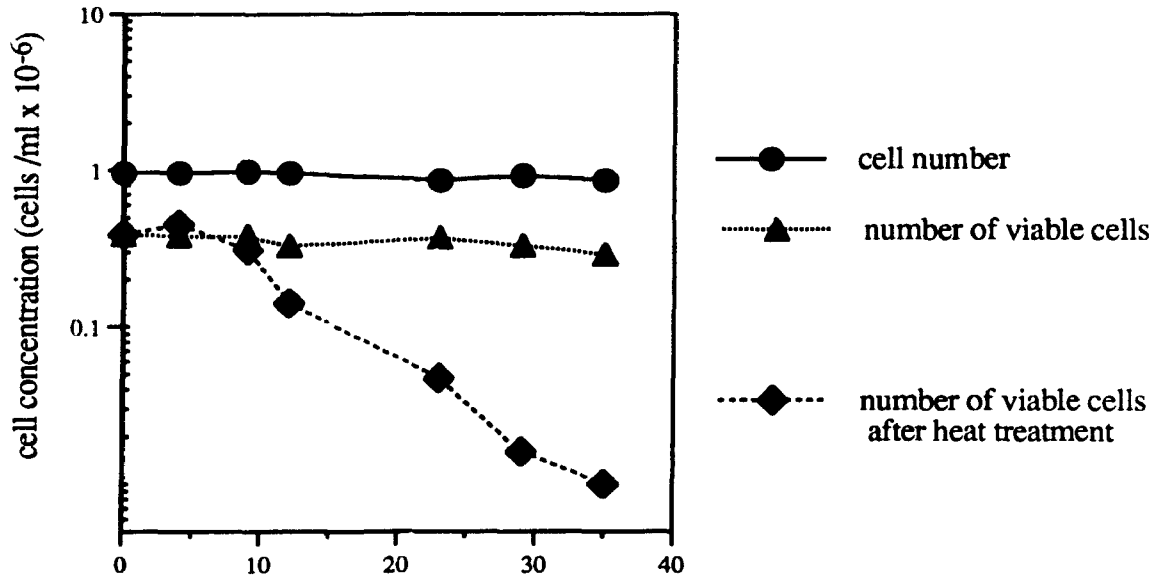
Figure 19. *gcs1-6* mutant cells respond to transfer to fresh medium by loss of thermotolerance.

(A) Stationary-phase *gcs1-6* null cell cultures described in Fig. 17A were monitored for cell concentration (closed circles), and for thermotolerance. Thermotolerance is shown as number of viable cells before (filled triangles) and after heat treatment (filled diamonds). To determine the number of viable cells before heat treatment, samples of cell culture were diluted in PBS, spread on YEPD solid medium, and incubated at 29°C (as described in Materials and Methods). The number of colonies formed at 29°C (averaged from at least two dilutions of the same culture sample) was multiplied by the dilution factor to determine the number of viable cells. For measurement of thermotolerance, samples of cell cultures were incubated at 55°C for 5 min (as described in Materials and Methods) and then assayed for number of viable cells (filled diamonds) as described above.

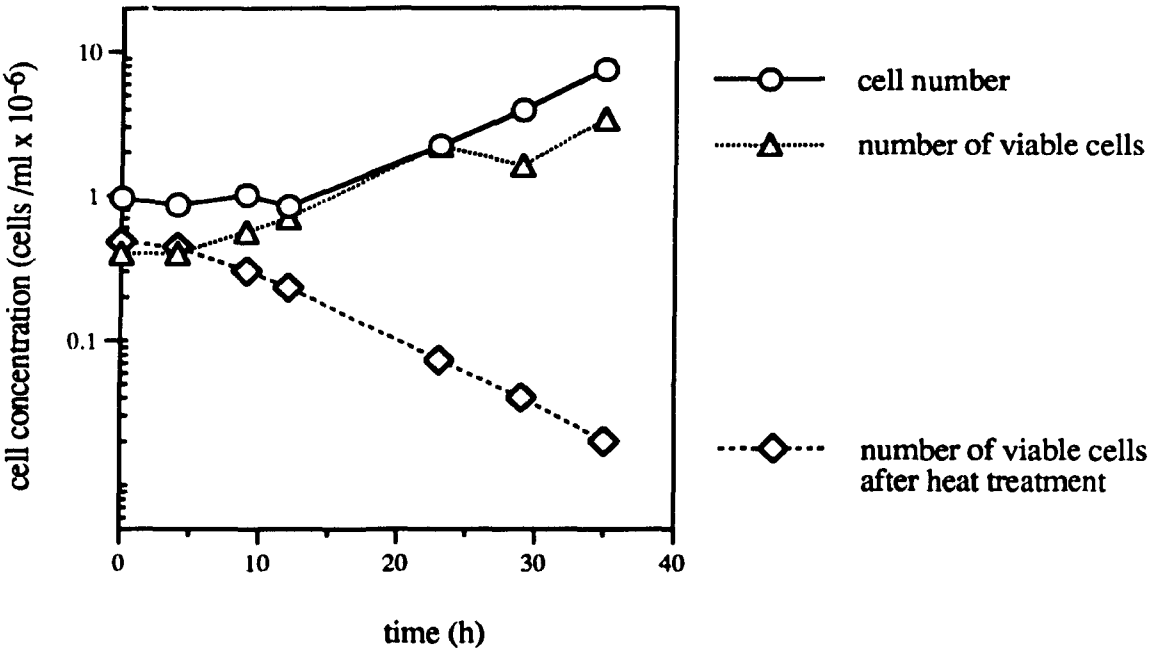
(B) Wild-type cell cultures described in Fig. 17A were monitored for cell concentration (open circles) and for thermotolerance as described above. Number of viable cells (open triangles) and number of viable cells after heat treatment (open diamonds) are plotted together with total cell number (reproduced from Fig. 17A).

Figure 19

A


cell concentration (cells /ml x 10-6)
10
1
0.1
0
10
20
30
40
cell number
number of viable cells
number of viable cells after heat treatment

B


cell concentration (cells /ml x 10-6)
10
1
0.1
0
10
20
30
40
time (h)
cell number
number of viable cells
number of viable cells after heat treatment

In the absence of heat shock, the viability of *gcs1-6* cells was not significantly affected by incubation under restrictive conditions. Stationary-phase *gcs1-6* cells transferred to fresh medium maintained viability (colony-forming ability at 29°C) in the absence of proliferation at 15°C; measurement of viable cell number (see Materials and Methods) showed that over 70% of *gcs1-6* cells were viable after 35 h of incubation at the restrictive temperature (Fig. 19A). This contrasts with previous results obtained with *gcs1-1 sed1-1* reentry-mutant cells, which indicated that these reentry-mutant cells rapidly lose viability under restrictive conditions (Drebot, 1987). Thus, the phenotype of *gcs1-6* null mutant cells demonstrates that loss of viability of stationary-phase cells under restrictive conditions is not involved in the reentry-mutant phenotype.

7. Mapping the 5' ends of *GCS1* transcripts by primer extension

Inspection of the *GCS1* upstream sequence revealed 3 short ORFs with predicted translation start sites (ATGs) at positions -39, -61 and -75 relative to the predicted translation start site for Gcs1 (Fig. 20). To determine if the *GCS1* transcript contains any of these upstream ORFs, the 5' ends of *GCS1* transcripts were mapped by primer extension, using two different primers (Fig. 20) and total RNA isolated from wild-type and *gcs1-6* null cells. The two primers gave identical sets of *GCS1*-specific primer-extension products (Fig. 21, lane 2, arrows b, c, d, e; Fig. 22, lane 2, arrows d, e, f, g), and mapped the 5' ends of the transcipts for the *GCS1* gene to positions -31, -27, -23 and -14 nucleotides upstream of the predicted translation start site (Fig. 20). Thus the leader sequences indicated for *GCS1* transcripts are relatively short compared to those of other yeast mRNAs (Cigan and Donaghue, 1987), and none of these *GCS1* transcripts includes even the proximal upstream ORF.

GCS1-specific transcripts from wild-type cells proliferating at 15°C were also identified from primer-extension experiments. The *GCS1*-specific transcripts were not

Figure 20. Mapping the 5' ends of *GCS1* transcripts

The DNA sequence of the *GCS1* gene is shown from the *Sau*3A cloning site 337 bp upstream of the presumptive ATG initiation codon to the end of the first 70 codons of the *GCS1* gene. Nucleotide numbering begins with the presumptive ATG codon specifying the initiator methionine which is also numbered as the first amino-acid residue. Amino-acid residues are numbered on the right, while nucleotide numbering is shown above the DNA sequence. The position of a 22-b palindrome is marked by the double-headed arrow. Primers 4A and 4B (underlined) were designed to hybridize to mRNA sequences upstream of the palindrome to avoid potential secondary structure artifacts with the primer-extension reactions. The boxed stars indicate the mapped positions of 5' ends of *GCS1* transcripts at -31, -27, -23 and -14 b upstream of the predicted initiator ATG codon. Potential ATG initiation codons for 3 short upstream ORFs at positions -39, -61, and -75, as well as the predicted ATG initiation codon of the *GCS1* gene are indicated (boxed ATGs).

Figure 20

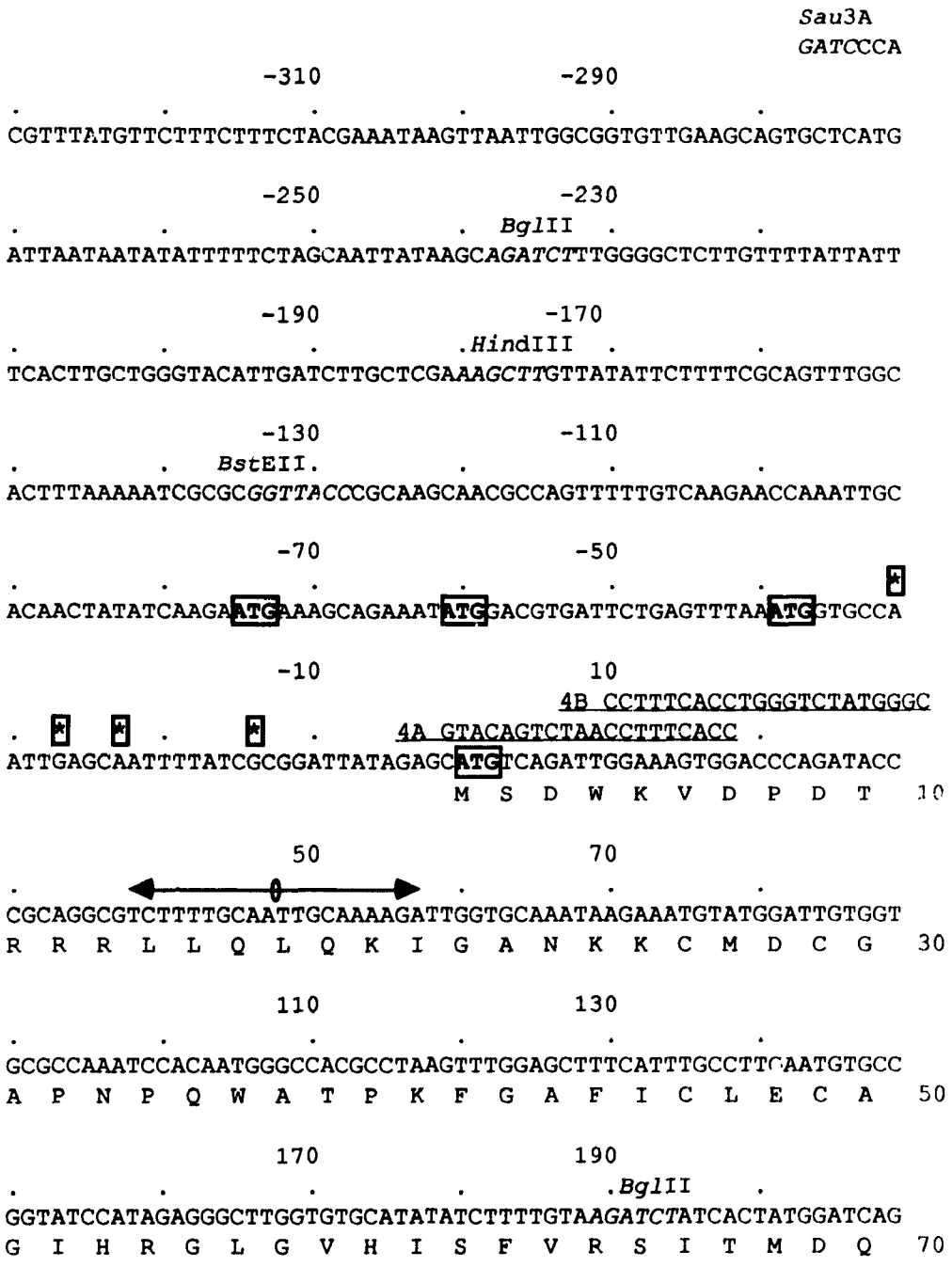

Sau3A
GATCCCA
-310
-290
CGTTTATGTTCTTTCTTTCTACGAAATAAGTTAATTGGCGGTGTTGAAGCAGTGCTCATG
-250
-230
BglII
ATTAATAATATATTTTTCTAGCAATTATAAGCAGATCTTTGGGGCTCTTGTTTTATTATT
-190
-170
HindIII
TCACTTGCTGGGTACATTGATCTTGCTCGAAAGCTTGTTATATTCTTTTCGCAGTTTGGC
-130
-110
BstEII
ACTTTAAAAATCGCGCGGGTTACCCGCAAGCAACGCCAGTTTTTGTCAAGAACCAAATTGC
-70
-50
ACAACTATATCAAGAATGAAAGCAGAAATATGGACGTGATTCTGAGTTTAAATGGTGCCA
-10
10
4B CCTTTCACCTGGGTCTATGGGC
4A GTACAGTCTAACCTTTCACC
ATTGAGCAATTTTATCGCGGATTATAGAGCATGTCAGATTGGAAAGTGGACCCAGATACC
M S D W K V D P D T 10
50
70
CGCAGGCGTCTTTTGCAATTGCAAAAGATTGGTGCAAATAAGAAATGTATGGATTGTGGT
R R R L L Q L Q K I G A N K K C M D C G 30
110
130
GCGCCAAATCCACAATGGGCCACGCCTAAGTTTGGAGCTTTCATTTGCCTTCAATGTGCC
A P N P Q W A T P K F G A F I C L E C A 50
170
190
BglII
GGTATCCATAGAGGGCTTGGTGTGCATATATCTTTTGTAAGATCTATCACTATGGATCAG
G I H R G L G V H I S F V R S I T M D Q 70

Figure 21. *GCS1*-specific primer-extension products

Primer-extension experiments were carried out as described in Materials and Methods using 40 μg of total RNA and primer 4B (see Fig. 20). Primer 4B was also used in a sequencing reaction with *GCS1* template for use in sizing the primer-extension products . Equal volumes of the primer-extension reactions were resolved by electrophoresis through an 8% polyacrylamide, 6 M urea sequencing gel. Lanes 1 and 8: sequencing ladder from sequencing reactions using primer 4B and *GCS1* template; T reaction (lane 1), C reaction (lane 8). Lanes 2, 4, 6: resolved primer-extension products from wild-type cells actively proliferating on glucose-based medium at 29°C (lane 2), galactose-based medium at 29°C (lane 4), or glucose-based medium at 15°C (lane 6). Lanes 3, 5, 7: resolved primer-extension products from *gcs1-6* null cells actively proliferating on glucose-based medium at 29°C (lane 3) or galactose-based medium at 29°C (lane 5). Resolved primer-extension products from stationary-phase non-proliferating *gcs1-6* null cells after 24 h incubation in glucose-based medium at 15°C are shown in lane 7. *GCS1*-specific products are indicated by arrows b, c, d, and e, and correspond to transcripts initiating at -31, -27,-23 and -14 respectively. Primer-extension products that are present in both wild-type and *gcs1-6* null lanes and thus cannot correspond to *GCS1*-specific transcripts are indicated by arrows a and f.

Figure 21

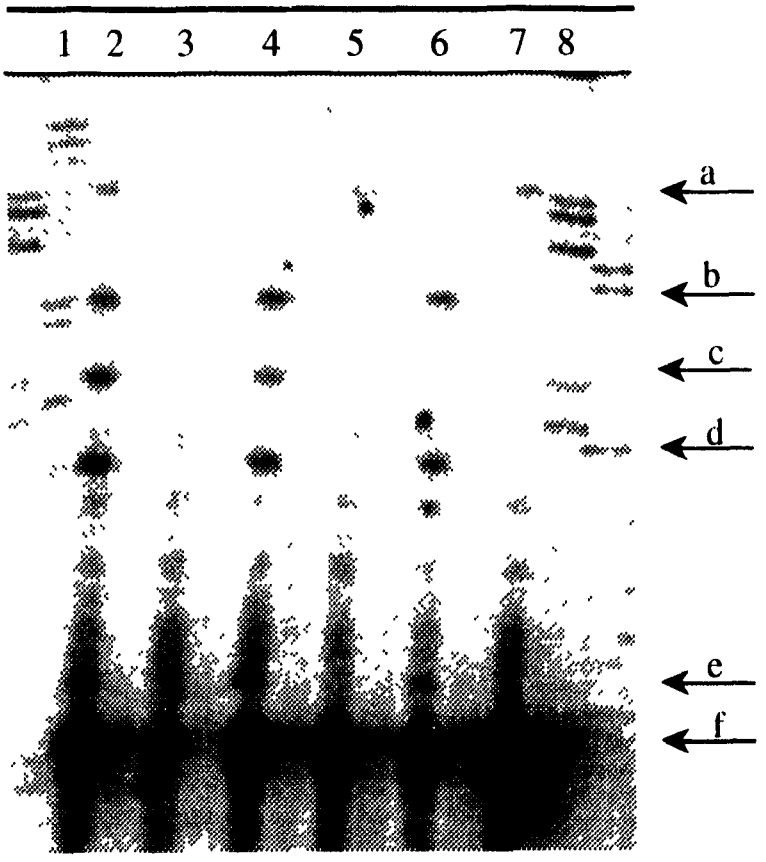


Figure 22. *GCS1*-specific and non-specific primer-extension products.

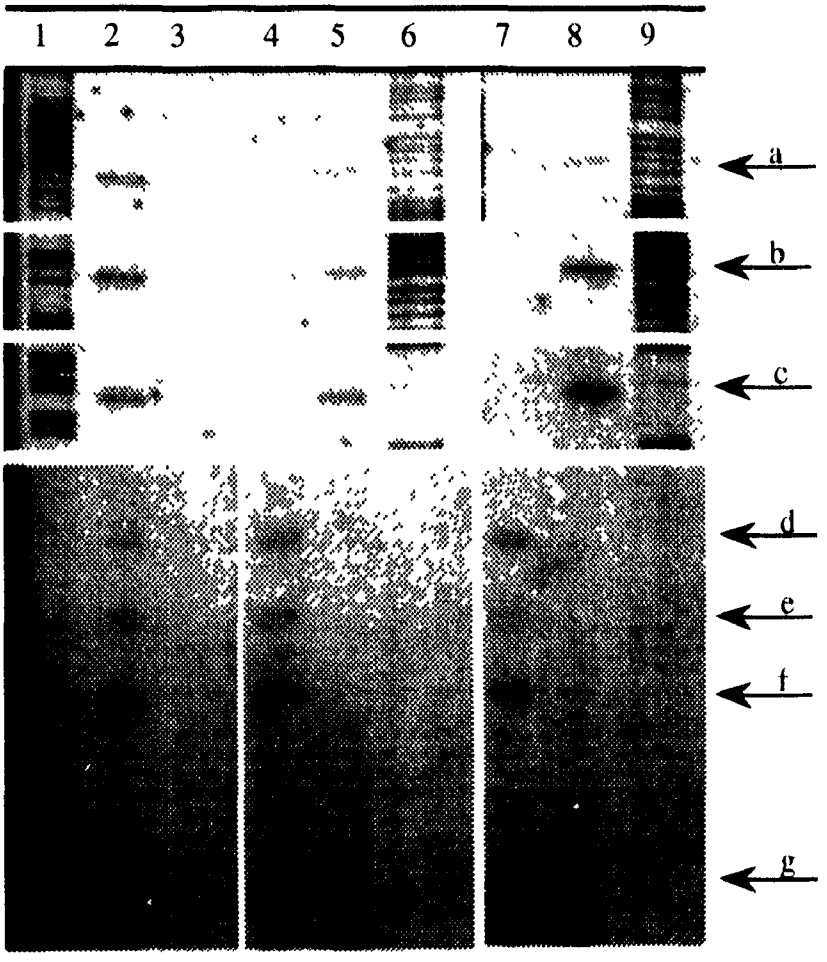
Primer extension reactions were performed as described in Materials and Methods using 40 μg of total RNA and primer 4A (see Fig. 20). Primer 4A was also used in a sequencing reaction using *GCS1* template to provide a sequencing ladder for sizing the primer extension products. Equal volumes of primer-extension reactions were resolved by electrophoresis through a 6% polyacrylamide, 6 M urea sequencing gel.

Lanes 1, 6, and 9: sequencing ladder from sequencing reaction using primer 4A and *GCS1* template; T reaction (lane 1), A reaction (lanes 6 and 9).

Lanes 2, 4, and 7: primer-extension products from wild-type cells. Experiments were performed using wild-type cells proliferating on glucose-based medium at 29°C (lane 2), galactose-based medium at 29°C (lane 4) or glucose-based medium at 15°C (lane7). Arrows d, e, f, and g correspond to *GCS1*-specific primer-extension products.

Lanes 3, 5, and 8: primer-extension products from *gcs1-6* null cells. Experiments were performed using RNA from *gcs1-6* null cells proliferating on glucose-based medium at 29°C (lane 3), galactose-based medium at 29°C (lane 5) or stationary-phase *gcs1-6* null cells after 24 h incubation at the restrictive temperature of 15°C (lane 8). Arrows a, b, and c correspond to non-*GCS1* primer-extension products.

Figure 22



altered in pattern or relative abundance by growth at 15°C (Fig. 21, compare lanes 2 and 6). Finally, primer-extension experiments were performed using on total RNA isolated from wild-type cells and *gcs1-6* null cells proliferating at 29°C on galactose-based growth medium. The *GCS1* transcripts were not altered in pattern or abundance by growth on YM-1 + galactose medium at 29°C (Fig. 21, compare lanes 2 and 4). In summary, the same four low-abundance *GCS1* transcripts were observed during growth on glucose at 15°C and at 29°C, and during growth on the alternative carbon source galactose at 29°C.

8. *GCS1* affects carbon-source and temperature-dependent gene expression

In the course of the primer-extension experiments described above, *GCS1*-dependent effects on gene expression were unexpectedly observed. These effects were discovered because of the fortuitous cross-hybridization of the *GCS1* primers to non-*GCS1* RNAs. Non-*GCS1* RNAs were identified in primer-extension experiments using total RNA from *gcs1-6* null cells. Any primer-extension products obtained using RNA from *gcs1-6* null cells must have resulted from primer hybridization to transcripts other than those from *GCS1*. Consistent with this conclusion was the finding that the non-*GCS1* primer-extension products did not correspond to the sizes of the *GCS1* DNA fragments in the sequencing ladder (Fig. 21, arrows a and f), as expected for extension products that do not correspond to *GCS1* transcripts, whereas the sizes of the *GCS1*-specific products (Fig. 21, arrows b, c, d, and e) correspond exactly to the sequencing ladder (prepared from *GCS1* templates). Different sets of non-*GCS1* primer-extension products (differing both in size and abundance) were obtained from two different *GCS1* primers (Fig. 21, arrows a and f; Fig. 22 arrows a, b, and c; Fig. 23A). Therefore these primers clearly identify non-*GCS1* mRNAs.

Figure 23. Primer-extension products not derived from *GCS1* transcripts.

(A) Non-*GCS1* primer-extension product from primer 4B (arrow a). Primer-extension reactions were performed as described in Fig. 21.

Lanes 1 and 8: sequencing ladder from sequencing reactions using primer 4B and *GCS1* template; T reaction (lane 1), C reaction (lane 8).

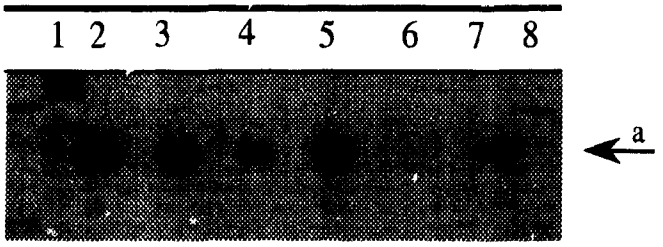
Lanes 2, 4, and 6: primer-extension products from wild-type cells. Experiments were performed using RNA from wild-type cells proliferating on glucose-based medium at 29°C (lane 2), galactose-based medium at 29°C (lane 4) or glucose-based medium at 15°C (lane 6).

Lanes 3, 5, and 7: primer-extension products from *gcs1-6* null cells. Experiments were performed using RNA from *gcs1-6* null cells proliferating on glucose-based medium at 29°C (lane 3), galactose-based medium at 29°C (lane 5), or using RNA from stationary-phase *gcs1-6* null cells after 24 h of incubation at the restrictive temperature of 15°C (lane 7).

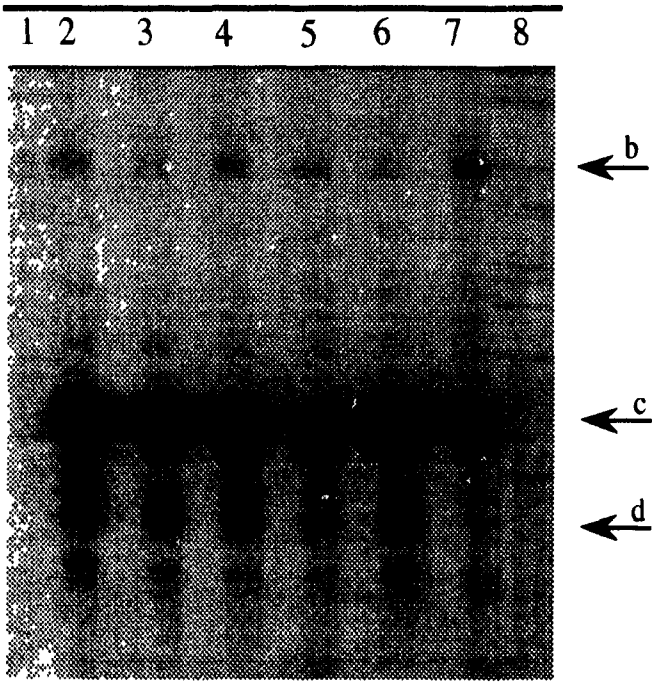
(B) Primer-extension experiments were performed as described above except that primer rp4-1 (5' GTCAATGGGTTCTTAGCCTTG 3') designed to hybridize to transcripts for ribosomal protein L4-1 (Arevalo and Warner, 1990) was used instead of primer 4B. The transcription start sites for L4-1 have been mapped (Yon *et al.*, 1991) and two primer-extension products (indicated by arrows c and d) corresponding to the major ribosomal L4-1 transcripts are indicated. A primer-extension product that is unlikely to be derived from a ribosomal L4-1 transcript is indicated by arrow b.

Figure 23

A



B



With one primer, three non-*GCS1* primer-extension products of 370, 228, and 117 nucleotides were observed (Fig. 22, arrows a, b, c), in addition to the *GCS1*-specific products of 51, 47, 44 and 34 nucleotides (Fig. 22, arrows d, e, f, g), in primer-extension reactions using total RNA isolated from wild-type cells growing on glucose at 29°C. However, none of these non-*GCS1* primer-extension products were observed using RNA from the same cells growing on galactose as the carbon source (Fig. 22, lane 4, arrows a, b, c). Thus, these non-*GCS1* transcripts are regulated by carbon source in wild-type cells.

Remarkably, this carbon-source regulation of these non-*GCS1* transcripts was reversed in *gcs1-6* null cells. In *gcs1-6* null cells growing at 29°C the three non-*GCS1* primer-extension products of 370, 228 and 117 nucleotides were present in primer-extension reactions using RNA from cells proliferating with galactose as the carbon source (Fig. 22, lane 5, arrows a, b, c) but were not observed using RNA from *gcs1-6* null cells proliferating on glucose at 29°C (Fig. 22, lane 3, arrows a, b, c). These differences in the patterns of primer-extension products obtained using RNA from wild-type and *gcs1* null cells suggest that the abundance of non-*GCS1* transcripts is altered in *gcs1* null cells. Since these altered transcript levels were observed in *gcs1-6* null cells proliferating at the permissive temperature, the lack of Gcs1 function may affect gene expression during ongoing cell proliferation. Thus Gcs1 may be required for wild-type gene expression during ongoing cell proliferation at the permissive temperature.

In this analysis, the *GCS1* transcripts served as an internal control for identification of RNA from wild-type cells, thus precluding any misidentification of wild-type and *gcs1* null samples.

In addition to regulation of non-*GCS1* primer-extension products by carbon source, a similar regulation of non-*GCS1* extension products by temperature was observed, and this temperature-dependent regulation was also reversed in *gcs1-6* null

cells. The three non-*GCS1* primer- extension products of 370, 228 and 117 nucleotides that were observed using RNA from wild-type cells proliferating on glucose at 29°C (Fig. 22; lane 2, arrows a, b, c) were not observed using RNA from wild-type cells proliferating at 15°C (Fig. 22, lane 7; arrows a, b, c). This temperature dependence was reversed in *gcs1-6* null-mutant cells. The non-*GCS1* primer-extension products were present in RNA from *gcs1-6* null cells at 15°C (Fig. 22; lane 8, arrows a, b, c), and absent in RNA from *gcs1-6* null cells proliferating at 29°C (Fig. 22; lane 3, arrows a, b, c). The putative transcripts that correspond to these non-*GCS1* primer-extension products are therefore cold-repressible in wild-type cells, but are cold-inducible in *gcs1-6* null cells. Thus, these temperature-regulated transcripts are positively regulated by Gcs1 when cells are proliferating at 29°C and are repressed by Gcs1 at 15°C. This pattern of altered gene expression in *gcs1* mutant cells at 15°C may be related to the phenotype of cold-sensitivity observed in *gcs1* mutant cells. A search of current databases in an effort to identify transcripts that might cross-hybridize to the primers used in the primer-extension reactions did not reveal any RNAs with untranslated leader sequences of the right sizes, suggesting that the particular transcripts that cross-hybridized to these primers have not been sequenced.

Temperature-dependent patterns of gene expression (as represented by different patterns of non-*GCS1* primer-extension products) were observed using three different primers in primer-extension experiments with total RNA isolated from proliferating wild-type cells . Non-*GCS1* primer-extension products hybridizing to primer 4B (Fig. 21, lanes 2 and 6, arrow a; Fig. 23A; lanes 2 and 6, arrow a) were present in wild-type cells proliferating at 29°C, but were absent in wild-type cells proliferating at 15°C. This temperature-dependent regulation was altered in *gcs1-6* null cells, such that these non-*GCS1* primer-extension products were present in *gcs1-6* null cells incubated at 15°C (Fig. 21, lane 7, arrow a; Fig. 23, lane 7, arrow a). This pattern of increased

abundance of non-*GCS1* primer-extension products from RNA isolated from *gcs1-6* null cells incubated at 15°C (compared to wild-type cells incubated at 15°C) was not observed for primer-extension products specific to the transcript for ribosomal protein L4-1 (Fig. 23B , lanes 6 and 7, arrows c and d). The abundance of products corresponding to ribosomal protein L4-1 transcripts are slightly higher in wild-type cells proliferating at 15°C (Fig. 23B, lane 6, arrows c and d) than in *gcs1-6* null cells incubated under restrictive conditions (Fig. 23B, lane 7, arrows c and d). However, non-L4-1 primer-extension products were also detected that were decreased in abundance with RNA from wild-type cells proliferating at 15°C, relative to RNA from *gcs1-6* null cells incubated at 15°C under restrictive conditions (Fig 23B, lanes 6 and 7, arrow b).

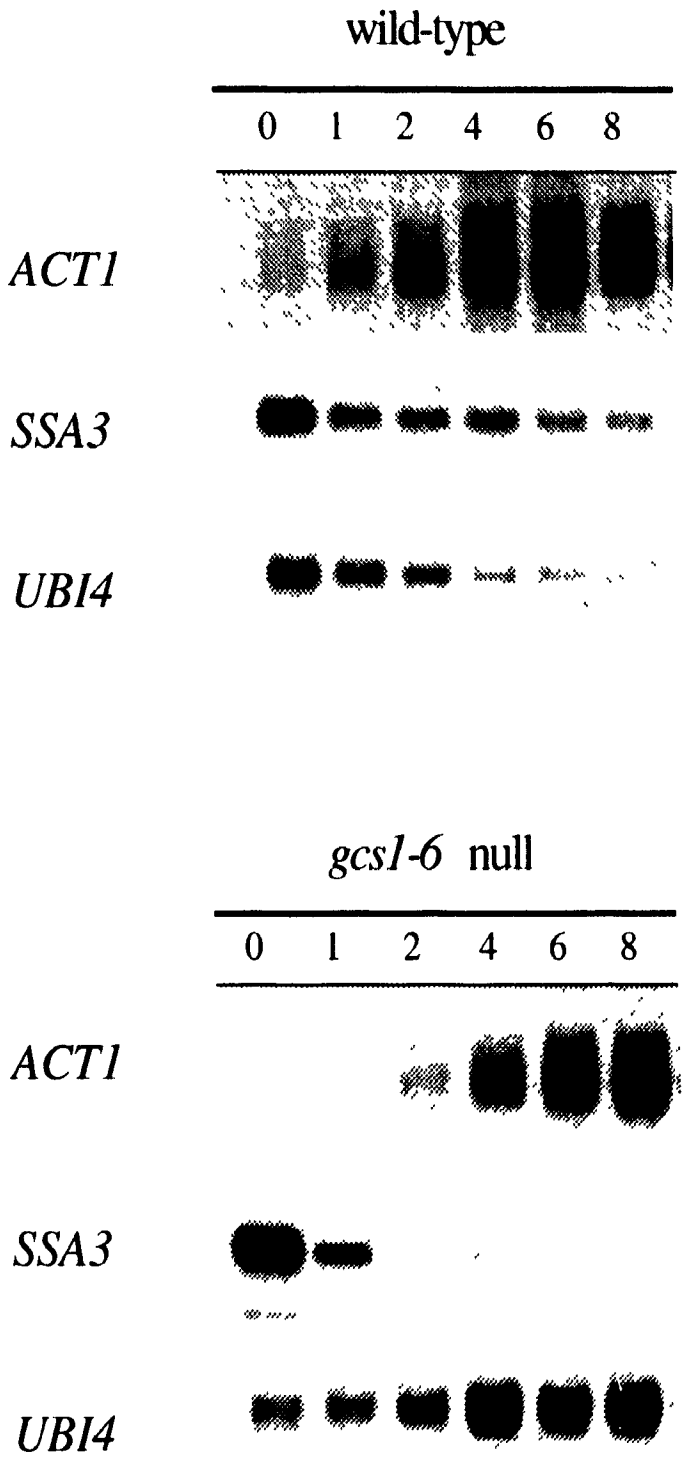
9. Differential gene expression in wild-type and *gcs1* null cells

A preliminary characterization of transcript abundance for mRNAs known to be abundant either in stationary-phase cells or in proliferating cells did not identify any transcripts that showed the regulation evident in the primer-extension analysis. However, differences in gene expression for certain transcripts were observed between wild-type and *gcs1-6* null cells. RNA isolated from wild-type and *gcs1-6* null stationary-phase cells after transfer to fresh medium and incubation at 15°C, was electrophoretically resolved on agarose gels and transferred to nylon membranes. These RNA blots were probed with radiolabelled DNA corresponding to two transcripts abundant in stationary-phase cells, those from *SSA3* and *UBI4*, and one transcript abundant in proliferating cells, from *ACT1*. The *ACT1* mRNA increased in abundance in *gcs1* mutant cells after transfer of stationary-phase cells to fresh medium, much like in wild-type cells (Fig. 24). Likewise, in both wild-type cells and mutant cells the *SSA3*

Figure 24. *UBI4* transcript levels are altered in *gcs1-6* mutant cells.

RNA isolated from wild-type or *gcs1-6* stationary-phase cells (lane 0), and 1, 2, 4, 6, and 8 h after transfer of stationary-phase cells to fresh medium and incubation at 15°C (lanes 1, 2, 4, 6, and 8) was electrophoretically resolved on agarose gels and transferred to nylon membranes. RNA blots were probed separately with radiolabelled restriction fragments from three different genes: *ACT1*, *SSA3* and *UBI4*. The *ACT1* probe was derived from plasmid pRS208 (provided by R. Storms) by purification of a 1-kbp *Hin*dIII/*Xho*I fragment. The *SSA3* probe was derived from plasmid pUC9-SSA3 by purification of a 750-bp RsaI fragment. The *UBI4* probe was derived from pUB200 (provided by D. Finley) by purification of a ~2.3-kbp EcoRI fragment.

Figure 24


wild-type
0 1 2 4 6 8
ACT1
SSA3
UBI4
gcs1-6 null
0 1 2 4 6 8
ACT1
SSA3
UBI4

transcript, normally abundant in stationary-phase cells (Werner-Washborne *et al.*, 1989; Boorstein and Craig, 1990), rapidly decreased in abundance upon transfer of stationary-phase cells to fresh medium (Fig. 24). These results indicate by yet another criterion that *gcs1* mutant cells, like wild-type cells, exit stationary phase upon incubation to fresh medium at the restrictive temperature.

UBI4 mRNA levels are normally high in stationary-phase cells, where Ubi4 supplies the ubiquitin required for ubiquitin-dependent protein degradation; levels of *UBI4* mRNA normally decline rapidly upon reentry of cells into the mitotic cell cycle (Finley *et al.*, 1987). As expected, the *UBI4* transcript decreased in abundance in wild-type cells upon transfer of stationary-phase cells to fresh medium. In *gcs1-6* null cells, however, the abundance of the *UBI4* transcript steadily increased (Fig. 24). This result is consistent with previous results showing that *gcs1-1 sed1-1* reentry-mutant cells increased expression of *UBI4* after incubation of stationary-phase cells in fresh medium at the restrictive temperature (Drebot, 1987).

UBI4 expression is normally increased in response to a requirement for increased protein degradation. For example, cells increase expression of *UBI4* in response to abnormal proteins generated by stress conditions such as heat shock or exposure to amino-acid analogues (Finley *et al.*, 1987). Thus, increased levels of *UBI4* mRNA in stationary-phase *gcs1* mutant cells attempting to resume proliferation may reflect increased levels of protein degradation in these cells.

10. Molecular construction of *gcs1-1* mutant strains

The cloned *gcs1-1* mutant allele allowed construction of *gcs1-1* mutant cells by molecular techniques. For this construction, *gcs1-6* null cells were transformed with the cloned mutant *gcs1-1* gene on a centromeric (low-copy) plasmid, and the phenotype of these cells was determined. The growth kinetics of *gcs1-6* null cells containing a

plasmid-borne *gcs1-1* mutant allele were compared to the growth kinetics of *gcs1-6* null cells transformed with the vector alone (Fig. 25A). Cells proliferating at the permissive temperature of 29°C were transferred to 15°C and growth kinetics were followed until the cells entered stationary phase and ceased proliferation. Comparison of the *gcs1-6* null cells transformed with the *gcs1-1* mutant allele to the *gcs1-6* null cells transformed with vector alone revealed no significant differences in rates of proliferation. That is, proliferating *gcs1-6* null cells transformed with the plasmid-borne *gcs1-1* mutant allele or with vector alone continued to proliferate after transfer to the restrictive temperature. The *gcs1-6* null cells also showed the same reentry-mutant phenotype when transformed with the plasmid-borne *gcs1-1* mutant allele: cells of the *gcs1-1* strain constructed by molecular techniques (with the W303 genetic background) remained cold-sensitive for resumption of proliferation from stationary phase (data not shown). This result shows that the phenotype conferred by the *gcs1-6* null allele is unaffected by the presence of mutant Gcs1 protein, and suggests that the mutant Gcs1 protein may be completely nonfunctional.

The cold-sensitivity phenotype of cells carrying the *gcs1-1* mutant allele constructed by molecular means (Fig. 25A) differs from the phenotype conferred by introduction of the *gcs1-1* mutant allele into the same W303 genetic background by a series of genetic crosses. Cells containing the genetically introduced *gcs1-1* mutant allele were cold-sensitive regardless of growth status (Fig. 25B), like *gcs1-1* mutant cells in the GR2 genetic background (Drebot, 1987). This more severe cold-sensitivity phenotype caused when the *gcs1-1* mutant allele was introduced genetically must be due to the interaction of the *gcs1-1* allele and alleles at additional loci that increase the severity of the cold-sensitivity phenotype from cold-sensitive only for resumption of proliferation to cold-sensitive regardless of growth status. Since the reentry-mutant *gcs1-1* strain characterized above (W303 genetic background) was constructed by

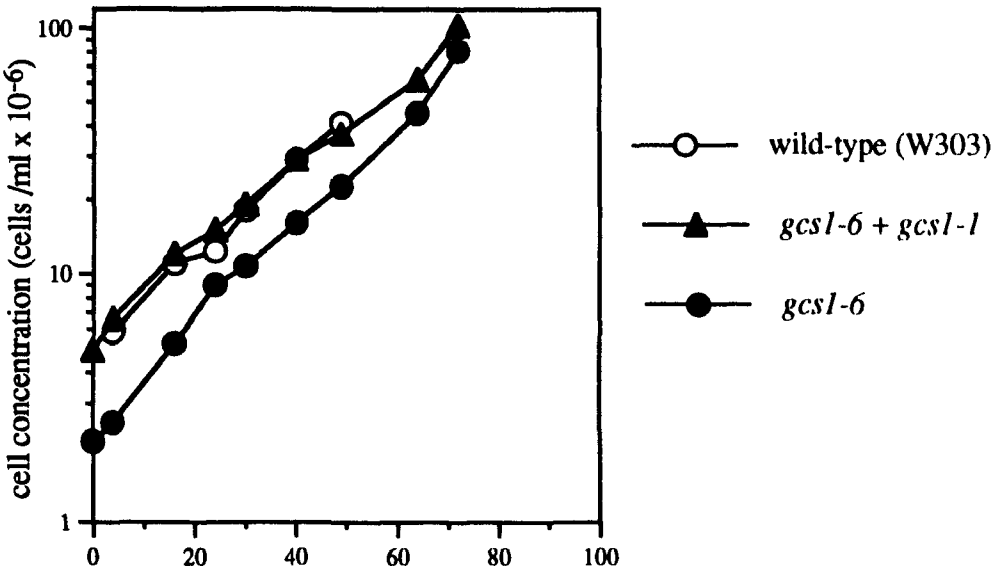
Figure 25. Growth kinetics of *gcs1-6* mutant cells containing a plasmid-borne *gcs1-1* mutant allele.

(A) Actively proliferating wild-type (open circles) and *gcs1* mutant cells (filled symbols) were transferred from 29°C to 15°C at time 0, and then incubated further at 15°C. *gcs1-6* null cells (strain GWK-8A), transformed with the plasmid-borne *gcs1-1* mutant allele (filled triangles), or with the same plasmid without insert (filled circles), were assayed for the ability to maintain ongoing proliferation at 15°C in YM1 selective medium. Selective medium (YM1-histidine) was used to maintain the plasmid-borne *gcs1-1* mutant allele carried in the *HIS3* vector, pRS313. Both wild-type (strain W303) and *gcs1-6* null cells were also transformed to histidine prototrophy with pRS313 (without insert) for direct comparison of the growth kinetics of all three strains in the same selective medium.

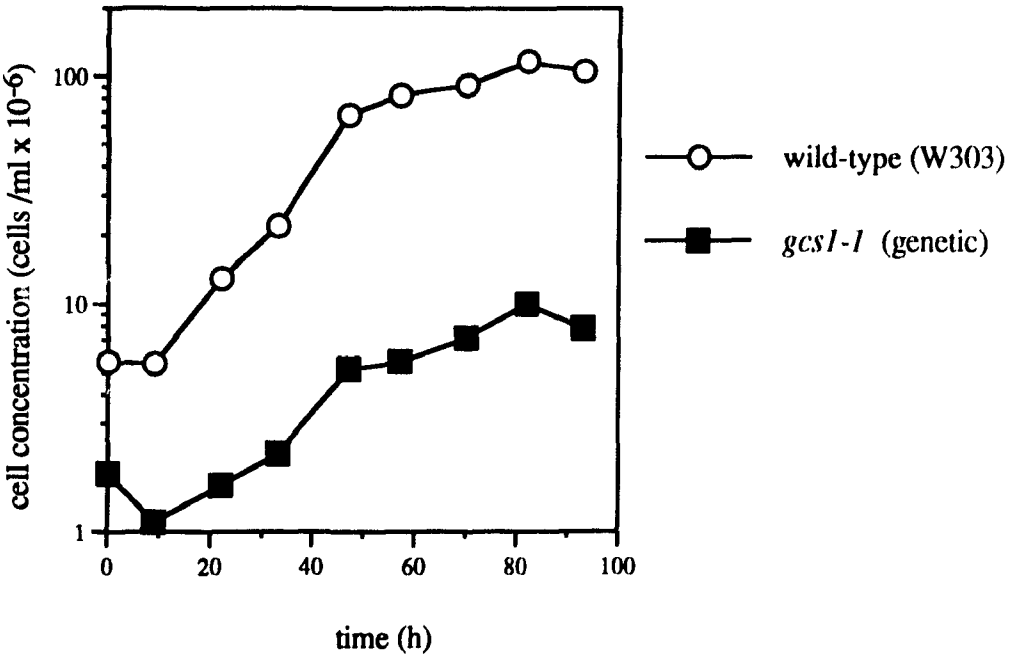
(B) Actively proliferating wild-type (open circles) and *gcs1-1* mutant cells (filled squares) were transferred from 29°C to 15°C at time 0 and incubated further at 15°C. At intervals, samples were removed for determination of cell concentration. These *gcs1-1* mutant cells (strain MDgcs1-3X) were constructed by introduction of the *gcs1-1* mutant allele (from strain MD-025-5) into the W303 genetic background by 3 successive backcrosses.

Figure 25

A


cell concentration (cells /ml x 10-6)
100
10
1
0
20
40
60
80
100
wild-type (W303)
gcs1-6 + gcs1-1
gcs1-6

B


cell concentration (cells /ml x 10-6)
100
10
1
0
20
40
60
80
100
time (h)
wild-type (W303)
gcs1-1 (genetic)

known molecular modifications, the difference in the phenotype conferred by the genetically introduced *gcs1-1* allele must reflect genetic differences that were introduced along with the *gcs1-1* allele, perhaps originating from the IIIXD strain from which the *gcs1-1* allele was isolated. These modifier alleles must be linked to *gcs1-1*, since the genetically constructed strain was constructed by four successive backcrosses. The probability that an unlinked gene would segregate with the mutant *gcs1* allele in four successive backcrosses is 1 in 16. Thus, there is a greater than 93% probability that the modifer alleles are linked to the *gcs1-1* mutant allele. The effect of linked modifier alleles on the phenotype of *gcs1-1* mutant cells shows that the phenotype of cells lacking Gcs1 function may be determined by the presence of other alleles present in the genetic background.

11. Effect of genetic background on the phenotype of *gcs1* mutant cells

As shown above, truncation or deletion of the *GCS1* gene in the W303 genetic background produces cells with a reentry-mutant phenotype. That is, cells containing the *gcs1-2* or *gcs1-3* truncation allele, or the *gcs1-6* null allele, were blocked for resumption of cell proliferation from stationary phase at the restrictive temperature. However, proliferating *gcs1-2*, *gcs1-3* or *gcs1-6* mutant cells continued to proliferate after transfer to the restrictive temperature, with doubling times that were similar to that of wild-type cells. This result contrasts with previous studies of strains with the GR2 genetic background that indicated that the reentry-mutant phenotype necessarily resulted from the combination of two mutant alleles, *gcs1-1* and *sed1-1*, and that cells containing only the *gcs1-1* mutant allele were unable to maintain ongoing proliferation at the restrictive temperature. The genetically constructed *gcs1-1* mutant alleles contain alleles at linked genetic loci that increase the severity of the cold-sensitivity phenotype of *gcs1-1* mutant cells with the W303 genetic background. Thus it is likely that the

phenotype of cold sensitivity regardless of growth status observed for *gcs1-1* mutant cells with the GR2 genetic background is also due to the presence of these linked modifier alleles. The reentry-mutant phenotype of *gcs1-1 sed1-1* mutants cells with the GR2 genetic background, may be due to suppression of the effects of the linked modifier allele by *sed1-1*.

The question of whether Gcs1 is required for maintenance of ongoing cell proliferation in other wild-type strains was not addressed in strain GR2, since strain GR2 lacked convenient markers for the molecular construction of *gcs1-6* null-mutant cells. Instead, an analysis was made of the phenotype of *gcs1-6* mutant cells constructed by disruption of the wild-type *GCS1* allele in the 21R genetic background. This analysis showed that strain 21R, unlike strain W303, requires Gcs1 for maintenance of ongoing cell proliferation. Furthermore, *gcs1-6* null-mutant cells and genetically constructed *gcs1-1* mutant cells have the same phenotype with respect to cold sensitivity; no effect of linked modifer alleles was observed in the 21R genetic background. Therefore the linked modifer alleles discussed above may represent certain alleles at polymorphic loci which are normally present in strain 21R, and may also be present in strain GR2.

11A. *gcs1-1* and *gcs1-6* mutant cells with the 21R genetic background. The *gcs1-6* mutant allele was constructed in strain 21R to determine the phenotype of *gcs1-6* null-mutant cells in the 21R genetic background. Haploid 21R wild-type cells were transformed with the *gcs1-6* disruption construct, linearized pBNΔ4 (Fig. 16A; Table 2), and transformants that acquired uracil prototrophy by integration of the *URA3* vector marker were assayed for loss of *GCS1* sequences by Southern blotting (Fig. 16B). The *gcs1-6* null cells with the 21R genetic background were then assayed for cold sensitivity. Ura^+ *gcs1-6* null cells were cold-sensitive when assayed by replica-plating

to solid medium and incubation at 15°C. To determine the phenotype of cold sensitivity with respect to growth phase, the growth kinetics of these *gcs1-6* null cells were determined in liquid culture assays. Stationary-phase *gcs1-6* null cells with the 21R genetic background were unable to proliferate after transfer to fresh medium and incubation at 15°C (Fig. 26A). However, approximately 40% of these *gcs1-6* mutant cells could reenter the cell cycle as determined by measurement of budding index (measured as percent unbudded cells) (Fig. 26B). Analysis of these *gcs1-6* null cells by DAPI staining of cell nuclei after incubation of stationary-phase cells in fresh medium at the restrictive temperature indicated that in addition to bud initiation, nuclear migration and medial nuclear division could also be observed (data not shown). Thus, reentry into the mitotic cell cycle by the criteria of bud initiation, nuclear migration, and nuclear division does not necessarily lead to resumption of cell proliferation from stationary phase.

gcs1-6 null cells with the 21R genetic background may not be blocked in reentry into the mitotic cell cycle from stationary phase, but may instead be blocked in continued proliferation following reentry into the mitotic cell cycle. Consistent with this hypothesis, proliferating *gcs1-6* null-mutant cells were impaired in maintenance of ongoing proliferation after transfer to the restrictive temperature (Fig. 26C). At 14°C the doubling time of 21R wild-type cells was 8 h, while the continued proliferation of *gcs1-6* mutant cells was severely impaired (approx. 50 h doubling time) (Fig. 26C). Thus, Gcs1 is critical for maintenance of ongoing cell proliferation at low temperatures in strain 21R.

The growth kinetics of *gcs1-6* mutant cells with strain 21R genetic background were also determined at 15°C for direct comparison to the growth kinetics (also determined at 15°C) of *gcs1-6* mutant cells with strain W303 genetic background. At 15°C, the doubling time of 21R wild-type cells was 5 h, while the doubling time of

Figure 26. Growth kinetics of *gcs1-6* mutant cells with the 21R genetic background.

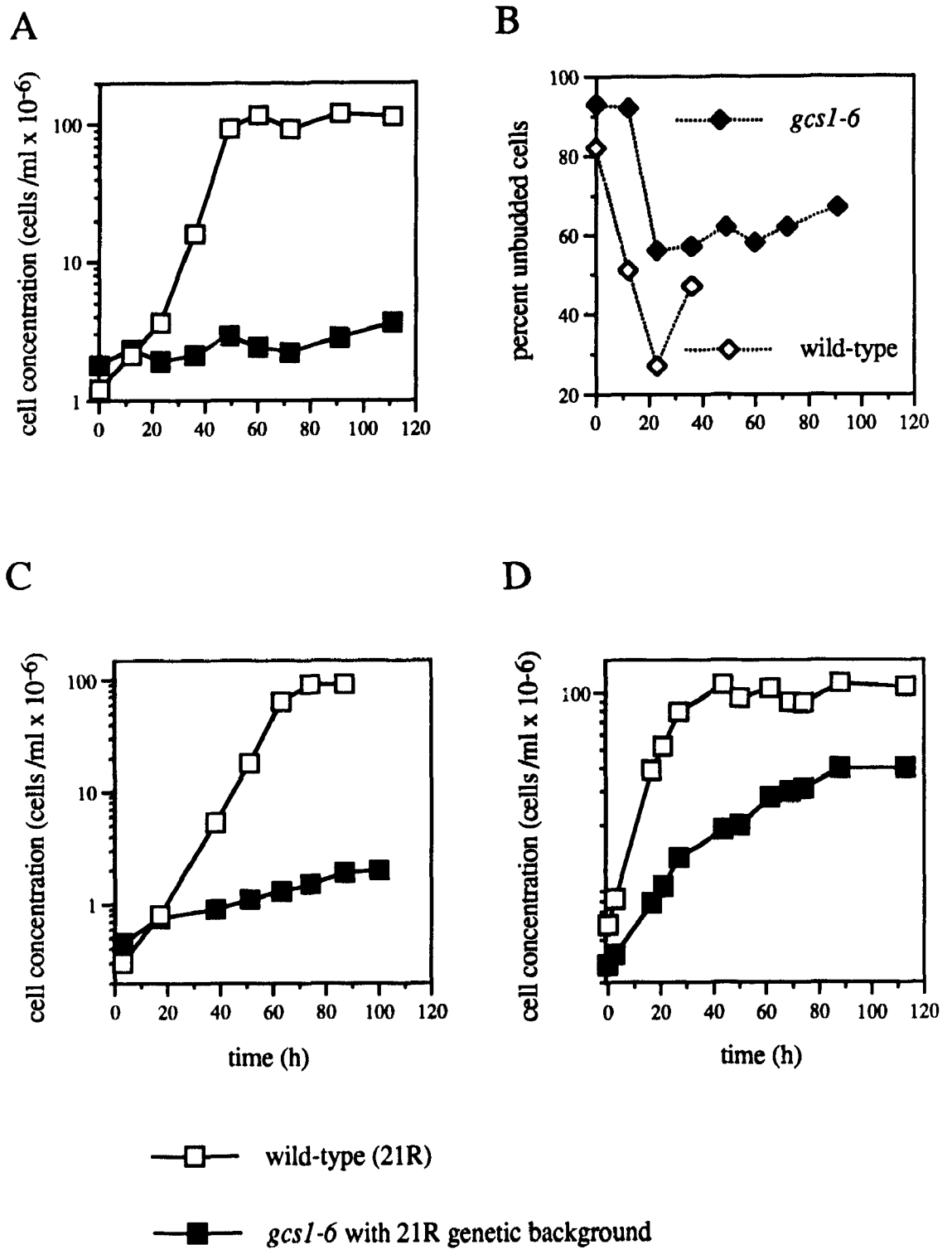
(A) Stationary-phase wild-type cells of strain 21R (open squares) and *gcs1-6* mutant cells with the 21R genetic background (filled squares) were diluted into fresh YEPD medium and incubated at 15°C. At intervals samples were removed to determine cell concentration.

(B) Wild-type and *gcs1-6* mutant cell cultures described in Fig. 27A were also monitored for percentage of unbudded cells as described in Materials and Methods.

(C) Actively proliferating wild-type (open squares) and *gcs1-6* mutant cells (filled squares) were transferred from 29°C to 14°C at time 0, and then incubated further at 14°C. At intervals samples were removed to determine cell concentration.

(D) Actively proliferating wild-type (open squares) and *gcs1-6* mutant cells (filled squares) were transferred from 29°C to 15°C at time 0, and then incubated further at 15°C. At intervals samples were removed to determine cell concentration.

Figure 26


A
B
C
D
cell concentration (cells /ml x 10-6)
percent unbudded cells
gcs1-6
wild-type
time (h)
wild-type (21R)
gcs1-6 with 21R genetic background

gcs1-6 mutant cells was increased 4-fold to 20 h (Fig. 26D). By comparison, the doubling time of W303 wild-type cells was 11 h. Thus strain W303 itself is clearly cold-sensitive in comparison to strain 21R (cells of strain W303 are unable to proliferate at 14°C). However, the absence of Gcs1 is clearly more deleterious to growth at low temperatures for the cold-resistant strain 21R than for the relatively cold-sensitive strain W303: the doubling time of isogenic *gcs1-6* mutant cells was approximately the same as that for wild-type W303 cells. Thus the phenotype of *gcs1* mutants is modified by polymorphisms that depend upon the genetic background.

The cold-sensitivity phenotype of the genetically transferred *gcs1-1* allele in the 21R genetic background was found to be similar to that of the *gcs1-6* null-mutant allele in the same 21R genetic background. The growth kinetics of cells containing the *gcs1-1* allele in the 21R genetic background were determined in liquid culture assays. Stationary-phase cells of strain MD-025-5 (Table 1), containing the *gcs1-1* mutant allele, were unable to resume proliferation after transfer to fresh medium and incubation at the restrictive temperature (Fig. 27A). Nevertheless, half of these cells reentered the mitotic cell cycle as determined by measurement of budding index (Fig. 27B). Thus, the *gcs1-1* mutant cells with the 21R genetic background, like *gcs1-6* mutant cells with the same background, may be capable of reentry into the mitotic cell cycle. Proliferating *gcs1-1* mutant cells were unable to continue cell proliferation after transfer to the restrictive temperature (Fig. 27C) and rapidly lost viablility; after 58 h of incubation at the restrictive temperature greater than 95% of *gcs1-1* mutant cells had lost viability (Fig. 27C). Thus *gcs1-1* mutant cells with the 21R genetic background are cold-sensitive for maintenance of ongoing proliferation, but may be capable of reentry into the mitotic cell cycle. The cold-sensitivity phenotype produced by the *gcs1-6* null allele in the 21R genetic background is similar to the phenotype of *gcs1-1* mutant cells,

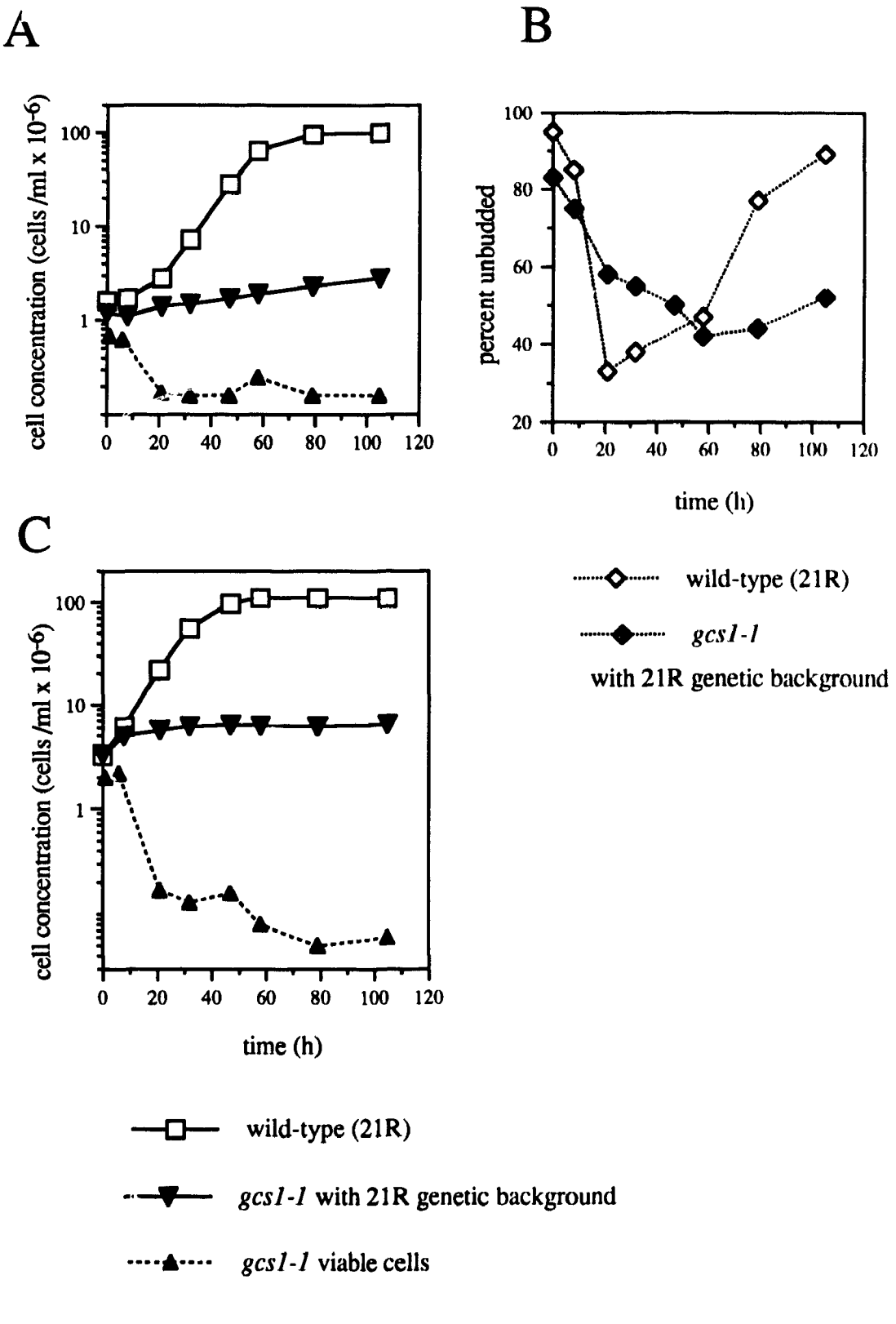
Figure 27. Growth kinetics of *gcs1-1* mutant cells with the 21R genetic background.

(A) Stationary-phase wild-type cells of strain 21R (open squares) and *gcs1-1* mutant cells with the 21R genetic background (filled triangles) were diluted into fresh YEPD medium and incubated at 15°C. At intervals samples were removed for determination of cell concentration and cell viability. The number of viable *gcs1-1* mutant cells (upward triangles) was determined by spreading diluted samples of cell culture on YEPD solid medium, followed by incubation at 29°C (as described in Materials and Methods). The number of colonies formed at 29°C (averaged from at least two dilutions of the same culture sample) was multiplied by the dilution factor to determine the number of viable cells.

(B) Wild-type and *gcs1-1* mutant cell cultures described in Fig. 26A were also monitored for percentage of unbudded cells as described in Materials and Methods.

(C) Actively proliferating wild-type (open squares) and *gcs1-1* mutant cells (filled triangles) were transferred from 29°C to 15°C and then incubated further at 15°C. At intervals samples were removed to determine cell concentration and cell viability as described in Fig. 26A.

Figure 27


A
B
C
cell concentration (cells /ml x 10-6)
100
10
1
0
20
40
60
80
100
120
percent unbudded
time (h)
wild-type (21R)
gcs1-1
with 21R genetic background
wild-type (21R)
gcs1-1 with 21R genetic background
gcs1-1 viable cells

indicating that the presence of linked modifer alleles does not appear to affect the phenotype of *gcs1* mutant cells with the 21R genetic background.

11B. *gcs1-2* and *gcs1-3* mutant cells with the 21R genetic background. In addition to the *gcs1-6* null allele, the *gcs1-2* and *gcs1-3* truncation alleles were also constructed in the 21R genetic background. These truncation alleles were constructed as previously described (Fig. 12A; Fig. 13A) by transformation of cells of strain 21R with the *gcs1-2* or *gcs1-3* disruption plasmids. Disruption of the *GCS1* chromosomal locus in Ura^+ transformants was confirmed by Southern analysis (Fig. 12B; Fig. 13B). As anticipated, both *gcs1-2* and *gcs1-3* mutant cells with the 21R genetic background were cold-sensitive when assayed by replica-plating to solid medium and incubation at 15°C for 5-7 days (data not shown).

Although *gcs1-2* and *gcs1-3* mutant cells are impaired in the resumption of proliferation from stationary phase, these mutant cells are capable of reentering the mitotic cell cycle from stationary phase as determined by measurement of budding index. As found for *gcs1-6* mutant cells (Fig. 26B), 40 - 50% of *gcs1-2* and *gcs1-3* mutant cells were budded after 23 h of incubation at 15°C (data not shown). Thus, the requirement for Gcs1 function in resumption of proliferation from stationary phase appears to be independent of the mitotic cell cycle. Perhaps the detrimental effects of absence of Gcs1 are not manifested in *gcs1* mutants with the 21R background until after the completion of Start. Thus, these *gcs1* mutant cells within the 21R background reenter the mitotic cell cycle from stationary phase at the restrictive temperature, but become impaired in continued proliferation.

After several weeks of incubation at 15°C, entire lawns of *gcs1-2* and *gcs1-3* mutant cells were observed to be slowly proliferating. This result was in contrast to the areas containing *gcs1-6* null cells, which only showed proliferation in isolated

papillations. This indicated that the cold-sensitivity phenotype of *gcs1-2* and *gcs1-3* cells may not be as severe as that of *gcs1-6* null mutant cells. Determination of the growth kinetics of *gcs1-2* and *gcs1-3* mutant cells in liquid medium indicated that, indeed, stationary-phase *gcs1-2* and *gcs1-3* mutant cells resumed proliferation after 50 h of incubation at 15°C (Fig. 28A), whereas cell proliferation in *gcs1-6* mutant cultures was observed only after 90 h of incubation (Fig. 28A). Thus the phenotype of cold sensitivity for stationary-phase cells appears to be less severe in the *gcs1* truncation mutants with the 21R genetic background than in the *gcs1* null mutants with the same genetic background. (These cultures were not tested for the presence of cold-resistant cells, and thus it is possible that the proliferation in *gcs1-2* and *gcs1-3* cultures was due to recombination at the *gcs1-2* and *gcs1-3* loci to regenerate the wild-type *GCS1* gene.)

Transfer of proliferating *gcs1-2* and *gcs1-3* mutant cells to the restrictive temperature revealed that *gcs1-2* and *gcs1-3* mutants continued to proliferate (Fig. 28B). This ability of *gcs1-2* and *gcs1-3* cells to maintain ongoing proliferation was also demonstrated in selective medium, where restoration of a wild-type *GCS1* by reversal of the integration event would lead to Ura$^-$, and thus non-growing, cells (G.C. Johnston, personal communication). Thus the *gcs1-2* and *gcs1-3* mutant alleles may have partial function in the 21R genetic background, suggesting in turn that the N-terminus of Gcs1 may be sufficient for maintenance of ongoing proliferation by cells of strain 21R.

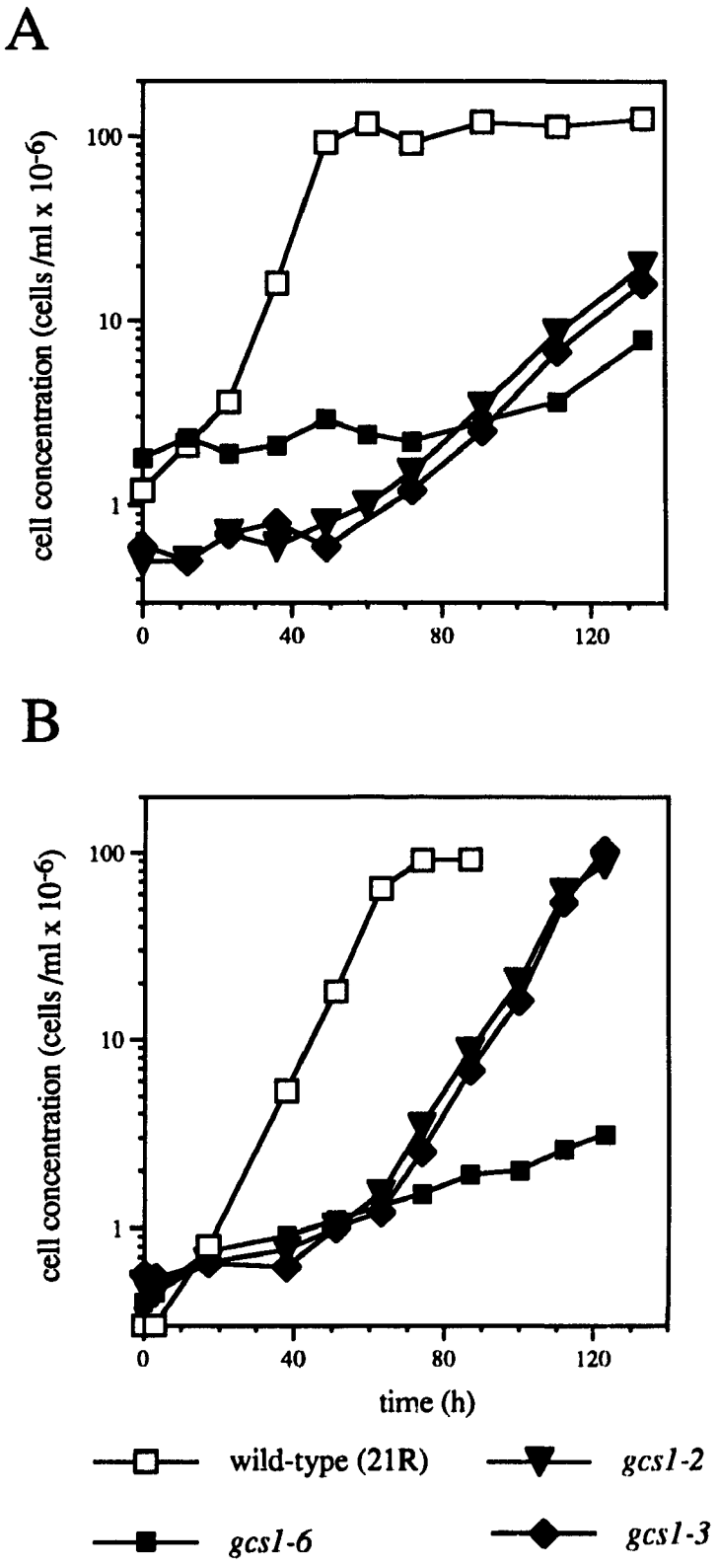
11C. The *gcs1-6* null allele in the FY56 genetic background does not cause cold sensitivity. The requirement for Gcs1 in both resumption of proliferation from stationary phase and in maintenance of ongoing cell proliferation was shown to be redundant in a third genetic background. Analysis of the phenotype of *gcs1* mutant cells with the FY56 genetic background indicated that Gcs1 is dispensable both for

Figure 28. Growth kinetics of *gcs1-2* and *gcs1-3* mutant cells with the 21R genetic background.

(A) Stationary-phase wild-type cells of strain 21R (open squares) and stationary-phase *gcs1* mutant cells (filled symbols) were diluted into fresh YEPD medium and incubated at 15°C. At intervals 0.1 ml samples were removed for determination of cell concentration. *gcs1-2* mutant cells of strain GRD-2 (triangles). *gcs1-3* mutant cells of strain GRT-2 (diamonds) and *gcs1-6* mutant cells of strain GRK4-7 (squares) were assayed for the ability to resume proliferation from stationary phase at 15°C. Because of the 0.1 ml sample size, cell concentrations below 6 x 10^6 cells/ml (reading of 600 on the Coulter Counter) may not be accurate.

(B) Proliferating wild-type cells of strain 21R (open squares) and *gcs1* mutant cells with the 21R genetic background (filled symbols) were transferred from 29°C to 15°C and then incubated further at 15°C. Cells were shifted from 29°C to 15°C at a relatively low cell density (< 5 x 10^5 cells /ml). At intervals, 0.1 ml samples were removed for determination of cell concentration. *gcs1-2* mutant cells of strain GRD-2 (triangles), *gcs1-3* mutant cells of strain GRT-2 (diamonds), and *gcs1-6* mutant cells of strain GRK4-7 (squares) were assayed for the ability to maintain ongoing proliferation at 15°C. Because of the 0.1 ml sample size, cell concentrations below 6 x 10^6 cells/ml (reading of 600 on the Coulter Counter) may not be accurate.

Figure 28


A
cell concentration (cells /ml x 10-6)
100
10
1
0
40
80
120
B
cell concentration (cells /ml x 10-6)
100
10
1
0
40
80
120
time (h)
wild-type (21R)
gcs1-2
gcs1-6
gcs1-3

resumption of proliferation from stationary phase and for maintenance of ongoing cell proliferation. *gcs1-6* null-mutant cells in the FY56 genetic background showed no cold sensitivity either for stationary-phase cells or for proliferating cells (data not shown), suggesting that, in certain genetic backgrounds, other genes can perform the functions of *GCS1*.

12. Suppression of the cold sensitivity of *gcs1* mutant cells that is specific for a given genetic background

Suppressors of the cold-sensitivity phenotype of *gcs1* mutant cells have been identifed that are specific for each genetic background. These suppressors of *gcs1* mutant phenotypes may alleviate the requirement for Gcs1 both in resumption of cell proliferation and in maintenance of ongoing proliferation, or only one of the requirements for Gcs1 function. Thus, suppressors such as *sed1-1*, which suppress only the requirement for Gcs1 in maintenance of ongoing proliferation, result in a reentry-mutant phenotype in combination with *gcs1* mutant alleles in genetic backgrounds where Gcs1 is required both for reentry into the mitotic cell cycle and for maintenance of ongoing cell proliferation. This requirement for Gcs1 in maintenance of ongoing proliferation may actually be due to modifier alleles, closely linked to *GCS1* that are present in the genetic backgrounds of certain strains. Suppressors have been identified which suppress only in the 21R genetic background, where Gcs1 is required both for resumption of proliferation from stationary phase and for maintenance of ongoing proliferation. These suppressor alleles may require the presence of the linked modifier alleles for suppressor function. Suppressors have also been identified which suppress only in the W303 genetic background, where Gcs1 is required only for reentry into the mitotic cell cycle from stationary phase.

12A. Suppression of the cold-sensitivity of *gcs1* mutant cells by cations. During an initial characterization of *gcs1-1* mutant cells with the 21R genetic background it was observed that the presence of 0.2 M KCl in rich growth medium suppressed the cold sensitivity of these cells (M. Drebot, personal communication). To further characterize this suppression, different cations were tested over a range of concentrations for effects on the proliferation of *gcs1-1* mutant cells in rich medium at 14°C. As shown in Fig. 29A, 0.1 M $CaCl_2$, 0.1 M KCl, and 0.1 M $MgCl_2$ all suppressed the cold sensitivity of stationary-phase *gcs1-1* mutant cells incubated in fresh medium at 14°C. In contrast, the presence of 0.1 M NaCl did not allow the *gcs1-1* mutant cells to proliferate at the restrictive temperature (Fig. 29A). Thus, the observed suppression was not due to any cation, or to chloride anion. Suppression must therefore be specific to certain cations.

Increased cation concentrations in the growth medium completely suppressed the *gcs1*-mediated cold sensitivity for resumption of proliferation, such that mutant cells resumed proliferation from stationary phase with the same lag as wild-type cells. However, *gcs1*-mediated cold sensitivity for maintenance of proliferation was not completely suppressed by cations. Mutant cells in the presence of increased external cation concentrations still proliferated more slowly than wild-type cells, which were unaffected by these growth conditions (data not shown). The doubling time of wild-type cells of strain 21R was 8 h, while the doubling time of *gcs1-1* mutant cells in growth medium supplemented with 0.1 M $CaCl_2$ or 0.1 M KCl was approximately 14 h. At lower cation concentrations, calcium ions were more effective than potassium or magnesium ions at suppressing the cold sensitivity of *gcs1-1* mutant cells: 0.01 M $CaCl_2$ allowed proliferation at approximatedly the same rate (doubling time of 26 h) as 0.03 M KCl or 0.03 M $MgCl_2$ (Fig. 29B). The rate of proliferation of *gcs1-1* mutant cells was a function of both the particular cation and the cation concentration, consistent

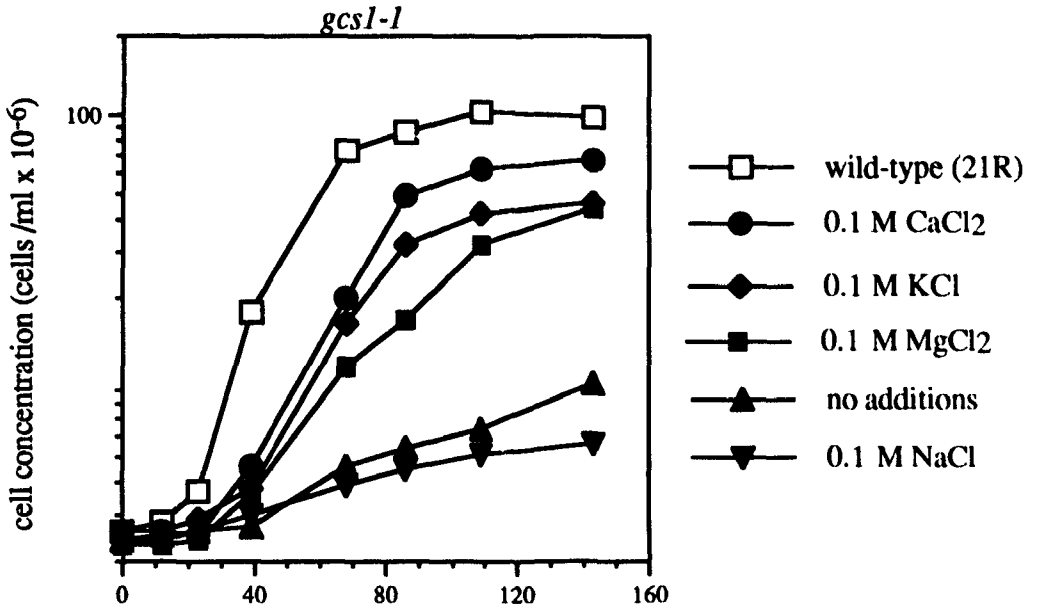
Figure 29. Suppression of the cold sensitivity of *gcs1-1* mutant cells by elevated cation concentrations.

(A) Stationary-phase wild-type cells of strain 21R (open squares) and *gcs1-1* mutant cells with the 21R genetic background (filled symbols) were diluted into fresh YEPD medium and incubated at 14°C. At intervals samples were removed for determination of cell concentration. *gcs1-1* mutant cells of strain MD-025-5 were diluted into fresh YEPD medium supplemented with 0.1 M $CaCl_2$ (filled circles), 0.1 M KCl (filled diamonds), 0.1 M $MgCl_2$ (filled squares), 0.1 M NaCl (downward triangles), or YEPD without supplementation (upward triangles).

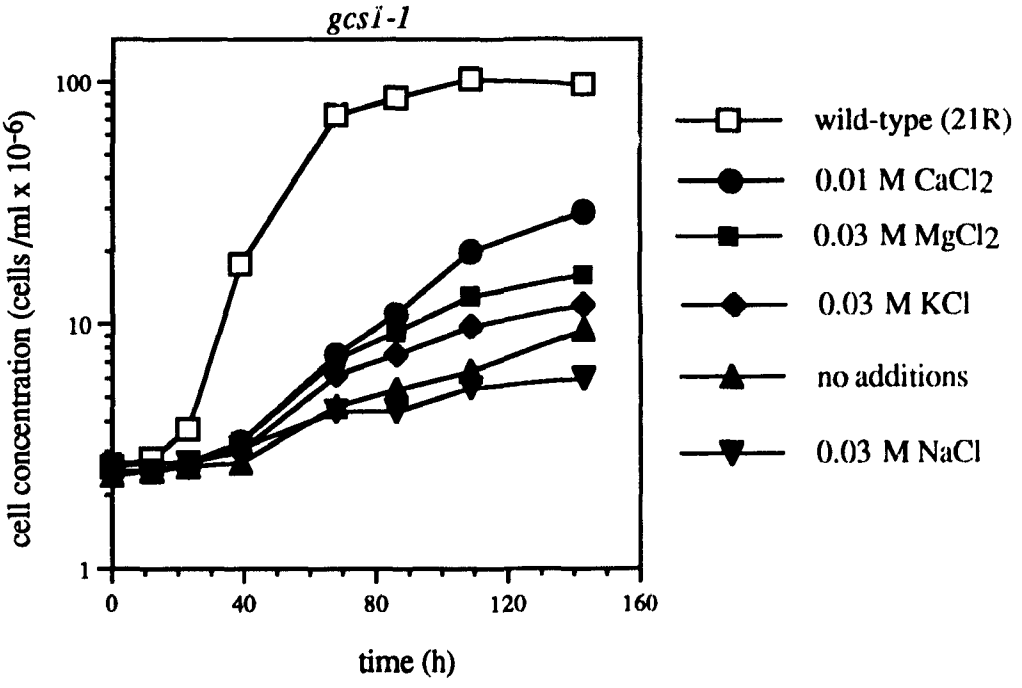
(B) Stationary-phase wild-type cells of strain 21R (open squares) and *gcs1-1* mutant cells with the 21R genetic background (filled symbols) were diluted into fresh YEPD medium and incubated at 14°C. At intervals samples were removed for determination of cell concentration. *gcs1-1* mutant cells of strain MD-025-5 were diluted into fresh YEPD medium supplemented with 0.01 M $CaCl_2$ (filled circles), 0.03 M $MgCl_2$ (filled squares), 0.03 M KCl (filled diamonds), 0.03 M NaCl (downward triangles) or YEPD without supplementation (upward triangles).

Figure 29

A


gcs1-1
cell concentration (cells /ml x 10-6)
100
0
40
80
120
160
wild-type (21R)
0.1 M CaCl2
0.1 M KCl
0.1 M MgCl2
no additions
0.1 M NaCl

B


gcs1-1
cell concentration (cells /ml x 10-6)
100
10
1
0
40
80
120
160
time (h)
wild-type (21R)
0.01 M CaCl2
0.03 M MgCl2
0.03 M KCl
no additions
0.03 M NaCl

with a continued requirement for elevated cation concentrations to maintain ongoing proliferation at 14°C.

In contrast, *gcs1* mutant cells with the GR2 genetic background showed different growth kinetics in the presence of increased concentrations of cations in the external growth medium. Stationary-phase *gcs1-1 sed1-1* reentry-mutant cells with the GR2 background transferred to fresh medium supplemented with 0.1 M $CaCl_2$ or 0.03 M $CaCl_2$ only resumed proliferation at the restrictive temperature after lags of 40 or 70 h, respectively (Fig. 30B). Thus the *gcs1*-mediated cold sensitivity for resumption of proliferation from stationary phase was not completely suppressed, and the resultant lag was dependent upon the cation concentration. However, the doubling time (12 h) of these *gcs1-1 sed1-1* mutant cells after resumption of proliferation was independent of the cation concentration (Fig. 30B). Thus, for these reentry-mutant cells, cations only affected resumption of cell proliferation, and not the subsequent rate of cell proliferation.

The increased cell proliferation after an extended lag observed for *gcs1-1 sed1-1* reentry-mutant cell cultures in the presence of cations was not due to the accumulation of genetically altered cells that had become cold-resistant for resumption of proliferation from stationary-phase. The *gcs1-1 sed1-1* reentry-mutant cells that proliferated at the restrictive temperature remained cold-sensitive for resumption of proliferation from stationary phase, as determined by the following assay. After 119 h at the restrictive temperature, the *gcs1-1 sed1-1* cell culture was tested for the presence of genetically altered cold-resistant cells within the population. Diluted *gcs1-1 sed1-1* cell culture was spread on YEPD solid medium and incubated at 29°C; colonies that formed were replica-plated to YEPD solid medium and incubated at the restrictive temperature. By this assay, greater than 99% of cells within the *gcs1-1 sed1-1* cell population remained cold-sensitive after 119 h of incubation at the restrictive

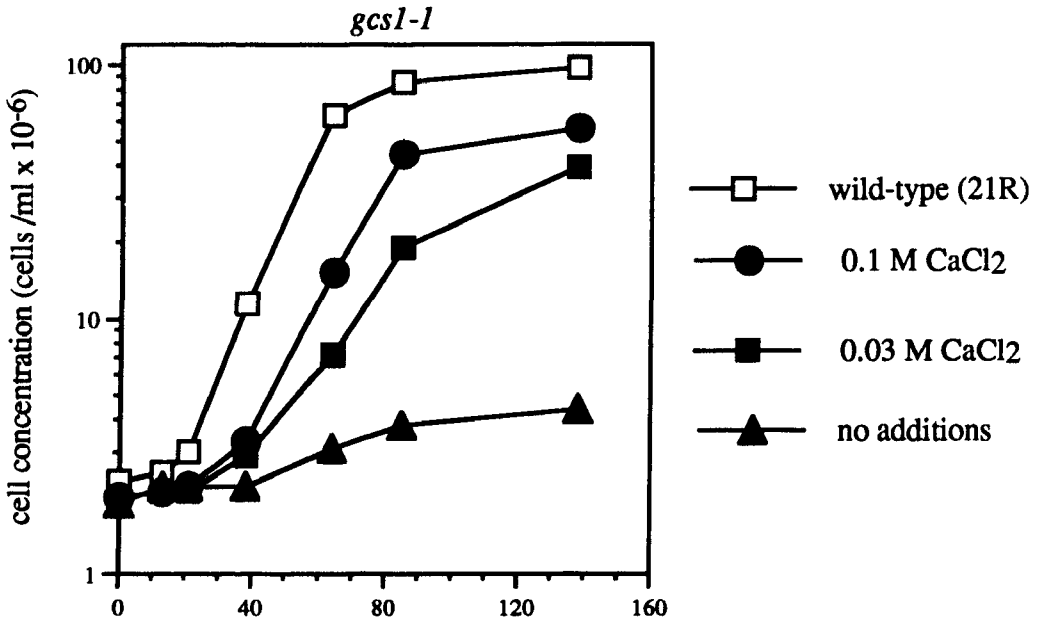
Figure 30. The effect of elevated cation concentrations on the growth kinetics of *gcs1* mutant cells with the 21R or GR2 genetic backgrounds.

(A) Stationary-phase wild-type cells of strain 21R (open squares) and *gcs1* mutant cells with the 21R genetic background (filled symbols) were diluted into fresh YEPD medium and incubated at 14°C. At intervals samples were removed for determination of cell concentration. *gcs1-1* mutant cells of strain MD-025-5 were diluted into fresh YEPD medium supplemented with 0.1 M $CaCl_2$ (filled circles), 0.03 M $CaCl_2$ (filled squares) or YEPD without supplementation (filled triangles).

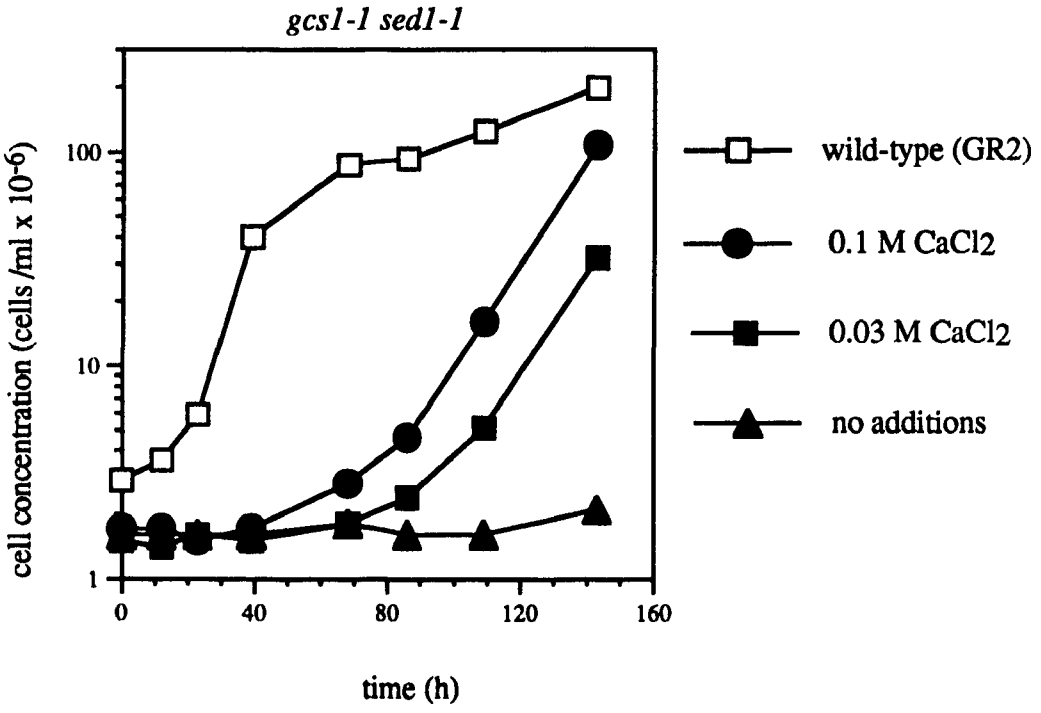
(B) Stationary-phase wild-type cells of strain GR2 (open squares) and *gcs1* mutant cells with the GR2 genetic background (filled symbols) were diluted into fresh YEPD medium and incubated at 14°C. At intervals samples were removed for determination of cell concentration. *gcs1-1 sed1-1* cells of strain MD-G02 were diluted into fresh YEPD medium supplemented with with 0.1 M $CaCl_2$ (filled circles), 0.03 M $CaCl_2$ (filled squares) or YEPD without supplementation (filled triangles).

Figure 30

A


gcs1-1
cell concentration (cells /ml x 10-6)
100
10
1
0
40
80
120
160
wild-type (21R)
0.1 M CaCl2
0.03 M CaCl2
no additions

B


gcs1-1 sed1-1
cell concentration (cells /ml x 10-6)
100
10
1
0
40
80
120
160
time (h)
wild-type (GR2)
0.1 M CaCl2
0.03 M CaCl2
no additions

temperature. In contrast, incubation of the same culture in the absence of cations resulted in the usual accumulation of cold-resistant cells even in the absence of detectable increase in cell number: 13% of *gcs1-1 sed1-1* cells were cold-resistant after 119 h incubation at the restrictive temperature. Thus, in the cation-suppressed cultures which resumed proliferation the genetically altered cold-resistant cells remained at less than 1% of the total cells in the population. However, in the absence of cations, the bulk of the population does not proliferate, and the genetically altered cold-resistant cells eventually become a significant proportion of the total cell population.

An unexpected effect of elevated cation concentrations was observed for W303 wild-type cells. The relatively slow rate of proliferation of wild-type cells of strain W303 at 15°C (11 h doubling time) was increased (to an 8 h doubling time) by elevated cation concentrations in the growth medium (data not shown). In contrast, as previously discussed, external cation concentrations had no effect on the proliferation of wild-type 21R or GR2 cells (data not shown). Preliminary results suggest that the effect of cations on *gcs1-3* mutant cells with the W303 genetic background is similar to that observed for mutant cells with the GR2 genetic background. Stationary-phase *gcs1-3* mutant cells with the W303 genetic background resumed proliferation in the presence of increased cation concentrations after a lag of 60 h (data not shown). Like reentry-mutant cells in the GR2 genetic background, the rate of proliferation of *gcs1-3* mutant cells after reentry into the mitotic cell cycle was the same or greater than that of W303 wild-type cells. Thus increased cation concentrations in the growth medium partially suppress the *gcs1*-mediated cold sensitivity for resumption of proliferation in cells of strain GR2 and W303.

In summary, suppression of *gcs1*-mediated cold sensitivity for resumption of proliferation from stationary phase was observed in three different genetic backgrounds.

The time required for resumption of proliferation from stationary phase was independent of cation concentration for *gcs1* mutant cells with the 21R genetic background; however, the rate of subsequent cell proliferation was dependent on cation concentration (Fig. 30A). This result is consistent with complete suppression of the *gcs1*-mediated cold sensitivity for resumption of proliferation from stationary phase, and partial suppression of the *gcs1*-mediated cold sensitivity for maintenance of ongoing proliferation in strain 21R. In contrast, stationary-phase *gcs1* reentry-mutant cells with the GR2 or W303 genetic backgrounds only resumed proliferation after an extended lag equivalent to 5 to 10 generation times of wild-type cells at 15°C. The lag before resumption of cell proliferation from stationary phase was dependent on cation concentration for *gcs1* mutants in the GR2 background; however, the rate of subsequent cell proliferation was independent of cation concentration (Fig. 30B). This result is consistent with partial suppression of the *gcs1*-mediated cold-sensitivity for resumption of proliferation from stationary phase, and the absence of *gcs1*-mediated cold sensitivity for maintenance of ongoing proliferation in reentry-mutant cells of strain GR2 and W303.

12B. A high-copy suppressor specific for *gcs1-1* mutant cells with the 21R genetic background. As described above, two different plasmids, p6d-3 and CSC-2A, had been shown to restore a cold-resistance phenotype to *gcs1* mutant cells. These experiments were done with *gcs1* mutant cells with the 21R genetic background. Since the plasmid p6d-3 contains the *GCS1* gene itself, the CSC-2A plasmid must contain a suppressor sequence (consistent with copy-suppression, CSC-2A was isolated from a high-copy YEp24-based yeast genomic library). This suppressor gene, which I term *CSS1* (cold sensitivity suppressed), suppressed in high copy the cold sensitivity of *gcs1-1* mutant cells with the 21R genetic background (Fig. 2). To determine if the *CSS1*

gene in high copy could suppress the cold sensitivity of *gcs1-1* mutant cells with the W303 genetic background, the *gcs1-1* allele from strain MD-025-5 (21R genetic background) was crossed into the W303 background by three successive backcrosses to produce strain MDgcs1-3X (M. Drebot, personal communication). Transformation of this strain with *CSS1* on a high-copy plasmid did not result in loss of cold sensitivity of transformed mutant cells when assayed by replica-plating of Ura^+ transformants to solid medium and incubation at 15°C (data not shown). Therefore the *CSS1* gene suppresses the cold sensitivity of *gcs1-1* mutants with the 21R genetic background, but not the cold sensitivity of *gcs1-1* mutants with the W303 genetic background. (Potential effects of linked modifier alleles on suppression were not involved: the genetic *gcs1-1* mutant allele in the W303 genetic background has the same linked modifer alleles as the genetic *gcs1-1* mutant allele in the 21R genetic background. As previously discussed, no effect from linked modifier alleles was observed on the cold-sensitivity phenotype of *gcs1-1* mutant cells with the 21R genetic background [Fig. 27].)

To localize the *CSS1* gene within the initial 9-kbp insert, the insert was restriction mapped as previously described (Fig. 1) and subcloned in high-copy plasmids as described in Fig. 31. Individual subclones were tested for suppression of the cold sensitivity of *gcs1-1* mutant cells; the smallest complementing subclone, pL47, contained a 3.4-kbp *Hpa*I fragment. Deletion of a 500-bp *Bgl*II fragment from pL47 was sufficient to eliminate suppression of the cold-sensitivity phenotype of *gcs1-1* mutant cells, showing that the *CSS1* gene is localized to the right half of the insert in pL47 (Fig. 32A). The sequence of the right half of pL47 was obtained as described in Fig. 32B; this sequence, which was based partially on sequence of only one strand (Fig. 32B), revealed a 1.6-kbp ORF. To confirm the existence of this ORF by identification of an mRNA of the appropriate size, a radiolabelled probe prepared from the internal

Figure 31. Localization of *CSS1* within the 9-kbp insert of plasmid CSC-2A.

The restriction map of the 9-kbp insert from plasmid CSC-2A is shown with the following restriction sites: *Bgl*II (G), *Cla*I (C), *Eco*RI (E), *Hin*dIII (H), *Hpa*I (A), *Kpn*I (K), *Pst*I (P), *Sst*I (S), *Xba*I (X). Derivatives of CSC-2A were constructed in high-copy vectors (Table 2) and tested for suppression of the cold sensitivity of *gcs1-1* mutant cells. These *URA3* plasmids; pK5.4, pK3.8, pL46, pL47, pL49, pSFG2, and pL48, were used to transform cells carrying the *gcs1-1* and *ura3-52* mutations (strain MD-025-5) to uracil prototrophy. Ura^+ transformants were replica-plated to solid YEPD medium and incubated at 14°C. + = suppression (colony formation at 14°C), − = no suppression (failure to form colonies at 14°C). The dashed lines in the diagram of the insert of pL49 indicate deletion of the 500-bp *Bgl*II fragment.

Figure 31

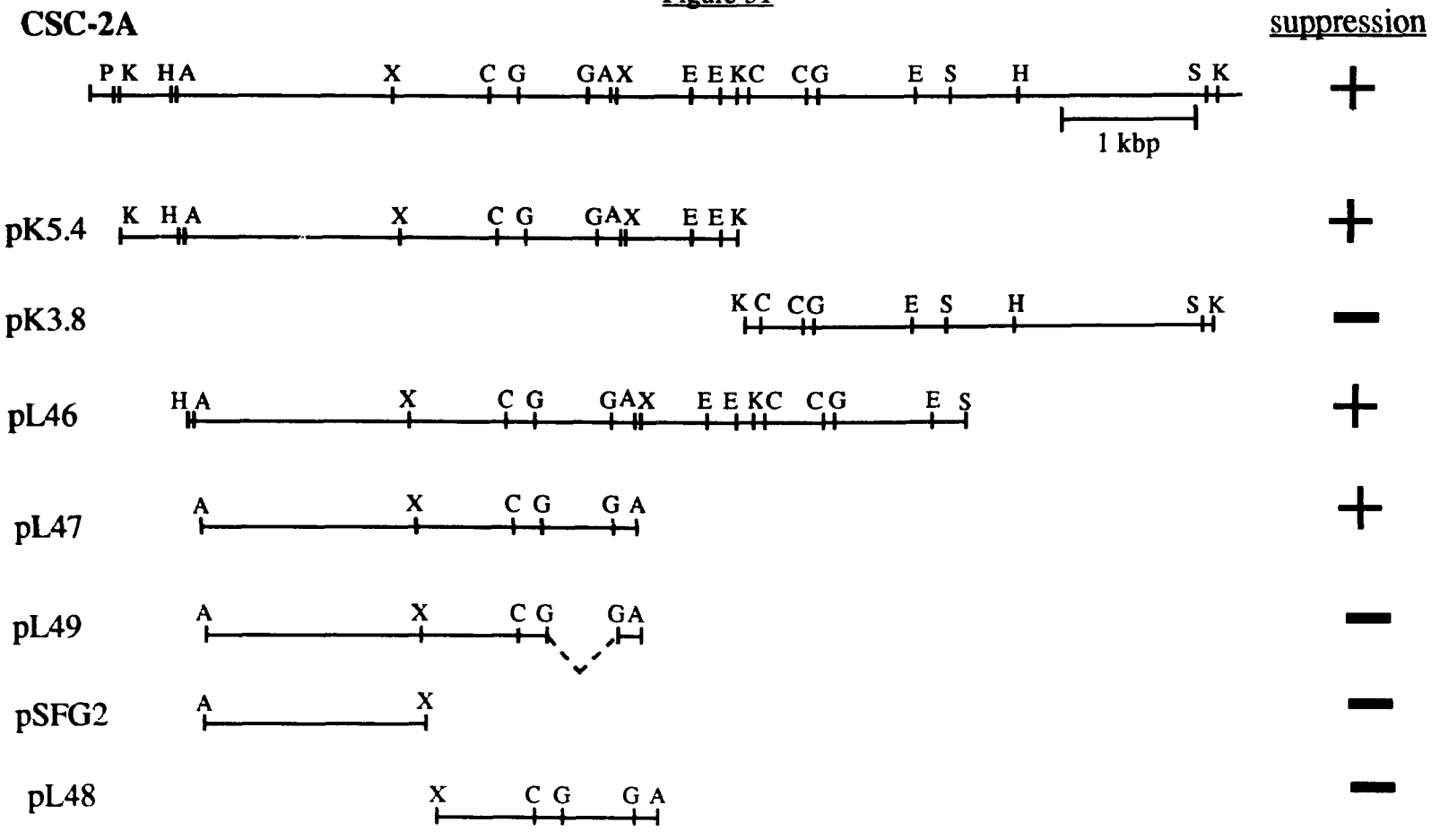

CSC-2A
suppression
P K H A X C G G A X E E K C C G E S H S K
1 kbp
pK5.4
K H A X C G G A X E E K
pK3.8
K C C G E S H S K
pL46
H A X C G G A X E E K C C G E S
pL47
A X C G G A
pL49
A X C G G A
pSFG2
A X
pL48
X C G G A

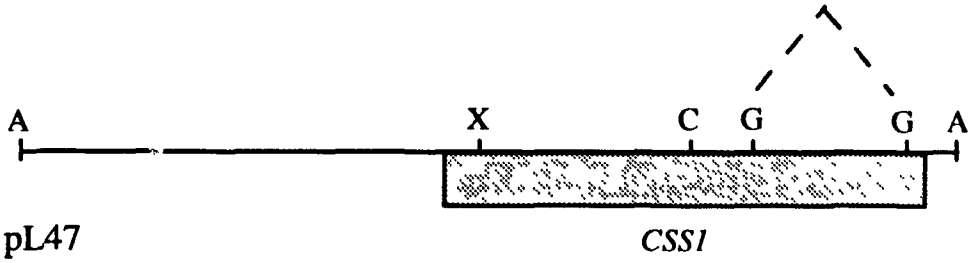
Figure 32. Strategy for sequencing the *CSS1* gene.

(A) Restriction map of the smallest suppressing subclone, pL47, is shown with the restriction sites for *Bgl*II (G), *Cla*I (C), *Eco*RI (E), *Hpa*I (A), *Xba*I (X). The *CSS1* gene was localized to the right end of the pL47 insert by the deletion analysis shown in Fig. 31. Deletion of the 500-bp *Bgl*II fragment from pL47 (dashed lines) was sufficient to eliminate suppression of the cold-sensitive phenotype of *gcs1-1* mutant cells.

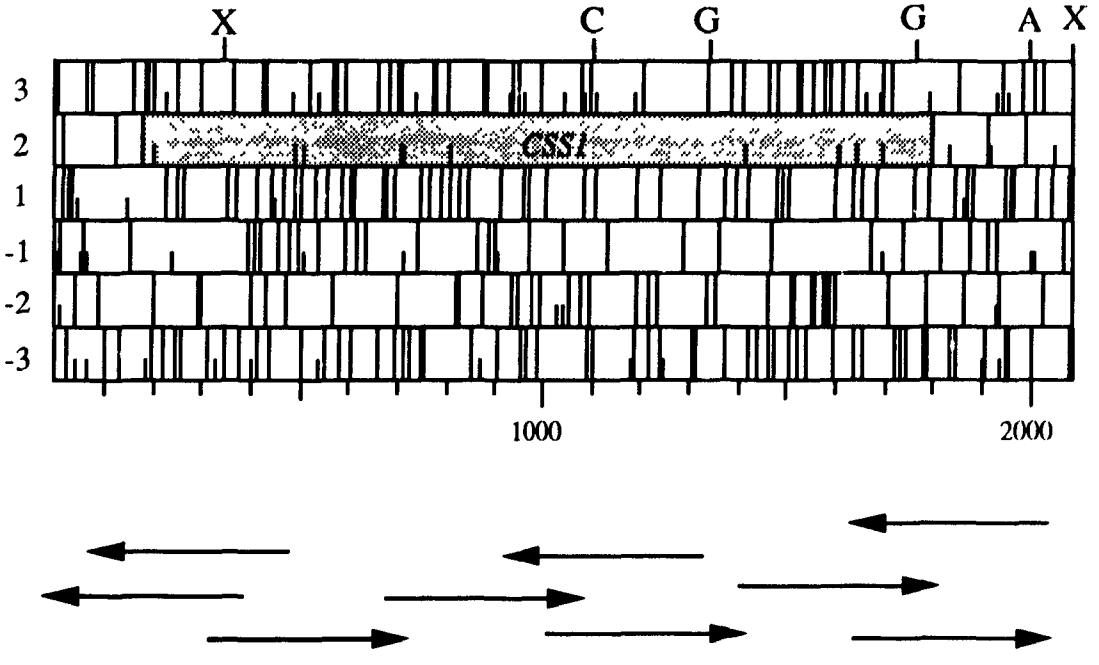
(B) The pL47 insert was partially sequenced through directed subcloning in M13 vectors to generate single-stranded templates (Table 3), as well as by using specifically designed 20-mer oligonucleotide primers on either single-stranded or double-stranded templates (as described in Materials and Methods). The sequence data is illustrated as an ORF map (generated with DNA Strider software), and the ORF corresponding to *CSS1* is shaded. The six possible reading frames are numbered from +3 to -3; positions of the putative methionine codons are marked with short bars, and positions of stop codons are marked with full-length bars. Arrows correspond to sequence obtained either from individual M13 subclones or from the specifically designed primers.

Figure 32

A



B



*Xba*I-*Bgl*II fragment within the ORF was used to probe total RNA from wild-type cells that had been resolved on an agarose gel and transferred to nylon membranes. A transcript of approximately 1.7 kb was detected, consistent with the DNA sequence predictions (data not shown).

To map the *CSS1* gene, a radiolabelled *CSS1* fragment from plasmid pL45 was used to probe a Southern blot of separated yeast chromosomes (see Materials and Methods). This analysis localized *CSS1* to chromosome V or VIII (data not shown). Since both chromosomes V and VIII have been physically mapped using 8 restriction enzymes (Tanaka and Isono, 1992), the restriction map of the 9-kb insert containing the *CSS1* gene was compared to the physical maps of chromosomes V and VIII. This comparison suggested that the *CSS1* gene is located on the right arm of chromosome VIII between *ste12* and *spo12*, and tightly linked to *cdc12* (Fig. 33A).

The unpublished sequence of *CDC12* was obtained (J.R. Pringle, Univ. of North Carolina, personal communication) and compared to the sequence of *CSS1*. Overlap was found between the downstream region of *CDC12* and the promoter region of *CSS1* (Fig. 33B). Thus *CSS1* represents a novel gene, since no other locus has been mapped to this position.

The sequence of the *CSS1* gene predicts a leucine-rich polypeptide of 531 amino acids (Fig. 34). Searches of protein databases revealed weak sequence similarity with kinesin-like protein*CIN8* (Hoyt et al., 1992), and myosin-like protein *MLP1* (Koelling *et al.*, 1992), proteins that may be involved in movement of vesicles and cytoskeletal functions (Hoyt et al., 1992). Furthermore, ongoing analysis of suppressors of *gcs1*-mediated cold sensitivity has implicated protein targeting as one process that may be affected by mutations in *GCS1* (X. Wang, personal communication). The *gcs1*-mediated defect in maintenance of ongoing cell proliferation at 15°C may therefore be related to some housekeeping activity such as protein targeting. The different

Figure 33. Physical mapping of the *CSS1* gene.

(A) The *CSS1* gene was localized to chromosome V or VIII by probing a blot of separated yeast chromosomes with a radiolabelled *CSS1* fragment (data not shown). The *CSS1* gene was further localized to the right arm of chromosome VIII by comparison of the restriction map of the CSC-2A 9-kb insert (containing *CSS1*) to the physical maps of chromosome V and VIII (Tanaka and Isono, 1992). This comparison localized *CSS1* to the right arm of chromosome VIII between *ste12* and *spo12*.

(B) Comparison of the physical and genetic maps of chromosome VIII (Mortimer, 1989) suggested that *CSS1* may be linked to *cdc12*. Inspection of the promoter sequence of *CDC12* revealed overlap with the promoter sequence of *CSS1*. The proximity of the *CDC12* and *CSS1* is illustrated by the ORF map (generated by DNA Strider software). The six possible reading frames are numbered from +3 to -3; positions of the putative methionine codons are marked with short bars, and positions of stop codons are marked with full-length bats. *CDC12* (hatched) and *CSS1* (shaded) are divergently transcribed (arrows) from a promoter region of ~300 bp.

Figure 33

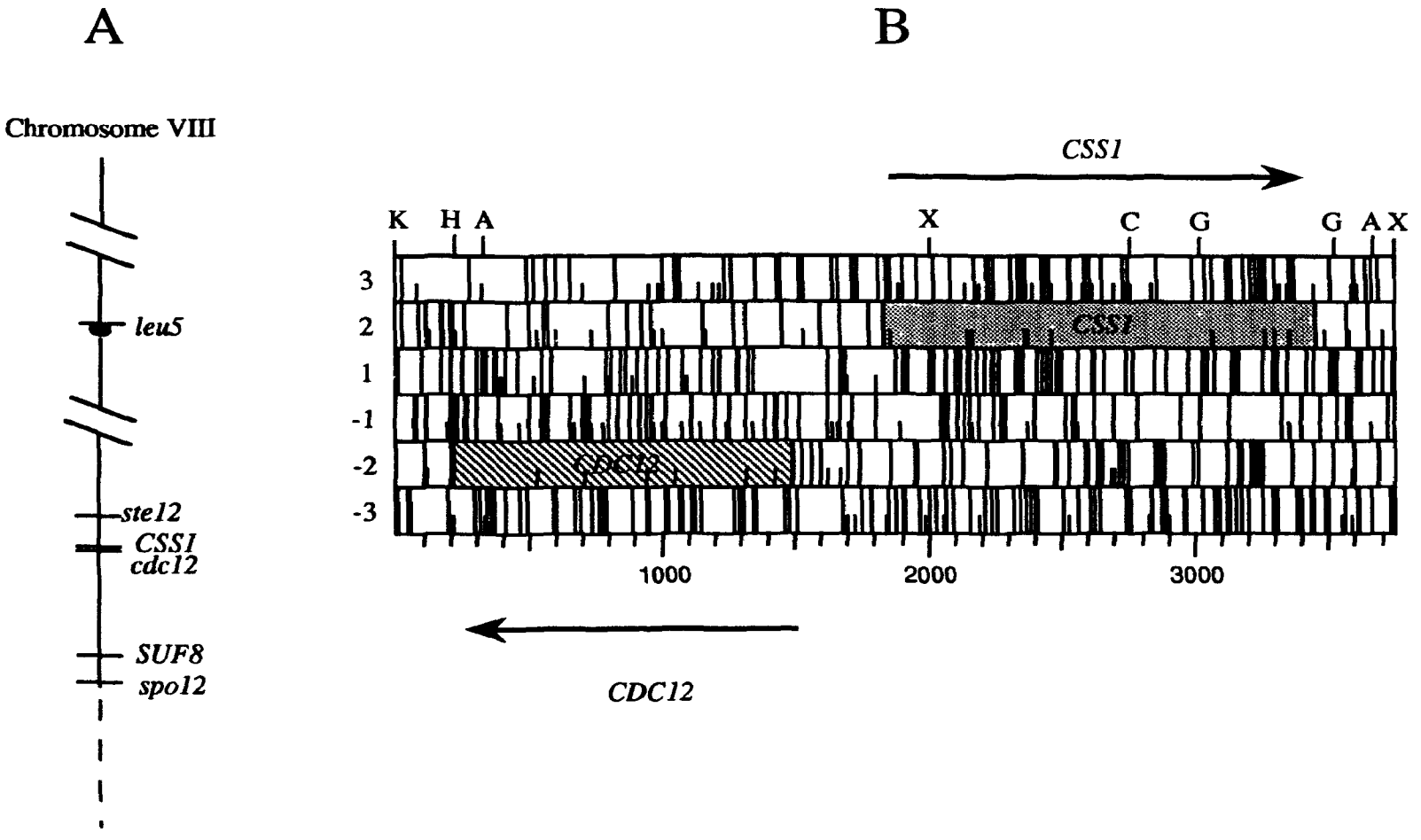

A
Chromosome VIII
leu5
ste12
CSS1
cdc12
SUF8
spo12
B
CSS1
K H A X C G G A X
3
2
1
-1
-2
-3
CSS1
CDC12
1000
2000
3000
CDC12

Figure 34. The *CSS1* gene and flanking sequences.

The nucleotide sequence of the *CSS1* gene is numbered from the presumptive ATG initiation codon of the *CSS1* ORF. The predicted amino-acid sequence is also numbered from the initiator methionine with amino-acid numbering indicated on the right. Nucleotide numbering is shown on the left and includes a dot scale above the nucleotide sequence that places a dot above every 10th nucleotide. The restriction sites described in Fig. 32 are boxed, and appear in the order: *Xba*I (TCTAGA), *Cla*I (ATCGAT), *Bgl*II (AGATCT), *Bgl*II (AGATCT), *Hpa*I (GTTAAC), *Xba*I (TCTAG/).

Figure 34

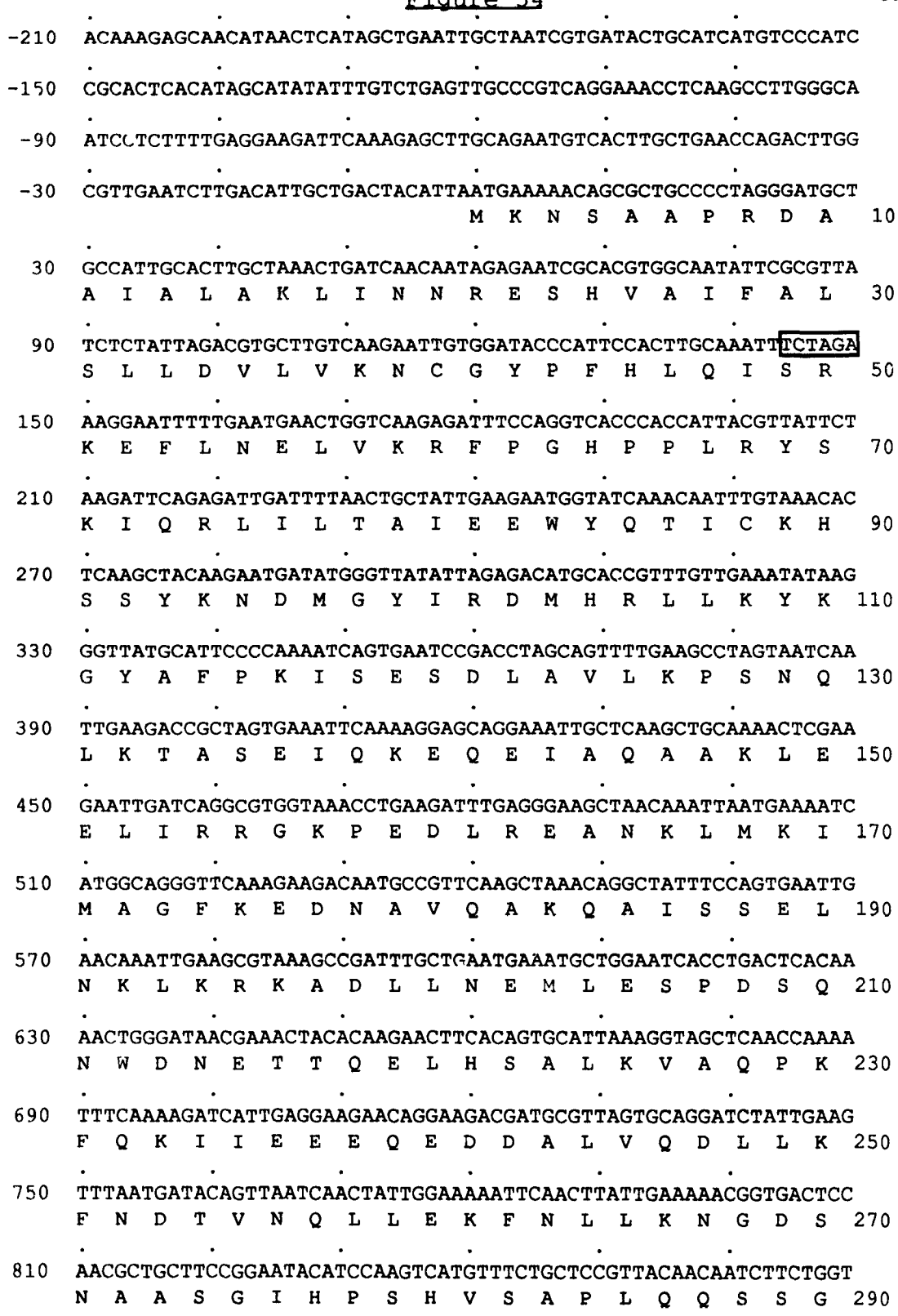

-210 ACAAAGAGCAACATAACTCATAGCTGAATTGCTAATCGTGATACTGCATCATGTCCCATC
-150 CGCACTCACATAGCATATATTTGTCTGAGTTGCCCGTCAGGAAACCTCAAGCCTTGGGCA
-90 ATCCTCTTTTGAGGAAGATTCAAAGAGCTTGCAGAATGTCACTTGCTGAACCAGACTTGG
-30 CGTTGAATCTTGACATTGCTGACTACATTAATGAAAAACAGCGCTGCCCCTAGGGATGCT
M K N S A A P R D A 10
30 GCCATTGCACTTGCTAAACTGATCAACAATAGAGAATCGCACGTGGCAATATTCGCGTTA
A I A L A K L I N N R E S H V A I F A L 30
90 TCTCTATTAGACGTGCTTGTCAAGAATTGTGGATACCCATTCCACTTGCAAATTTCTAGA
S L L D V L V K N C G Y P F H L Q I S R 50
150 AAGGAATTTTTGAATGAACTGGTCAAGAGATTTCCAGGTCACCCACCATTACGTTATTCT
K E F L N E L V K R F P G H P P L R Y S 70
210 AAGATTCAGAGATTGATTTTAACTGCTATTGAAGAATGGTATCAAACAATTTGTAAACAC
K I Q R L I L T A I E E W Y Q T I C K H 90
270 TCAAGCTACAAGAATGATATGGGTTATATTAGAGACATGCACCGTTTGTTGAAATATAAG
S S Y K N D M G Y I R D M H R L L K Y K 110
330 GGTTATGCATTCCCCAAAATCAGTGAATCCGACCTAGCAGTTTTGAAGCCTAGTAATCAA
G Y A F P K I S E S D L A V L K P S N Q 130
390 TTGAAGACCGCTAGTGAAATTCAAAAGGAGCAGGAAATTGCTCAAGCTGCAAAACTCGAA
L K T A S E I Q K E Q E I A Q A A K L E 150
450 GAATTGATCAGGCGTGGTAAACCTGAAGATTTGAGGGAAGCTAACAAATTAATGAAAATC
E L I R R G K P E D L R E A N K L M K I 170
510 ATGGCAGGGTTCAAAGAAGACAATGCCGTTCAAGCTAAACAGGCTATTTCCAGTGAATTG
M A G F K E D N A V Q A K Q A I S S E L 190
570 AACAAATTGAAGCGTAAAGCCGATTTGCTGAATGAAATGCTGGAATCACCTGACTCACAA
N K L K R K A D L L N E M L E S P D S Q 210
630 AACTGGGATAACGAAACTACACAAGAACTTCACAGTGCATTAAAGGTAGCTCAACCAAAA
N W D N E T T Q E L H S A L K V A Q P K 230
690 TTTCAAAAGATCATTGAGGAAGAACAGGAAGACGATGCGTTAGTGCAGGATCTATTGAAG
F Q K I I E E E Q E D D A L V Q D L L K 250
750 TTTAATGATACAGTTAATCAACTATTGGAAAAATTCAACTTATTGAAAAACGGTGACTCC
F N D T V N Q L L E K F N L L K N G D S 270
810 AACGCTGCTTCCGGAATACATCCAAGTCATGTTTCTGCTCCGTTACAACAATCTTCTGGT
N A A S G I H P S H V S A P L Q Q S S G 290

Figure 34 (cont.)

```
                .         .         .         .         .         .
 870  GCTTTAACGAATGAAATCAACTTAATCGATTTCAATGATTTGGACGAAGCGCCCTCTCAA
      A  L  T  N  E  I  N  L  I  D  F  N  D  L  D  E  A  P  S  Q  310
                .         .         .         .         .         .
 930  GGCAACAACAATACCAACGGCACGGGTACACCAGCAGCAGCGGAAACATCTGTCAATGAC
      G  N  N  N  T  N  G  T  G  T  P  A  A  A  E  T  S  V  N  D  330
                .         .         .         .         .         .
 990  TTATTGGGCGATTTGACCGATTTATCTATTTCGAACCCTTCAACCGCTAACCAAGCATCC
      L  L  G  D  L  T  D  L  S  I  S  N  P  S  T  A  N  Q  A  S  350
                .         .         .         .         .         .
1050  TTTGGTCTGGGAGGCGATATCGTGTTGGGGTCCTCTCAACCCGCACCACCAGTTACTACT
      F  G  L  G  G  D  I  V  L  G  S  S  Q  P  A  P  P  V  T  T  370
                .         .         .         .         .         .
1110  ACTAACAACTCGAACAACACTTTAGATCTTTGGGACTTTCAACTCCTCAATCGCCAACT
      T  N  N  S  N  N  T  L  D  L  L  G  L  S  T  P  Q  S  P  T  390
                .         .         .         .         .         .
1170  AATTCCCAAGCAGTAAACAGTAGTGGGTTCGATCTTTTGATGGGATTTAATCCAACAACA
      N  S  Q  A  V  N  S  S  G  F  D  L  L  M  G  F  N  P  T  T  410
                .         .         .         .         .         .
1230  GGTACTACTACTGCGCCCGCAAGAACCCTAGTCAATCAGTCTCCTAACTTGAAGATTGAG
      G  T  T  T  A  P  A  R  T  L  V  N  Q  S  P  N  L  K  I  E  430
                .         .         .         .         .         .
1290  TTCGAAATATCTAGGGAGTCAAACTCAGTTATAAGGATAAAATCGTTCTTTACAAACTTA
      F  E  I  S  R  E  S  N  S  V  I  R  I  K  S  F  F  T  N  L  450
                .         .         .         .         .         .
1350  AGTTCGTCACCGATCTCCAACTTAGTGTTCTTATTAGCAGTCCCTAAGTCAATGTCTTTG
      S  S  S  P  I  S  N  L  V  F  L  L  A  V  P  K  S  M  S  L  470
                .         .         .         .         .         .
1410  AAATTGCAACCCCAATCGAGTAACTTTATGATTGGCAACGCTAAAGATGGTATCTCTCAA
      K  L  Q  P  Q  S  S  N  F  M  I  G  N  A  K  D  G  I  S  Q  490
                .         .         .         .         .         .
1470  GAAGGTACAATTGAAAATGCGCCCATGAACCCTTCAAAGGCTTTGAAAGTCAAGTGGAAG
      E  G  T  I  E  N  A  P  M  N  P  S  K  A  L  K  V  K  W  K  510
                .         .         .         .         .         .
1530  GTCAACTATTCTGTCAACTCCACCCAAGCTGAAGAAACTGCTGTTTTTACGTTACCTAAT
      V  N  Y  S  V  N  S  T  Q  A  E  E  T  A  V  F  T  L  P  N  530
                .         .         .         .         .         .
1590  GTATAATCCTCTGGCGTTTCTTATCAATCCTTTCTCTTCTCTATGTATATTTTTAGAAGG
      V  *                                                        531
                .         .         .         .         .         .
1650  ATAATACCTCTATGTAGACAATCTTATAAAGATCTTCGCAACAATACATATTTCACTTAC
                .         .         .         .         .         .
1710  TAATATATGTTGCGTTTTCATGCATCAAAAGTAGTTAGCAATGCTAGATCGTGTAAAGGA
                .         .         .         .         .         .
1770  AAAAAAAGAGAAATAGTATAATTCGCCATCCTCATAACCACTGAAAAATCGAAGTTAACC
                .         .         .         .         .         .
1830  AAAAGGAAAGTTGAAATGGAGGAAGTGTTCCGTTTTTACTCGAACTCTAGAAATATCTTC
                .         .         .         .
1890  ATTCATAAAAGCCTTTCATTAAAACCATCCACCATTGA
```

phenotypes of *gcs1* mutants in different genetic backgrounds may reflect different constellations of interacting gene products required to fulfill a housekeeping function under different conditions.

Both elevated cation concentrations and increased copy number of the *CSS1* gene preferentially suppress *gcs1*-mediated cold sensitivity for resumption of proliferation from stationary phase for cells with the 21R genetic background. The relationship, if any, between these two mechanisms of suppression is unclear.

12C. A suppressor specific to the W303 genetic background. The *LEU2* gene was identified as a suppressor of the cold-sensitivity phenotype of *gcs1* mutant cells with the W303 genetic background. During attempts to construct another disruption of the *GCS1* gene using the *LEU2* gene as a selectable marker it became apparent that the presence of the *LEU2* marker suppressed the cold sensitivity of *gcs1* mutant cells during growth on rich medium. The *LEU2*-disrupted truncation allele, *gcs1-4,* contains the *LEU2* gene inserted into the unique *HpaI* site within the *GCS1* ORF. This allele was constructed using the disruption plasmid, pL51, containing the *LEU2* gene inserted into the *HpaI* site within the cloned *GCS1* ORF. Cleavage of the disruption plasmid with the appropriate restriction enzymes generated a linear fragment containing the *GCS1* gene and flanking sequences, interrupted by the *LEU2* gene inserted within the *GCS1* ORF. Transformation of W303 diploid cells (homozygous for *leu2*) with the linearized disruption construct, and selection for growth in the absence of leucine, generated Leu^+ diploid transformants. Diploids shown by Southern analysis to contain a disrupted *GCS1* gene were sporulated and asci were dissected; four viable segregants were produced from each dissected ascus. None of these segregants was cold-sensitive when assayed by replica-plating to solid YEPD medium and incubation at 15°C for 9 to 12 days; the growth of Leu^+ *gcs1* mutant cells and Leu^- wild-type cells was

indistinguishable with respect to cold sensitivity (data not shown). This result was surprising, since truncation of the *GCS1* gene had previously been shown to result in a cold-sensitivity phenotype. One explanation for the loss of cold sensitivity in *gcs1-4* mutant segregants was that the *LEU2* gene suppresses the *gcs1*-mediated cold-sensitive defect.

Direct evidence of the effect of the *LEU2* gene on *gcs1* mutant cells was obtained by transformation of *gcs1-2* mutant cells with the *LEU2* gene on a plasmid. The cold sensitivity of *gcs1-2* mutant cells, assayed by replica-plating to YEPD solid medium and incubation at 15°C was suppressed by transformation with the *LEU2*-based vector YEp13 (data not shown). The simplest interpretation of this result is that leucine prototrophy suppresses the *gcs1*-mediated cold sensitivity in *gcs1* mutant cells with the W303 genetic background.

Leucine prototropy was also found to suppress the slow-growth phenotype of W303 wild-type cells at 15°C (M. Drebot, personal communication). These two effects of leucine prototrophy, suppression of the slow-growth phenotype of W303 wild-type cells and suppression of the cold-sensitivity of *gcs1* mutant cells, may be related. In the absence of a slow-growth phenotype that is relieved by leucine prototrophy, no effect of leucine prototrophy was observed for *gcs1* mutant cells. For example, cells of strain 21R are also auxotrophic for leucine, but conversion of these cells to leucine prototrophs had no effect on the growth rate of wild-type or *gcs1* mutant cells (data not shown). Thus, suppression of the cold-sensitivity phenotype of *gcs1* mutant cells and the slow-growth phenotype of W303 wild-type cells by leucine prototrophy is specific to the W303 genetic background.

Suppression of the slow-growth phenotype of W303 wild-type cells by leucine prototrophy suggests that W303 wild-type cells may be defective in leucine uptake at 15°C. Thus, suppression of the slow-growth phenotype of W303 cells may involve

alleviation of a slowed rate of protein synthesis in W303 cells. Furthermore, suppression of the *gcs1*-mediated cold-sensitivity for resumption of proliferation by leucine prototrophy suggests that the rate of protein synthesis may be important for stationary-phase *gcs1* mutant cells attempting to resume cell proliferation (see Discussion).

IV. DISCUSSION

1. General remarks

This work has extended our characterization of the reentry-mutant phenotype. As previously described, reentry-mutant cells are cold-sensitive for the resumption of cell proliferation from stationary phase, but are unimpaired for maintenance of ongoing proliferation at a temperature that is restrictive for stationary-phase cells. Thus, the reentry-mutant phenotype shows that the requirements for resumption of proliferation from stationary phase are distinct from those for maintenance of ongoing cell proliferation. Previously, the reentry-mutant phenotype was shown to require the interaction of two mutations, *gcs1-1* and *sed1-1* (Drebot, 1987); however, the present study has shown that in certain genetic backgrounds (eg. W303), the *gcs1-1* mutation by itself confers a reentry-mutant phenotype, indicating that *sed1-1* is not an essential feature of this phenotype. Since *sed1-1* is not essential for the reentry-mutant phenotype, it is reasonable that the analysis of this phenotype has focused on the *GCS1* gene.

The *GCS1* gene was sequenced (Fig. 5), and mapped to a novel locus upstream of the *HO* gene on chromosome IV (Fig. 9). Inspection of the derived amino-acid sequence of the *GCS1* gene revealed a putative C-x-x-C-x_{16}-C-x-x-C metal-binding motif localized at the Gcs1 N-terminus. The importance of this C2-C2 motif to Gcs1 function was demonstrated by localization of the *gcs1-1* mutation to the codon encoding the second cysteine in the C2-C2 motif (Fig. 11). Further experiments were required to show that *gcs1-1* mutant cells were phenotypically the same as *gcs1* null cells (Fig. 25A), suggesting that the *gcs1-1* mutation results in complete loss of function of the Gcs1 protein. Thus, the *gcs1-1* mutation that results in substitution of tyrosine for

cysteine at the second cysteine of the four cysteine C2-C2 motif may render the Gcs1 protein completely nonfunctional. Further evidence for the importance of the C2-C2 motif and C-terminal flanking residues has come from the identification of two other genes that contain sequences homologous to *GCS1*.

2. The *GCS1* gene family

Submission of the derived Gcs1 protein sequence to the yeast protein database assembled by Mark Goebl revealed that *GCS1* may be part of a larger *S. cerevisiae* gene family, and that homologs of this *GCS1* gene family exist in the evolutionarily distant yeast, *Schizosaccharomyces pombe*. Evidence for the existence of a *GCS1* gene family comes from the derived protein sequence of another *S. cerevisiae* gene (Fig. 35). The derived protein sequence of *SPS18* (J. G. Coe, L. E. Murray, and I. W. Dawes, personal communication) shows 31% overall identity to Gcs1, and 48% identity over a 48-amino-acid region (Fig. 36) spanning the C2-C2 motif and C-terminal flanking region (which I term the C2-C2 domain). The finding of a high degree of sequence homology between Gcs1 and Sps18 over the C2-C2 domain suggests that this domain plays an important function in members of the *GCS1* gene family.

Further support for the functional importance of the C2-C2 domain in members of the *GCS1* gene family has come from the identification of a homologous domain in a derived protein sequence from the evolutionarily distant yeast, *Schizosaccharomyces pombe*. The derived protein sequence from the *S. pombe* gene, *TAS2* (N. Walworth, personal communication), is also 48% identical to Gcs1 over the same 48-amino-acid region encompassing the C2-C2 motif and C-terminal flanking region (Fig. 36). The presence of this C2-C2 domain in two proteins from evolutionarily distant yeasts

Figure 35. Comparison of the predicted amino-acid sequence of Gcs1 and Sps18.

The predicted amino-acid sequences of Gcs1 and Sps18 were compared using Clustal V sequence analysis software (Higgins *et al.*, 1992)

Amino-acid identities and similarities are highlighted in heavy and light shading respectively. The following pairs of amino acids were designated as similar; (K, R), (D, E), (S, T), (F, W), (L, I). The positions of the *gcs1-2* and *gcs1-3* truncations are indicated by arrows. The putative nuclear localization sequences and metal-binding domains are also indicated.

Figure 35

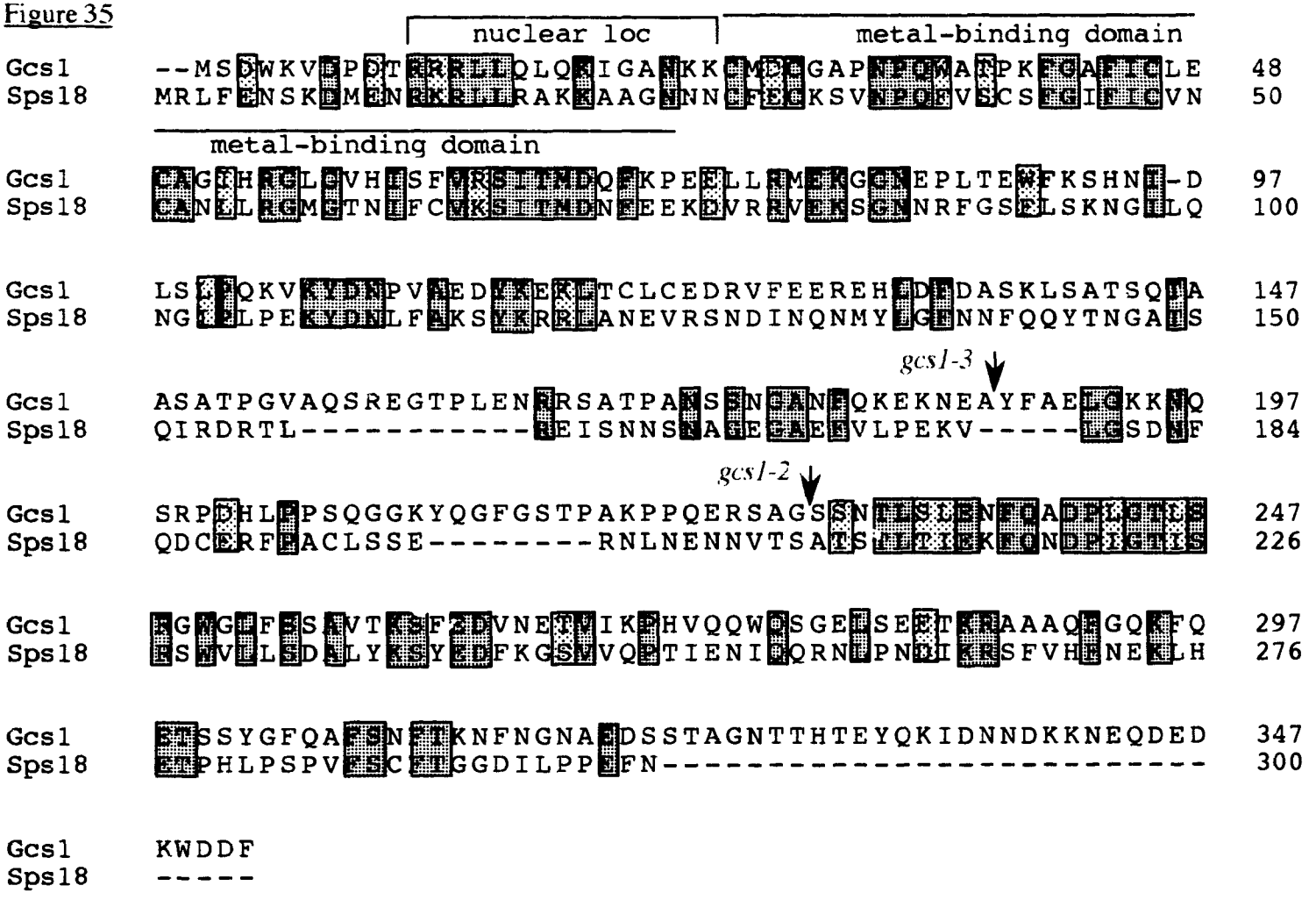

nuclear loc
metal-binding domain
metal-binding domain
gcs1-3
gcs1-2
Gcs1
Sps18

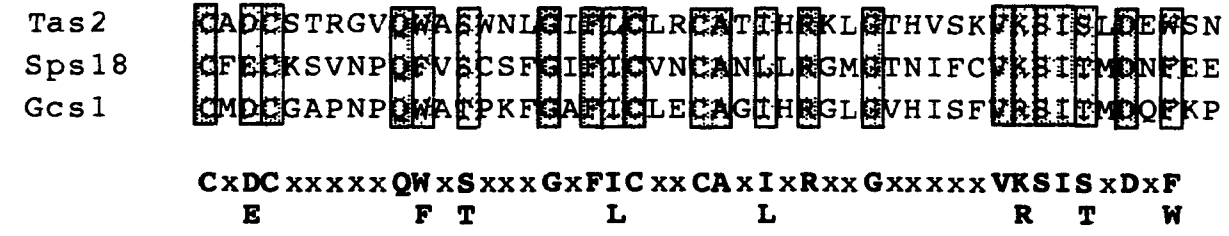
Figure 36. Comparison of GST and GATA C2-C2 domains.

(A) The C2-C2 domains of Gcs1, Sps18 and Tas2 are aligned to highlight similarities (light shading) and identities (heavy shading). Amino-acid similarities are designated as previously described (Fig. 35). A consensus sequence for the GST C2-C2 domain is illustrated in bold type.

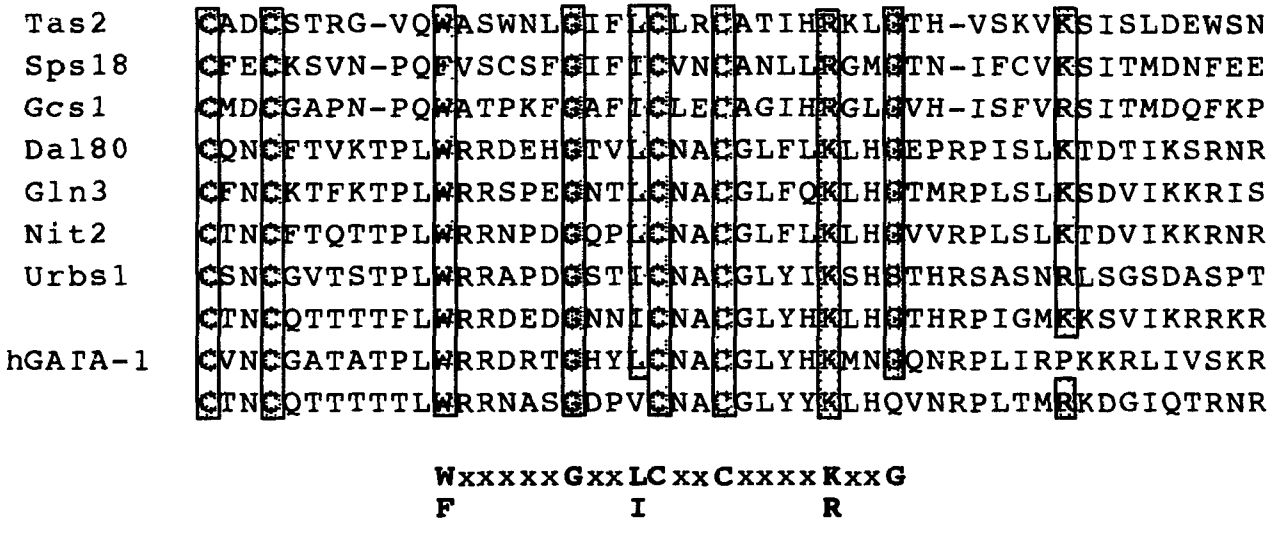
(B) The C2-C2 domains of selected fungal GATA proteins; Dal80 (Cunningham and Cooper, 1991), Gln3 (Minehart and Magasanik, 1991), Nit2 (Fu and Marzluf, 1990), Urbs1 (Voisard *et al*, 1991), and a mammalian GATA protein, hGATA-1 (Martin and Orkin, 1990), are compared to the C-C2 domains of Tas2, Sps18, and Gcs1. C2-C2 domains are aligned to highlight similarities (light shading) and identities (heavy shading). Amino-acid similarities are designated as previously described (Fig. 35). A consensus sequence is illustrated in bold type.

Figure 36

A


Tas2
Sps18
Gcs1
CxDCxxxxxQWxSxxxGxFICxxCAxIxRxxGxxxxxVKSISxDxF
E
F T
L
L
R T W

B


Tas2
Sps18
Gcs1
Da180
Gln3
Nit2
Urbs1
hGATA-1
WxxxxxGxxLCxxCxxxxKxxG
F
I
R

suggests that the function of this domain is conserved, and that this domain is important for protein function.

Both *SPS18* and *TAS2* encode gene products of unknown function, however sequences similar to the Gcs1/Sps18/Tas2 (GST) C2-C2 domain may be present in other proteins of known function. Identification of related sequences in proteins of known function may help to indicate the function of the GST C2-C2 domain. To determine if domains related to the GST C2-C2 domain are present in other proteins, the PIR protein database was first searched for all proteins containing C-x-x-C-x_{16-17}-C-x-x-C motifs. This search produced approximately 100 proteins, that were then inspected for further similarity to the consensus sequence for the GST C2-C2 domain. A weak similarity was detected in this way between the GST C2-C2 domain and the C2-C2 domain found in the GATA family of DNA-binding proteins (Fig. 36).

The GATA-protein family consists of a number of DNA-binding proteins, from phylogenetically diverse organisms, that all bind to the same [A/T]GATA[A/G] sequence (Orkin, 1992). This conservation of binding to the same *cis*-acting sequence by evolutionarily diverse GATA proteins is reflected in the highly conserved metal-binding consensus C2-C2 sequence: C-x-N-C-x4-T-P/T-L-W-R-R-x3-G-x3-C-N-A-C (Trainor *et al.*, 1990) found in all GATA proteins. In addition to the C2-C2 consensus sequence encompassing the sequence between the cysteine pairs, the DNA-binding motif of GATA proteins also includes additional basic amino acids just downstream of the C2-C2 motif. Studies of a 66-amino-acid peptide containing a GATA DNA-binding motif from the human GATA-1 protein have shown that basic amino acids as distant as 20-amino acids C-terminal from the C2-C2 motif are required for stable DNA binding (Omichinski *et al.*, 1993). Thus, C2-C2 motifs of the GATA type that function in DNA binding show conservation of both the C2-C2 motif and the C-terminal flanking regions.

Comparison of the GST C2-C2 domains with the C2-C2 domains of the GATA proteins shows an overall sequence similarity of 21% with two gaps included in the sequence alignments (Fig. 36). Although this similarily is not that high relative to the 46% similarity of the GST C2-C2 domains to each other, the similarity between the GATA C2-C2 domains and the GST C2-C2 domains does extend beyond the C2-C2 motif and includes C-terminal flanking amino-acids. As previously described, conservation of both the C2-C2 motif and the C-terminal flanking region would be expected for metal-binding domains that are functionally related to the GATA C2-C2 domain. The sequence similarity between the GST C2-C2 domains and the GATA C2-C2 domains is increased if only the sequence without gaps is considered, since the greatest sequence similarity is seen in the sequence surrounding the second cysteine pair in the four cysteine C2-C2 motif. However, the significance of this similarity between the GATA and GST C2-C2 domains is unclear. Since functional similarity between the GST and GATA C2-C2 domains would imply that the GST domain functions in DNA binding, further experiments are required to determine if Gcs1 is a DNA-binding protein.

3. Temperature-dependent gene regulation is altered in *gcs1* mutant cells

Experiments designed to map the 5' ends of *GCS1* transcripts by primer extension fortuitously detected additional non-*GCS1* transcripts that were present in total RNA from wild-type cells proliferating at 29°C, and were absent in total RNA from the same cells proliferating at 15°C (Fig. 22, lanes 2 and 7). Transcripts that showed this type of temperature-dependent gene regulation were detected with different primers (Fig. 23, lanes 2 and 6), indicating that cell proliferation at 15°C is accompanied by quantitative changes in transcript levels for a number of genes in wild-type cells. That is, growth at 15°C is normally associated with a quantitatively different pattern of gene expression.

The decreased abundance of non-*GCS1* transcripts in total RNA isolated from cells proliferating at 15°C was only observed in wild-type cells. In *gcs1* mutant cells incubated at 15°C, decreased abundance of non-*GCS1* transcripts was not observed (Fig. 22, lanes 3 and 8). Thus the quantitatively different pattern of gene expression present in wild-type cells is not observed in *gcs1* mutant cells, suggesting that Gcs1 is required for the normal regulation of gene expression during cell proliferation at 15°C.

The observation that the regulation of gene expression is altered in *gcs1* mutant cells at the restrictive temperature is particularly suggestive, since Gcs1 has been shown to be essential only for growth at low temperatures. Although the requirements for growth at low temperatures such as 15°C are not well understood, it has been suggested that the expression of certain genes is essential for relieving an innate cold sensitivity of some fundamental process in *Saccharomyces cerevisiae*. For example, genes found to be essential only at low temperaures include the *SAC1* gene, thought to encode a component of the actin cytoskeleton (Novick *et al.*, 1989). The hypothesis has been made that actin filament assembly, stability or function has an intrinsic cold sensitivity which is relieved by the *SAC1* gene product (Novick *et al.*, 1989). The altered expression of genes required for growth at low temperatures in *gcs1* mutant cells could therefore result in a cold-sensitive phenotype.

The argument could be made that the altered gene regulation observed in *gcs1* mutant cells at the restrictive temperature is a consequence of the inability to proliferate at the restrictive temperature. However, this is unlikely to be the case, since altered gene regulation was also observed at the permissive temperature under conditions in which *gcs1* mutant cells were engaged in active proliferation. Non-*GCS1* transcripts were present in total RNA from wild-type cells proliferating at 29°C, but were absent from total RNA of *gcs1* mutant cells proliferating at 29°C (Fig. 22, lanes 2 and 3).

Thus, the regulation of the non-*GCS1* transcripts is actually reversed in *gcs1* mutant cells.

This reversed regulation of temperature-dependent gene expression suggests a possible basis for the reentry-mutant phenotype. The reentry-mutant phenotype depends upon maintenance of ongoing proliferation in *gcs1* mutant cells at 15°C. A model for how ongoing proliferation might be maintained in *gcs1* mutant cells is suggested by the coincident patterns of gene expression in *gcs1* mutant cells proliferating at 29°C and wild-type cells proliferating at 15°C. It is possible that maintenance of ongoing proliferation at 15°C by *gcs1* mutant cells is dependent upon maintenance of the temperature-dependent gene expression observed in *gcs1* mutant cells proliferating at 29°C. In other words, *gcs1* mutant cells proliferating at 29°C may not switch their temperature-dependent gene expression after transfer to 15°C. The ability of proliferating *gcs1* mutant cells to maintain temperature-dependent gene expression appropriate to growth at 15°C would result in the continued proliferation of *gcs1* mutant cells after transfer to 15°C.

A model for the reentry-mutant phenotype which invokes inappropriate temperature-dependent gene expression in stationary-phase cells attempting to resume proliferation at 15°C may explain the differences in *gcs1*-mediated cold sensitivity in different genetic backgrounds. Possibly, in some genetic backgrounds, the temperature-dependent gene expression in proliferating *gcs1* mutant cells transferred to 15°C is switched to the pattern of gene expression that is incompatible with growth at 15°C (i.e. the pattern of gene expression observed in stationary-phase cells). This results in the inability of proliferating *gcs1* mutant cells to maintain ongoing proliferation at 15°C. Suppression of this switch would maintain the temperature-dependent gene regulation appropriate for growth at 15°C without necessarily affecting gene expression in stationary-phase *gcs1* mutant cells attempting to resume proliferation at 15°C. Further

exploration of the requirements for growth at 15°C and the molecular basis of the switch between different patterns of gene expression at different temperatures may improve our understanding of the reentry-mutant phenotype.

4. Reversed regulation of gene expression in *gcs1* mutant cells

The reversed regulation of non-*GCS1* transcripts is not limited to conditions in which *gcs1* mutant cells are unable to proliferate. A similar reversed regulation of some non-*GCS1* transcripts was observed during proliferation of wild-type and mutant cells at 29°C when using a different carbon source, galactose. In wild-type W303 cells these non-*GCS1* transcripts were absent from cells proliferating on galactose-based medium at 29°C although they were present in total RNA from cells proliferating on glucose-based medium at 29°C (Fig. 22, lanes 2 and 4). Thus proliferation of wild-type cells on galactose at 29°C is normally associated with a quantatively different pattern of gene expression when compared to that of cells proliferating on glucose at 29°C.

The decreased abundance of non-*GCS1* transcripts in total RNA isolated from cells proliferating on galactose was only observed in wild-type cells. In *gcs1* mutant cells, the non-*GCS1* transcripts were abundant during proliferation on galactose, but were absent or decreased in abundance during proliferation on glucose (Fig. 22, lanes 3 and 5). Thus, the regulation of the expression of the non-*GCS1* transcripts is reversed in *gcs1* mutant cells proliferating on galactose, in the same way that the expression of non-*GCS1* transcripts was reversed in *gcs1* mutant cells incubated at 15°C.

It is difficult to postulate a simple model to explain the reversed regulation of gene expression in *gcs1* mutant cells that does not require two competing forms of regulation. That is, in the absence of Gcs1 a different type of regulation is imposed. In a model in which Gcs1 functions as a DNA-binding protein, competition between different types of regulation could be imposed through competition by different DNA-

binding proteins for the same *cis*-acting promoter sequence. Precedent for different DNA-binding factors competing for the same sites has been established by the discovery of *cis*-acting promoter elements that contain overlapping sites for different DNA-binding proteins (Willet *et al.*, 1993, reviewed in Guarente, 1993). Thus in the absence of Gcs1, reversed regulation could be effected at the promoter level by the binding of a different transcription factor to the same site, with the new factor imposing the reversed regulation.

5. The phenotypes of *gcs1* mutant cells with different genetic backgrounds

The construction of *gcs1-6* null cells in different genetic backgrounds has demonstrated that *GCS1* is not always essential for cell proliferation at low temperatures; the requirement for Gcs1 function is dependent on genetic background. The most extreme example of this was observed in cells of strain FY56. That is, *gcs1-6* null cells with the FY56 genetic background are cold-resistant both for resumption of proliferation from stationary phase and for maintenance of ongoing cell proliferation, indicating that both of these requirements for Gcs1 function can be bypassed. This result may not be surprising in light of increasing evidence for a family of Gcs1-like proteins. In cells of strain FY56, the function of Gcs1 may be provided by these *GCS1*- related gene products.

In cells of strain W303, the requirement for Gcs1 function during cell proliferation at low temperatures is determined by growth status of the cells. For these cells, Gcs1 is required for resumption of cell proliferation from stationary phase; but not for maintenance of ongoing cell proliferation. The absence of a requirement for Gcs1 function for maintenance of ongoing cell proliferation results in a reentry-mutant phenotype. That is, *gcs1-6* null cells with the W303 genetic background are cold-sensitive only for the resumption of proliferation from stationary phase (Fig. 17).

However, this reentry-mutant phenotype can be modified to a cold-sensitivity phenotype in which Gcs1 is required for maintenance of ongoing cell proliferation. The introduction of the *gcs1-1* mutant allele together with linked modifier alleles (from other genetic backgrounds) into the W303 genetic background results in a phenotype of cold-sensitivity regardless of growth status (Fig. 25B). Thus in the presence of these modifier alleles, Gcs1 is required for maintenance of ongoing cell proliferation.

The presence of these *gcs1*-linked modifier alleles in other strain genetic backgrounds could make Gcs1 essential for maintenance of ongoing cell proliferation at low temperatures in those strains. This expectation may be realized in cells of strain 21R, since Gcs1 is required for maintenance of ongoing cell proliferation in these cells. That is, *gcs1-6* null cells with the 21R genetic background are cold-sensitive for maintenance of ongoing cell proliferation, and thus do not show a reentry-mutant phenotype (Fig. 26C). However, the requirement for Gcs1 function during maintenance of ongoing cell proliferation in cells of strain 21R can be satisfied by the gene product of the *gcs1-2* or *gcs1-3* truncation alleles (Fig. 28B). Thus, *gcs1-2* and *gcs1-3* truncation alleles with the 21R genetic background are cold-sensitive only for resumption of proliferation from stationary phase. The full-length Gcs1 protein is apparently required for resumption of proliferation from stationary phase in both the 21R and W303 genetic backgrounds. Thus the requirement for Gcs1 function for maintenance of ongoing proliferation is less stringent than the requirement for Gcs1 function during resumption of proliferation from stationary phase.

The influence of other genetic loci on the requirement for Gcs1 during maintenance of ongoing cell proliferation was recognized in earlier work. Previous work in the GR2 genetic background indicated that a requirement for Gcs1 during maintenance of ongoing cell proliferation was conferred by the *gcs1-1* mutation, but this requirement could be overcome by the presence of the unlinked *sed1-1* allele

(Drebot, 1987). However, the *sed1-1* allele did not alleviate the requirement for Gcs1 in resumption of proliferation from stationary phase. Thus the reentry-mutant phenotype required the combination of both the *gcs1-1* and *sed1-1* alleles. Reinterpretation of these results in light of the present work suggests an alternative explanation of the role of the *sed1-1* allele in the reentry-mutant phenotype. The *gcs1-1* mutant cells with the GR2 genetic background which are cold-sensitive regardless of growth status may contain *gcs1*-linked modifier alleles which modify the phenotype of these *gcs1-1* mutant cells from cold-sensitive only for resumption of proliferation from stationary phase to the more severe phenotype of cold-sensitivity regardless of growth status. Thus, the requirement for Gcs1 during maintenance of ongoing proliferation in strain GR2 may also be conferred by *gcs1*-linked modifier alleles. In this case, *sed1-1* may function to suppress the effects of the *gcs1*-linked modifier alleles on the phenotype of *gcs1-1* mutant cells resulting the production of cells with a reentry-mutant phenotype.

This sensitivity of the reentry-mutant phenotype to modifier alleles that may be present in the genetic backgrounds of laboratory strains, can be restated in terms of a model in which Gcs1 functions in transcription regulation. That is, Gcs1 may function to regulate gene expression during the resumption of proliferation from stationary phase at low temperatures. However, in the presence of linked modifier alleles, Gcs1 is also required at low temperatures for the appropriate expression of genes during maintenance of ongoing proliferation. This requirement for Gcs1 function during maintenance of ongoing proliferation can be suppressed by the unlinked modifier allele, *sed1-1*. Thus, Gcs1 is almost always required for the appropriate expression of genes during resumption of proliferation from stationary phase, but the requirement for Gcs1 function for maintenance of ongoing proliferation depends upon a combination of different factors that are determined by the strain genetic background.

6. Gcs1 function is independent of the mitotic cell cycle

The phenotype of *gcs1* mutants with the 21R genetic background suggests that the requirement for Gcs1 function is independent of the mitotic cell cycle. That is, stationary-phase cells containing the *gcs1-6* null mutation in the strain 21R genetic background were unable to resume proliferation after transfer to the restrictive temperature (Fig. 26A); however, by the criteria of bud formation, nuclear migration and medial nuclear division, these mutant cells were able to reenter the mitotic cell cycle from stationary phase. Thus in strain 21R, Gcs1 is required for resumption of cell proliferation from stationary phase, but may not be required for reentry into the mitotic cell cycle. This result implies that the requirement for Gcs1 function may be directly related to requirements for growth, and only indirectly related to requirements for the mitotic cell cycle. Thus, stationary-phase cells may be selectively impaired in some growth-related function by mutations in *GCS1*; however, cells with some genetic backgrounds can initiate the cell cycle before the growth-related function becomes impaired enough to halt cell-cycle activity.

Actively proliferating cells may be able to bypass the requirement for Gcs1 and maintain this hypothesized growth-related function in the absence of Gcs1 due to differences in the protein complement of proliferating and stationary-phase cells. That is, actively proliferating cells may have higher levels of gene products that can substitute for Gcs1. Alternatively, actively proliferating cells may be able to continue proliferation in the absence of the Gcs1-mediated function, because the function is not always required in proliferating cells.

7. Stationary-phase *gcs1* mutant cells are specifically impaired for resumption of proliferation

Stationary-phase *gcs1-6* null cells with the W303 genetic background are unable to resume proliferation or bud during incubation in fresh medium at 15°C. Defects in a hypothesized growth-related function that are severe enough to prevent the initiation of new cell cycles might result in decreased rates of mass accumulation. Consistent with this, the rate of protein synthesis in stationary-phase *gcs1* mutant cells attempting to resume proliferation at the restrictive temperature is markedly decreased (~4-fold) in both reentry-mutant *gcs1-1 sed1-1* cells with the GR2 genetic background (Drebot *et. al,* 1987) and in reentry-mutant *gcs1-6* null cells with the W303 genetic background (G.C. Johnston, personal communication). Thus one (possibly indirect) consequence of the reentry-mutant phenotype is a decreased rate of protein synthesis during resumption of proliferation from stationary phase. This *gcs1*-mediated defect in rate of protein synthesis is not observed in actively proliferating reentry-mutant cells transferred to the restrictive temperature: a reduced rate of protein synthesis would result in an increased doubling time, and these cells continue to proliferate with the same doubling time as wild-type cells (Fig. 17B).

Further support for the importance of rates of protein synthesis in reentry-mutant cells with the W303 genetic background comes from the observation that leucine prototrophy suppresses both the slow growth phenotype of W303 wild-type cells and the cold sensitivity of reentry-mutant cells. One explanation of these results is that W303 wild-type cells are cold-sensitive for leucine uptake resulting in decreased rates of protein synthesis and a slow-growth phenotype for Leu$^-$ W303 wild-type cells incubated at 15°C. In this hypothesis, leucine prototrophy restores intracellular leucine pools resulting in increased rates of protein synthesis at 15°C and suppression of the slow-growth phenotype of wild-type cells. More importantly, the suppression of *gcs1*-mediated cold-sensitivity by leucine prototrophy suggests that the defect in resumption of proliferation from stationary phase at 15°C is directly related to the decreased rates

of protein synthesis observed in these cells. Leucine prototrophy does not restore the growth rate of *gcs1* mutant cells to that of wild-type leucine prototrophs, but instead permits a slow-growth phenotype for *gcs1* mutant cells (equivalent to leucine-auxotrophic cells of strain W303) incubated at 15°C. Thus, leucine prototrophy only partially alleviates the *gcs1*-mediated defect in resumption of proliferation from stationary phase.

The hypothesis that leucine protrophy increases the rate of protein synthesis in stationary-phase *gcs1* mutant cells resuming proliferation at 15°C is easily tested; however, the relationship between increased rates of protein synthesis and suppression of the *gcs1*-mediated defect in resumption of proliferation may be very indirect. That is, the decreased rate of protein synthesis observed in *gcs1* mutant cells under restrictive conditions may be an indirect consequence of inappropriate gene expression during resumption of cell proliferation from staticnary phase.

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