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A member of a novel family of yeast 'Zn-finger' proteins mediates the transition from stationary phase to cell proliferation

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The cloning and molecular characterization of the *GCS1* gene from the budding yeast *Saccharomyces cerevisiae* show that stationary phase is in fact a unique developmental state, with requirements to resume cell proliferation that can be distinct from those for maintenance of proliferation. Deletion of the *GCS1* gene produces a novel phenotype: stationary-phase mutant cells do not resume proliferation at a restrictive temperature of 15°C, but mutant cells lacking Gcs1p that are proliferating at the permissive temperature of 29°C continue to proliferate after transfer to 15°C as long as nutrients are available. The *GCS1* gene sequence predicts a 39 kDa polypeptide with a novel 'Zn-finger' motif. A point mutation within the finger motif produces a phenotype that mimics that of deletion of the *GCS1* gene, showing that the finger motif is essential for full Gcs1p activity. Gcs1p and the products of two newly identified genes, *SPS18* and *GLO3*, constitute a family of novel Zn-finger proteins.

Key words: Gcs1p/Glo3p/*Saccharomyces cerevisiae*/ stationary phase/Zn-finger proteins

Introduction

For the budding yeast *Saccharomyces cerevisiae*, depletion of the nutrient supply causes cells to enter a non-proliferating state referred to as stationary phase (see Werner-Washburne *et al.*, 1993). Stationary-phase cells contain an unreplicated complement of DNA and are uniformly unbudded; both properties indicate that these cells have halted progress through the cell cycle and have yet to perform the regulatory step Start that initiates the mitotic cell cycle (Hartwell, 1974). In addition to this regulated cessation of proliferation, stationary-phase cells display a constellation of physiological and transcriptional alterations that distinguish them from actively proliferating cells (Werner-Washburne *et al.*, 1993). The stimulation of renewed growth and cell proliferation that is brought about by replenishing the nutrient supply causes a prompt loss of these stationary-phase properties. By these criteria the stationary-phase state is different than a simple suspension of events that characterize active cell proliferation.

The isolation and characterization of a yeast mutant with a novel conditional phenotype have revealed that starvation-induced stationary phase in fact represents a unique developmental state (Drebot *et al.*, 1987, 1990a,b; Werner-Washburne *et al.*, 1993). The novel phenotype depends on the physiological status of the mutant cell population at the time that restrictive conditions are imposed, so that mutant cells in stationary phase are impaired for the resumption of cell proliferation at 15°C when stimulated by the addition of fresh medium, and fail to resume cell proliferation at this restrictive temperature. Although stationary-phase mutant cells are defective for resumption of proliferation at 15°C, actively proliferating mutant cells are able to continue proliferation indefinitely when transferred to the restrictive temperature, as long as nutrients are provided. That is, the defect in these mutant cells is only manifested during the resumption of cell proliferation from stationary phase; mutant cells are unimpaired during active cell proliferation. This novel phenotype, which we have termed the re-entry mutant phenotype (Drebot *et al.*, 1987), is summarized in Table I (column B). This mutant phenotype is distinct from that of cold sensitivity at all times (Table I, column C), and thus demonstrates by genetic means that the requirements to resume cell proliferation from stationary phase are genetically distinguishable from the requirements to continue cell proliferation.

The failure of re-entry mutant cells (Table I, column B) to resume cell proliferation from stationary phase when stimulated at 15°C does not reflect an unresponsiveness to the stimulation brought about by fresh growth medium. Indeed, stationary-phase re-entry mutant cells and wild-type cells display the same physiological and transcriptional alterations when incubated in fresh growth medium at 15°C (Drebot *et al.*, 1987; Filipak *et al.*, 1992). Therefore, the genetic impairment in these re-entry mutant cells affects cellular physiology or development at a later time, after initial responses are triggered but before cell proliferation can resume.

Our initial studies (Drebot *et al.*, 1987) suggested that this novel re-entry mutant phenotype (Table I, column B) resulted from the interaction of mutations termed *gcs1-1* and *sed1-1*. In those experiments the *gcs1-1* mutation (plus an undefined linked mutation; see Results) imparted cold sensitivity under all growth conditions (Table I, column C). The presence of the *sed1-1* mutation allowed already proliferating mutant cells to continue to proliferate at 15°C, but did not allow the resumption of cell proliferation from stationary phase at 15°C (the re-entry mutant phenotype; Table I, column B). We show here that the *sed1-1* mutation is not always necessary to generate the novel re-entry mutant phenotype. Therefore the *gcs1-1*

Table I. Mutant phenotypes

Growth conditions initial	Test	Growth phenotype under test conditions		
		A wild-type	B re-entry mutant	C cold-sensitive
Log phase 29°C	log phase 29°C	+	+	+
Log phase 29°C	log phase 15°C	+	+	−
Stationary phase 29°C	fresh medium 29°C	+	+	+
Stationary phase 29°C	fresh medium 15°C	+	−	−

allele is the major genetic determinant of the mutant phenotype of these cells.

To understand the re-entry mutant defect and address the role of the Gcs1 protein (Gcs1p), we have characterized wild-type and mutant versions of the *GCS1* gene. Our molecular analysis reveals that the predicted Gcs1p contains a novel finger motif whose integrity is critical for Gcs1p function, and a C-terminal domain that is also important for Gcs1p function. Furthermore, we find that the absence of the *GCS1* gene alone can impose the novel re-entry mutant phenotype (Table I, column B), a conditional block only for resumption of proliferation from stationary phase.

Results

GCS1 is a novel gene

A genomic clone of the *GCS1* gene was isolated by complementation of the cold sensitivity imposed by the *gcs1-1* mutation (Figure 1A); subcloning localized *GCS1* to a 2.8 kbp fragment of plasmid p6d-3. We positioned the *GCS1* locus on the yeast genetic map by using a radiolabeled fragment from plasmid p6d-3 to probe a blot of separated yeast chromosomes, which localized the *GCS1* gene to chromosome IV (data not shown). Genetic crosses then positioned the *GCS1* gene between the *HO* and *CDC13* genes on the left arm of chromosome IV, within 1 cM of the *HO* locus (Table II). This tight genetic linkage reflects physical proximity; as shown in Figure 1A, significant overlap exists between the restriction maps of the *GCS1* genomic insert in plasmid p6d-3 and the *HO* 5′ flanking region (Jensen *et al.*, 1983) which positions the *HO* gene 3 kbp centromere-proximal to *GCS1*. Similar comparisons also showed that *GCS1* is distinct from *SSB1*, another gene localized to this region (Slater and Craig, 1989). Thus *GCS1* is a novel gene.

Sequence analysis of GCS1

As shown in Figure 1B, nucleotide sequence analysis of the 2.8 kbp complementing fragment from p6d-3 revealed a 1056 bp open reading frame (ORF) capable of encoding a 352 amino acid polypeptide with a predicted molecular weight of 39 000. To verify that this ORF defines the *GCS1* gene, a subclone (Figure 1A) carried on a low-copy vector was shown to allow mutant cells to resume proliferation from stationary phase at 15°C, while *gcs1-1* mutant cells transformed with vector alone remained unable to proliferate at 15°C (data not shown). This finding indicates that the ORF displayed in Figure 1B encodes the wild-type Gcs1 protein. Database searches showed that the *GCS1* gene has not been sequenced previously.

GCS1 transcripts display several 5′ ends

Inspection of the *GCS1* nucleotide sequence revealed two short in-frame ORFs upstream of the large ORF at positions −75 and −39 with respect to the predicted AUG translation start site (Figure 1B). Since some yeast transcripts contain upstream ORFs that have been shown to function in translational regulation (Hinnebusch, 1990), we wanted to determine if the *GCS1* transcript encompasses these upstream ORFs. The 5′ ends of the *GCS1* transcript(s) were therefore mapped by primer extension using two different primers (Figure 1B). These studies localized the transcription start sites for the *GCS1* gene to positions −31, −27, −23 and −14 relative to the initiator AUG codon (Figure 1B). The untranslated leader sequences for *GCS1* transcripts therefore exclude both upstream ORFs, suggesting that the *GCS1* gene is not subject to translational regulation by these upstream ORFs.

Gcs1p contains a 'Zn-finger' motif

Indications of Gcs1p function were provided by inspection of the Gcs1p polypeptide sequence. Two interesting sequence motifs were identified near the N-terminus of Gcs1p. The first motif, comprising amino acid residues 11–25, is a basic motif that resembles a nuclear localization sequence (Dingwall and Laskey, 1991), and suggests that Gcs1p may function within the nucleus. The second feature of the Gcs1p predicted polypeptide is a $CxxCx_{(16)}CxxC$ motif, encompassing amino acids 26–49 (Figure 1B), that resembles a 'Zn-finger' structure.

The gcs1-1 mutation alters the Zn-finger motif

The significance of the Zn-finger motif became evident through a molecular analysis to identify the genetic alteration characterizing the *gcs1-1* allele. Integration at the *gcs1-1* chromosomal locus of a 5′ fragment of *GCS1* generated two adjacent chromosomal versions of the *GCS1* gene: a truncated version of the resident *gcs1-1* locus equivalent to the truncated *GCS1* sequences on the transforming plasmid, and an intact but chimeric *GCS1* gene containing 3′ sequences derived from the *gcs1-1* locus (Figure 2). Transformants with this chromosomal configuration were cold-resistant, suggesting that the intact but chimeric version of the *GCS1* gene is functional. The 3′ end of the *gcs1-1* allele that makes up the 3′ end of the functional chimeric gene therefore does not contain mutations that can themselves affect Gcs1p function; the deleterious mutation most probably resides in the 5′ part of the *gcs1-1* mutant allele, within the first 230 codons of the *GCS1* ORF. The *gcs1-1* mutation was localized further within the N-terminal coding region by use of PCR. By this procedure the entire mutant *gcs1-1* gene was amplified, cloned and sequenced. These results showed

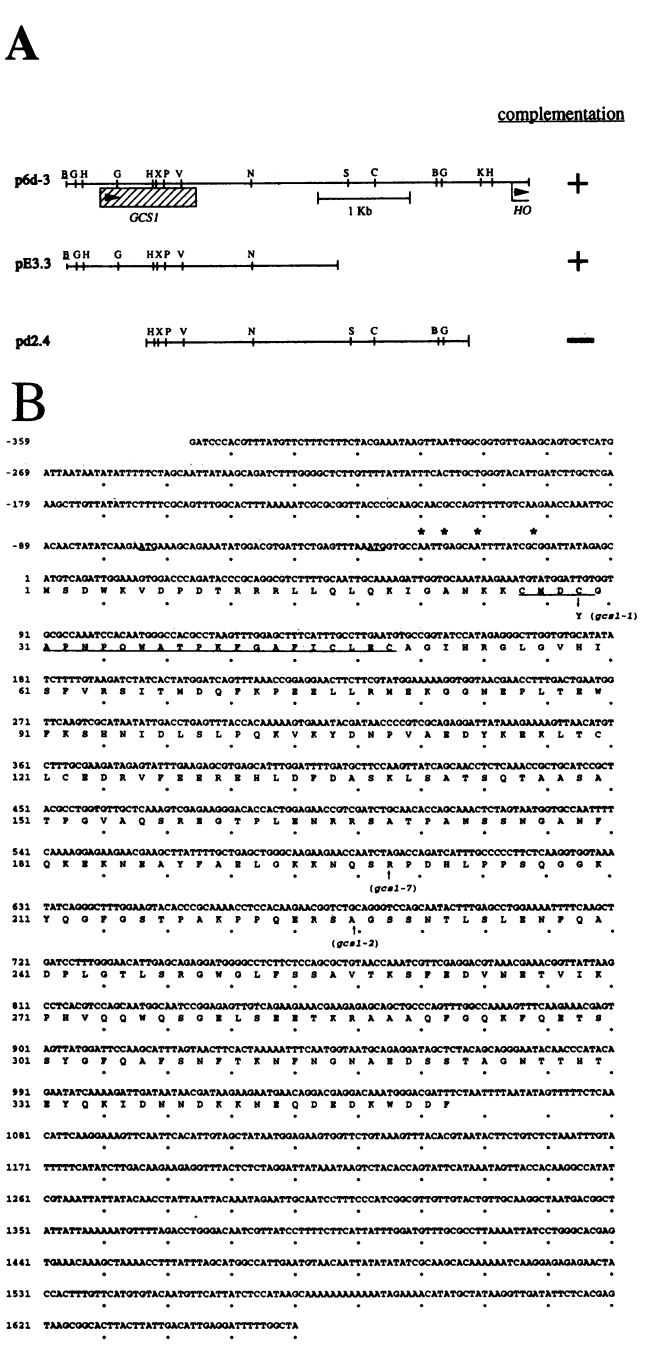

Fig. 1. Restriction map, complementation analysis and nucleotide sequence of the *GCS1* gene. (**A**) The restriction maps of the 6 kb complementing insert from plasmid p6d-3 and two subclones are shown along the top of each fragment. The position of the *GCS1* ORF is represented by the hatched box and the 5′ end is indicated by the solid arrow. For reference, the restriction map of the 5′ end of the flanking *HO* gene is included and the open box represents the 5′ portion of the *HO* ORF. +, complementation (colony formation at 15°C); −, no complementation (failure to form colonies at 15°C); B, *Bam*HI (the underlined *Bam*HI site defines the vector/insert boundary but is not present in the *GCS1* chromosomal locus); C, *Cla*I; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; P, *Pst*II; S, *Sst*I; V, *Pvu*II; X, *Xba*I. (**B**) The nucleotide sequence of the *GCS1* gene, numbered from the presumptive ATG codon specifying the initiator methionine, which is numbered as the first amino acid residue. The Zn-finger motif and the two upstream ATGs are underlined. Each major transcription start site is marked by an asterisk over the nucleotide. The amino acid at position 29 in the *gcs1-1* mutant gene and the sites of the truncations that generate the *gcs1-2* and *gcs1-7* alleles are indicated below the corresponding amino acid sequence.

Table II. Genetic linkage

Genetic interval	Ascus type PD	NPD	TT	Map distance (cM)[a]
gcs1−cdc13	30	0	2	3.1
gcs1−HO::URA3	44	0	1	1.1

[a]Genetic distances were calculated as specified by Mortimer and Schild (1985).

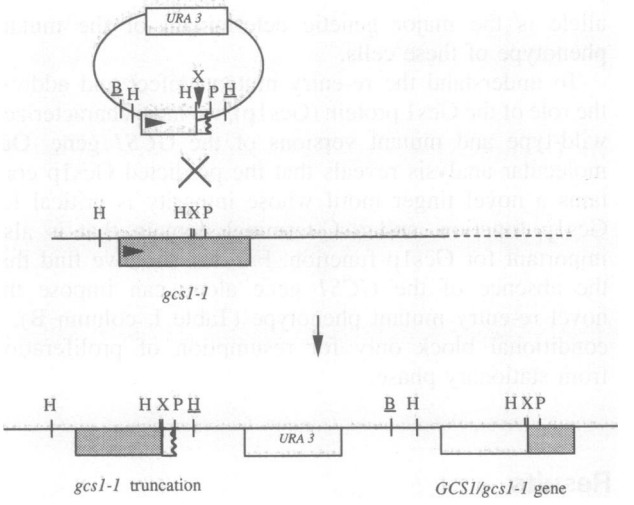

Fig. 2. Production of a chimeric *GCS1* gene. A *URA3*-based plasmid carrying an insert truncated at the *Pst*I site was linearized at the unique *Xba*I site within the *GCS1* nucleotide sequence. Integration at the *gcs1-1* mutant locus resulted in adjacent chromosomal versions of the *GCS1* gene: a truncated version similar to the plasmid-borne sequences, and an intact chimeric *GCS1* gene with 3′ sequences derived from the *gcs1-1* locus. Open boxes, plasmid-derived yeast sequences; stippled boxes, chromosomal sequences; restriction sites are designated as in the legend to Figure 1A (vector-derived restriction sites are underlined). The chromosomal structure was confirmed by Southern analysis.

that in fact two significant sequence alterations were present, and that both cause missense changes in the encoded Zn-finger motif. One alteration causes a conservative substitution of isoleucine for methionine at position 27, but the second alteration causes a non-conservative change of cysteine to tyrosine at position 29 (Figure 1B).

The amino acid sequence alterations within the Zn-finger motif of Gcs1p could reflect either mutational changes that actually contribute to the mutant phenotype or naturally occurring polymorphisms (the yeast DNA library used to clone the wild-type *GCS1* gene was derived from a strain unrelated to the original *gcs1-1* mutant strain). To determine the origins and significance of these two amino acid substitutions, we employed PCR to amplify the relevant *GCS1* region from the parent strain in which the *gcs1-1* allele was generated. Sequencing this amplified DNA showed that the conservative amino acid substitution at position 27 of *gcs1-1* (Figure 1B) is also encoded in the wild-type *GCS1* gene of the parent strain, and is thus a silent polymorphism not responsible for the re-entry mutant phenotype. In contrast, the Cys to Tyr alteration encoded by *gcs1-1* (Figure 1B) is not found in the parental *GCS1* allele and is therefore responsible for the *gcs1-1* mutant phenotype. This analysis shows that the Zn-finger motif in Gcs1p is essential for Gcs1p function.

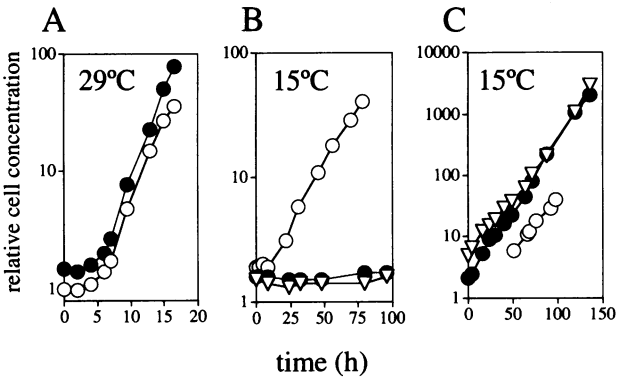

Fig. 3. Effect of temperature and growth status on the proliferation of *gcs1* mutant cells. Stationary-phase wild-type (○), *gcs1-6* null mutant (●) and *gcs1-1* mutant cells (Δ) were stimulated by the addition of fresh medium at 29 (**A**) or 15°C (**B**), or proliferating cells were transferred from 29 to 15°C (**C**). At intervals samples were removed for the determination of cell concentration. All strains were congenic with strain W303-1A; the *gcs1-1* mutant allele was present on a CEN plasmid in *gcs1-6* null mutant cells.

gcs1-6 null mutant cells are viable and display a re-entry mutant phenotype

The phenotype of cells harboring the *gcs1-1* mutation (Table I) suggests that Gcs1p function, under some conditions, may be necessary for cell growth (Drebot *et al.*, 1987). To assess the role of Gcs1p we created cells in which the *GCS1* gene had been replaced by the *URA3* gene, thereby creating the *gcs1-6* null allele. This gene replacement was carried out in *ura3-1* diploid cells, which allowed us to replace one homolog of the *GCS1* gene while the other intact *GCS1* gene in the diploid cell provided Gcs1p function. Diploid transformants identified by uracil prototrophy were then sporulated to yield haploid meiotic segregants. Each meiosis produced four haploid spores, two Ura^+ (that is, *gcs1-6*) and two Ura^- (*GCS1*), and all spores germinated and grew into colonies at 29°C. Moreover, *gcs1-6* null mutant cells at 29°C were able to resume proliferation from stationary phase and continue to proliferate with kinetics indistinguishable from the wild-type parent (Figure 3A). These results show that the *GCS1* gene is not essential at 29°C.

The importance of Gcs1p at the restrictive temperature of 15°C was assessed first by replica plating; using this test the *gcs1-6* null mutant segregants failed to proliferate. Most cells transferred by replica plating are in stationary phase at the time of transfer, which means that this replica plating assay cannot determine if the *gcs1-6* mutant cells are defective for long-term cell proliferation and colony formation, or simply for the resumption of proliferation from stationary phase (i.e. it cannot distinguish between the phenotypes described in columns B and C of Table I). To distinguish between these and other possibilities, we determined the *gcs1-6* null mutant phenotype in liquid culture. Mutant cells grown to stationary phase at 29°C and then incubated in fresh medium at 15°C failed to proliferate (Figure 3B; consistent with the phenotypes described in either columns B or C in Table I). However, when mutant cells that were proliferating exponentially in liquid medium at 29°C were challenged to continue to proliferate after transfer to the restrictive temperature of 15°C, these *gcs1-6* null mutant cells continued to proliferate with a population doubling time indistinguishable from that of wild-type cells (Figure 3C). That is, the *gcs1-6* null mutation imposes the re-entry mutant phenotype (Table I, column B; Drebot *et al.*, 1987, 1990a). We conclude from this experiment that Gcs1p is needed at 15°C for the resumption of cell proliferation, although not necessarily for ongoing cell proliferation.

Previously we have shown that the re-entry mutant phenotype was only revealed when mutant cells had been in stationary phase; mutant cells arrested in proliferation for other reasons, such as the presence of the mating pheromone α-factor or the S-phase inhibitor hydroxyurea, were not impaired for the resumption of proliferation at 15°C when these inhibitors were removed (Drebot *et al.*, 1990a). *gcs1-6* null mutant cells, first arrested for cell proliferation by α-factor or hydroxyurea and subsequently released from blocking conditions, were also able to resume proliferation promptly at 15°C, confirming our previous findings (data not shown). Thus the re-entry mutant phenotype is specific for the resumption of cell proliferation after a cessation of proliferation due to nutrient depletion (stationary phase).

Another facet of the re-entry mutant phenotype was revealed when *gcs1-6* null mutant cells proliferating at the restrictive temperature of 15°C eventually exhausted the liquid nutrient medium. These mutant cells that had been proliferating at 15°C ceased proliferation and acquired the stationary-phase properties of a uniform unbudded morphology and increased thermotolerance (Werner-Washburne *et al.*, 1993). By these criteria, mutant cells entered stationary phase where they became impaired for the resumption of proliferation at 15°C (data not shown). Gcs1p is thus dispensable at 15°C (and 29°C) for both active cell proliferation and entry into stationary phase, and plays a role only in the resumption of cell proliferation.

gcs1-6 null mutant cells respond to the stimulus of fresh growth medium

The resumption of cell proliferation from a starved, stationary-phase state entails various physiological responses by a stationary-phase cell to the presence of fresh growth medium (reviewed by Werner-Washburne *et al.*, 1993). To assess this responsiveness in *gcs1-6* null mutant cells, we monitored global biosynthetic activity, the loss of thermotolerance and altered patterns of gene expression that characterize the response to fresh growth medium by stationary-phase wild-type cells (Werner-Washburne *et al.*, 1993). Stimulated stationary-phase *gcs1-6* re-entry mutant cells did not synthesize DNA (Figure 4D) or proliferate (Figure 3B) at 15°C, and remained as a population of unbudded cells with single nuclei (Figure 4A). Nevertheless, these same stimulated re-entry mutant cells responded to the stimulation provided by fresh growth medium at this temperature by loss of thermotolerance (Figure 4B) and increased biosynthetic activity (Figure 4E and F), as monitored by the incorporation of radiolabeled precursors into acid-precipitable material. Measurement of RNA content 8 h after stimulation showed that mutant cells had undergone an ~3-fold increase in low molecular weight RNA (tRNA) and a 2-fold increase in high molecular weight RNA (rRNA). Although these values were somewhat less than the 5-

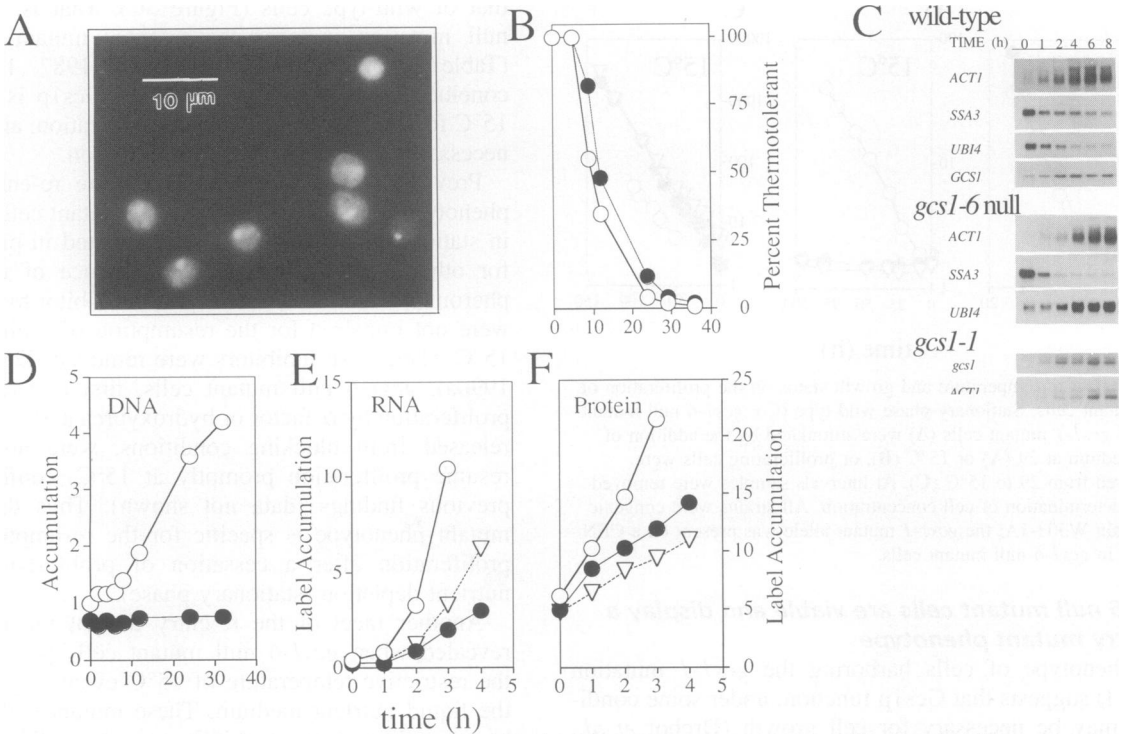

Fig. 4. Responses of stationary-phase *gcs1* mutant cells to the stimulation of fresh medium. Stationary-phase wild-type (○), *gcs1-6* null mutant (●) and *gcs1-1* mutant cells (Δ) were stimulated by the addition of fresh medium at 15°C. At intervals, samples were removed for the determination of thermotolerance (**B**), mRNA abundance (**C**), and DNA (**D**), RNA (**E**) and protein (**F**) accumulation. DNA accumulation is expressed on a per ml basis normalized to the starting value for the wild-type population. 48 h after the addition of fresh growth medium, *gcs1-6* null mutant cells were stained with DAPI and photographed (**A**). All cells maintained viability during the course of these experiments, as measured by colony formation on solid medium.

and 7-fold increases, respectively, for wild-type cells (data not shown), stimulated re-entry mutant cells can accumulate significant amounts of RNA. In addition, the changes in abundance of specific mRNAs in *gcs1-6* mutant cells paralleled those found in wild-type cells (Figure 4C). The *gcs1-6* null mutant cells became depleted for the stationary-phase *SSA3* transcript (Boorstein and Craig, 1990) and activated expression of the *ACT1* actin gene. Thus, Gcs1p is not necessary for these initial physiological and transcriptional responses of stationary-phase cells to the stimulation produced by fresh growth medium, even though Gcs1p is needed (at 15°C) for later activities that allow the resumption of cell proliferation.

gcs1-6 null mutant cells undergo physiological stress

Wild-type yeast cells express the *UBI4* polyubiquitin gene in stationary phase and also under conditions considered to bring on physiological stress (Finley *et al.*, 1987). Stationary-phase *gcs1-6* null mutant cells contain normal amounts of *UBI4* mRNA, but unlike wild-type cells maintain significant levels of *UBI4* mRNA after transfer to fresh growth medium (Figure 4C). Apparently Gcs1p is needed at 15°C to develop normal cellular physiology during the time that starved, stationary-phase cells respond and adapt to satisfactory growth conditions.

The gcs1-1 Zn-finger mutation confers a re-entry mutant phenotype

Our original genetic analysis of the re-entry mutant phenotype (Drebot *et al.*, 1987) led us to conclude that *gcs1-1* and another mutation, *sed1-1*, were involved in this novel phenotype. Thus the creation of a re-entry mutant phenotype by the absence of Gcs1p alone, as demonstrated above for *gcs1-6* null mutant cells, was unexpected. The involvement of another mutation, as indicated earlier (Drebot *et al.*, 1987), could indicate that the *gcs1-1* mutation imposes additional defects compared with the complete absence of Gcs1p, an unlikely situation given the recessive nature of the *gcs1-1* mutation (Drebot *et al.*, 1987). More likely, there may be accompanying mutation(s) in yet other genes that exacerbate the effects of *gcs1-1* and cause the phenotype described in column C of Table I. To clarify this situation we constructed equivalent *gcs1-1* mutant strains by molecular and standard genetic procedures, and compared phenotypes.

To construct a *gcs1-1* mutant strain by molecular means we introduced the cloned *gcs1-1* mutant allele on a low-copy centromere-based plasmid into *gcs1-6* null mutant cells. In these cells the only Gcs1p is encoded by the *gcs1-1* mutant gene; as shown in Figure 4C, the *gcs1-1* mutant gene is expressed upon stimulation of stationary-phase cells. Nevertheless, *gcs1-1 gcs1-6* mutant cells displayed a typical re-entry mutant phenotype, as shown in Figure 3; stationary-phase *gcs1-1 gcs1-6* null mutant cells transferred to fresh growth medium failed to proliferate at 15°C (Figure 3B), but mutant cells that were already proliferating at 29°C continued to proliferate after transfer to 15°C (Figure 3C). This is the phenotype of *gcs1-6* null mutant cells described above. (Selective conditions were maintained to ensure retention of the *gcs1-1* plasmid.) The *gcs1-1* allele, like the complete absence of Gcs1

polypeptide, therefore produces a re-entry mutant phenotype (Table I, column B).

Different results were obtained when the *gcs1-1* allele was introduced by genetic procedures. With multiple backcrosses we placed the *gcs1-1* mutant allele into the same genetic background (strain W303-1A) used to study the *gcs1-6* null allele. From the fifth backcross, the growth patterns of the 10 *gcs1-1* segregants from five complete tetrads were assessed in liquid medium and compared with the growth of the highly related *gcs1-1* mutant strain constructed as described above by molecular means. Remarkably, each *gcs1-1* meiotic segregant showed not the re-entry mutant phenotype but the cold-sensitive phenotype (Table I, column C; data not shown). The different phenotypes imposed by the *gcs1-1* mutation are probably not due to the subtle effects of *gcs1-1* gene dosage; the introduction of a high-copy plasmid harboring the *gcs1-1* mutant gene into a *gcs1-1* meiotic segregant did not relieve (or exacerbate) the cold-sensitive phenotype (Table I, column C; data not shown). Increased *gcs1-1* mutant gene expression in this situation was confirmed by Northern analysis (data not shown). Thus, there is likely to be at least one additional, uncharacterized mutation, introduced by genetic means along with the *gcs1-1* allele, that alters the *gcs1-1* phenotype from re-entry mutant to cold-sensitive. Complete nucleotide sequencing of the mutant *gcs1-1* gene showed that the only amino acid changes were those within the Zn-finger motif. Thus, any additional mutation is extragenic. Our limited genetic analysis shows that this additional mutation may be linked (<5 cM) to the *gcs1-1* mutant allele (data not shown) and therefore difficult to separate by recombination. Alternatively, a non-Mendelian determinant may affect the *gcs1-1* mutant phenotype.

The gcs1-1 Zn-finger mutation severely attenuates Gcs1 function

As described above, *gcs1-6* null mutant cells that harbor a plasmid-borne *gcs1-1* allele still display the re-entry mutant phenotype (Table I, column B). This finding suggests that the *gcs1-1* mutant polypeptide has little effect on *gcs1-6* null mutant cells. Another test of *gcs1-1* function was the incorporation of RNA and protein precursors upon stimulation of stationary-phase cells by fresh growth medium. Here the plasmid-borne *gcs1-1* mutant allele conferred only slight differences in the accumulation of RNA and protein compared with cells without the *gcs1-1* mutant allele (Figure 4E and F). This assay demonstrates that the activity of the *gcs1-1* mutant polypeptide is attenuated significantly.

The C-terminal third of Gcs1p is also required for Gcs1p activity

As shown above, the N-terminal region of Gcs1p, containing the Zn-finger motif, has an important role in Gcs1p function. To test the possibility that the C-terminal domain of Gcs1p also has a role in Gcs1p function, we generated *gcs1* mutant alleles encoding polypeptides truncated for C-terminal residues (Figure 1B). These studies showed that removal of 125 or 152 amino acids from the C-terminus of Gcs1p, as caused by the *gcs1-2* or *gcs1-7* truncation mutations, respectively, made mutant cells unable to resume cell proliferation from stationary phase at 15°C (Table I, column B; data not shown). The C-terminal residues therefore are necessary for Gcs1p function during that physiological transition.

Gcs1p is part of a family of Zn-finger proteins

In the course of a search for copy-suppressor genes capable of bypassing the absence of the *GCS1* gene we identified a novel gene, which we have termed *GLO3* (**G**cs1p-**l**ike **O**RF), that encodes a polypeptide with remarkable similarity to Gcs1p. As shown in Figure 5, the *GLO3* gene encodes a 408 amino acid protein with a predicted molecular weight of 45 695 Da, and Northern blot analysis reveals an ~1.4 kb transcript (data not shown). The Glo3p protein contains the Zn-finger motif that characterizes Gcs1p (Figure 5) and a C-terminal region with similarity to that of Gcs1p (see Discussion). To determine if *GLO3* has been identified previously, we mapped *GLO3* using the yeast lambda phage clone grid (Riles *et al.*, 1993). The *GLO3* gene is located on the right arm of chromosome V between *RAD51* and *BEM2*. This physical mapping and our nucleotide sequence analysis suggest that the *GLO3* gene is a previously unidentified gene in *S.cerevisiae*.

Discussion

Gcs1p plays a novel role in the resumption of yeast cell proliferation from stationary phase. In contrast, Gcs1p is dispensable for continued proliferation of actively growing cells, as shown by the phenotype (Figure 3) of *gcs1-6* null mutant cells lacking *GCS1* coding sequences. The fact that the re-entry mutant phenotype can be caused by the *gcs1-6* null allele rules out hypotheses based on quantitative differences in Gcs1p activity, and emphasizes

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   1    TAT AAT ACC AGT ACC TAG ATG TTA CGA ATG CTG TTC CCC TGT TAC TCT CTA AGC TGC CCT
  61    TTT CAA TTC ACC TAT AGA ATG TTT AAA ATT TTA CTT TCA ATA TTG GTA TTA TCT ATG ACA
 121    CAA AAG GAA AAG TAA CAT CGT TTG GTC ATT TTC GAC GAC ACG GTA AAG TTT CGA AAA AAA
 181    AAA AAA AAA AAG AAA ACA ACT ATT CTT CGT AAA CAC GAT TTA GCT GGC ATT TTA TAA AAG
 241    GAG ATC AAG GCG ATA GCA AAG AAA GGG TAT TTG TTA AGT ACT TGT TGA TCC GGT CTT CTT
 301    GAG CAA TTT AGT ATA CAC ATA GCG ACA ATG AGT AAC GAT GAA GGA GAA ACA TTT GCC ACG
                                          M  S  N  D  E  G  E  T  F  A  T
361/ 12 GAA CAG ACT ACT CAA CAA GTT TTT CAG AAG CTA GGT AGC AAC ATG GAA AAT CGA GTT TGT
        E  Q  T  T  Q  Q  V  F  Q  K  L  G  S  N  M  E  N  R  V  C
421/ 32 TTT GAT TGT GGT AAC AAG AAT CCT ACT TGG ACT TCG GTA CCT TTT GGT GTG ATG CTG TGT
        F  D  C  G  N  K  N  P  T  W  T  S  V  P  F  G  V  M  L  C
481/ 52 ATT CAA TGC TCT GCT GTG CAT AGA AAC ATG GGT GTT CAT ATC ACC TTT GTA AAG TCT TCT
        I  Q  C  S  A  V  H  R  N  M  G  V  H  I  T  F  V  K  S  S
541/ 72 ACA TTA GAT AAA TGG ACA ATT AAC AAT TTA AGA AGG TTT AAG TTG GGT GGG AAC CAT AAG
        T  L  D  K  W  T  I  N  N  L  R  R  F  K  L  G  G  N  H  K
601/ 92 GCT CGT GAT TTT TTC TTA AAG AAC AAT GGG AAA CAG CTA CTA AAT ACA GCA AAT GTT GAC
        A  R  D  F  F  L  K  N  N  G  K  Q  L  L  N  T  A  N  V  D
661/112 GCG AAA ACA AAA TAT ACG AGC CCT GTT GCT AAG AAA TAT AAG ATA CAT CTC GAC AAG AAG
        A  K  T  K  Y  T  S  P  V  A  K  K  Y  K  I  H  L  D  K  K
721/132 GTT CAA AAG GAT ATG GAG TTA TAC CCA TCA GAA TTA GTC TTG AAC GGT CAA GAT TCT TCG
        V  Q  K  D  M  E  L  Y  P  S  E  L  V  L  N  G  Q  D  S  S
781/152 GAC TCA CCG TTG GAC ACC GAT TCA GAC GCT TCC AGG TCG ACT TCC AAA GAA AAT AGT GTT
        D  S  P  L  D  T  D  S  D  A  S  R  S  T  S  K  E  N  S  V
841/172 GAC GAT TTT TTC TCT AAT TGG CAA AAA CCA AGC TCC AAT TCC AGT TCT AAA CTC AAT GTA
        D  D  F  F  S  N  W  Q  K  P  S  S  N  S  S  S  K  L  N  V
901/192 AAT ACA GGC TCT CTC GCC CCA AAA AAC AAC ACC ACA GGC AGC ACA CCC AAG ACC ACT GTC
        N  T  G  S  L  A  P  K  N  N  T  T  G  S  T  P  K  T  T  V
961/212 ACT AAA ACG AGA AGT TCC ATC TTA ACG GCA TGC AGA AAG AAA CCG GTA TTA AAC TCG CAA
        T  K  T  R  S  S  I  L  T  A  C  R  K  K  P  V  L  N  S  Q
1021/232 GAT AAA AAG AAA CAT TCA ATT TTG TCT TCG TCA AGA AAG CCC ACT AGA CTA ACC GCA AAG
        D  K  K  K  H  S  I  L  S  S  S  R  K  P  T  R  L  T  A  K
1081/252 AAA GTC GAC AAA TCA CAG GCA GAA GAC TTG TTT GAT CAA TTT AAA AAA GAG GCT CAA CAA
        K  V  D  K  S  Q  A  E  D  L  F  D  Q  F  K  K  E  A  Q  Q
1141/272 GAA AAG GAG GAC GAG TTC ACT AAT TCG TCT TCT AGC ACC AAA ATT AGA CAA AAT GAT TAC
        E  K  E  D  E  F  T  N  S  S  S  S  T  K  I  R  Q  N  D  Y
1201/292 GAT TCC CAA TTT ATG AAT AAC AGT AAA GGC AAC AAT AAT AAC AGT ATA GAT GAT ATT AAT
        D  S  Q  F  M  N  N  N  S  K  G  N  N  N  N  S  I  D  D  I  N
1261/312 ACC CAG CCT GAT GAA TTC AAT GAT TTT TTA AAT GAC ACT TCA AAC AGC TTC GAC ACA ACT
        T  Q  P  D  E  F  N  D  F  L  N  D  T  S  N  S  F  D  T  T
1321/332 AGA AAA GAA CAG CAA GAT ACT TTA ACA CCC AAG TTT GCC AAA TTG GGA TTT GGT ATG ACC
        R  K  E  Q  Q  D  T  L  T  P  K  F  A  K  L  G  F  G  M  T
1381/352 ATG AAT GAC GCC AAT GAT TTG GCA AAG CAA CAA AAA GAA TCT CAG AAA ATA GCT CAG GGA
        M  N  D  A  N  D  L  A  K  Q  Q  K  E  S  Q  K  I  A  Q  G
1441/372 CCA AGA TAT ACG GGC AGA ATC GCG GAA AGG TAC GGC ACA CAA AAA GCT ATA TCA TCT GAT
        P  R  Y  T  G  R  I  A  E  R  Y  G  T  Q  K  A  I  S  S  D
1501/392 CAA TTA TTT GGT CGT GGC TCA TTT GAT GAA GCA GCA AAC AGG GTA AGC GCA TGA CAA GTT
        Q  L  F  G  R  G  S  F  D  E  A  A  N  R  V  S  A  *
1561    GAA AAC ATT TGA CAA TGC TAC ATC AAT TTC CTC TTC TTC ATA TTT GGG AGA AGA TAA AGA
1621    AGT GGA CGA ATT TGG TAA TCC CAT CAA TAG TAG TGG AAG TGG AGC AGG TAA TTT TGA TGG
1681    CCG TAA TTC TAA TAA CGG TTT CAT CGA TTT TAA TGC CTC CGC AGA TGA TGA ATT GCA GAT
1741    GTT GAG AGA CGT GTA GAA CAG GGT GCT GAA AAA CTC GGT AGT TAT TTA AGG GAC TAC TTA
1801    AGA AAA TAA
```

Fig. 5. The *GLO3* gene. The nucleotide and derived amino acid sequences are numbered as in Figure 1.

the qualitative distinction between ongoing cell proliferation and the resumption of proliferation from stationary phase. At a restrictive temperature where Gcs1p is necessary for resumption of cell proliferation from stationary phase (Figure 3), the usual physiological responses of stationary-phase cells to the stimulation provided by fresh growth medium still take place in cells without Gcs1p (Figure 4), suggesting that Gcs1p function becomes important for the resumption of cell proliferation from stationary phase late in that developmental process.

The GCS1 yeast gene family

Genetic mapping and database searches indicated that the *GCS1* gene has not been reported previously. However, two other genes from the budding yeast (Figure 6) and a novel gene identified in the fission yeast *Schizosaccharomyces pombe* (N.Walworth and D.Beach, personal communication) encode polypeptides with significant structural similarity to Gcs1p. One of these gene products, Sps18p (Coe *et al.*, 1994), is 49% identical to Gcs1p in the Zn-finger region spanning residues 13–82. In addition, Gcs1p is 37% identical to Sps18p in the C-terminal region spanning residues 225–311; a third gene, *GLO3*, encodes a protein with striking similarity to Gcs1p in the Zn-finger region (51% identity between residues 13 and 82). The function of each of these gene products remains undefined, but the *SPS18* transcript is only evident during sporulation (Coe *et al.*, 1994), a developmental process that, like entry into stationary phase, is triggered by starvation. Thus, Gcs1p may belong to a family of proteins with functions related to progression from a starved, stationary-phase state: Sps18p for meiosis and sporulation, and Gcs1p for the resumption of mitotic cell proliferation.

Functional domains of Gcs1p

Our *in vitro* mutagenesis and mutational analyses show that Gcs1p contains at least two functional domains. A Gcs1p C-terminal domain was shown by gene-truncation studies to be necessary for the resumption of proliferation from stationary phase. N-terminal sequences critical for Gcs1p activity were localized by the identification at position 29 of a substitution that severely attenuates *gcs1-1* polypeptide function. The *gcs1-1* amino acid substitution replaces a cysteine residue in a Zn-finger motif (Figure 1B). These findings suggest that the Zn-finger domain, including the cysteine at position 29, is important for Gcs1p activity.

The Gcs1p finger

The Gcs1p Zn-finger motif is highly conserved among Gcs1p, Sps18p, Glo3p (Figure 6A) and a *S.pombe* protein (N.Walworth and D.Beach, personal communication), and

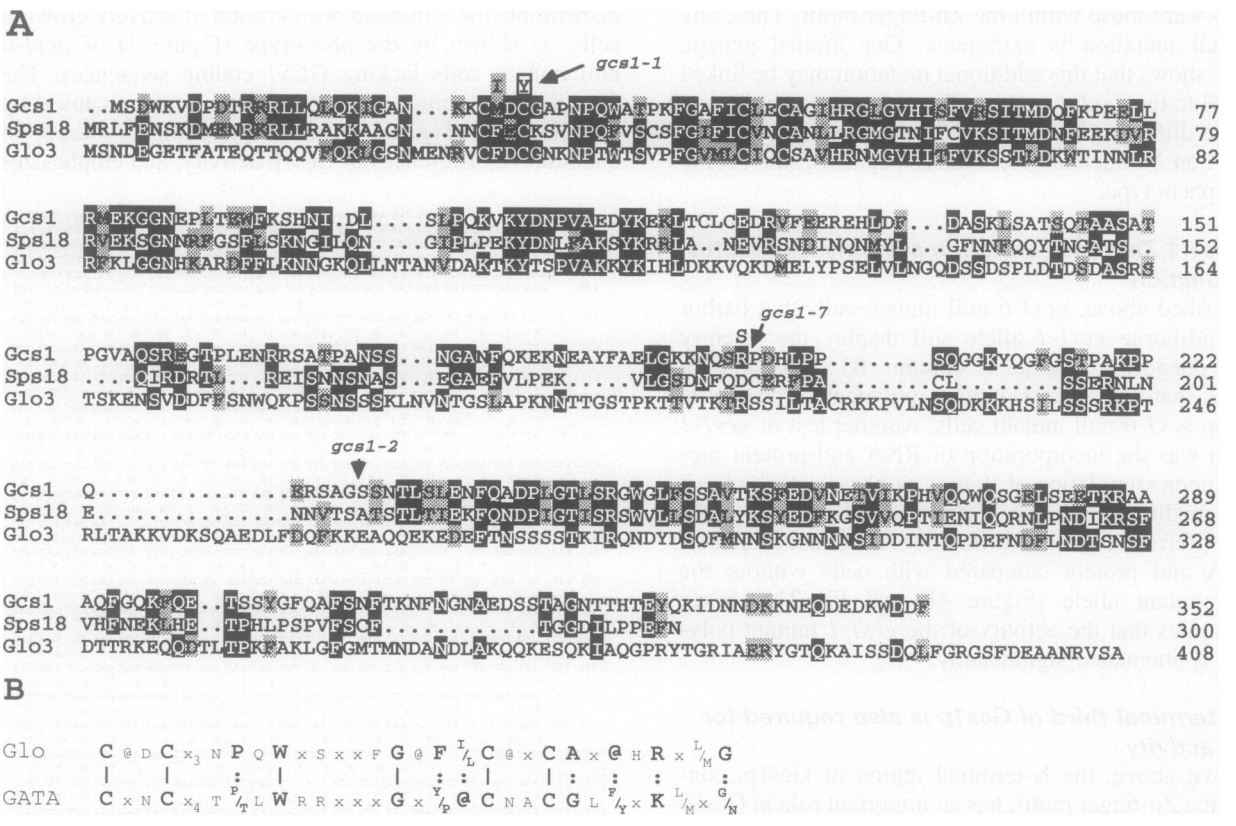

Fig. 6. Nucleotide sequence comparisons. (**A**) The predicted amino acid sequences of Gcs1p, Sps18p and Glo3p are compared using the University of Wisconsin Computer Group sequence analysis software (Devereaux *et al.*, 1984). Amino acid identities and similarities are highlighted in black and gray, respectively. The following groups of amino acids were designated as similar: (K, R), (M, V, L, I, F), (F, Y, W), (S, T), (E, D). The positions of the *gcs1-1* Zn-finger mutation and the *gcs1-2* and *gcs1-7* truncations are indicated by arrows. The amino acid changes above positions 27 and 29 in the Gcs1 sequence represent a polymorphism and the *gcs1-1* missense mutation, respectively. (**B**) The amino acid consensus sequence for the Zn-finger regions from all GATA proteins is aligned with the consensus sequence derived from the three Glo proteins, Gcs1p, Sps18p and Glo3p. @ indicates an aliphatic amino acid. Amino acid identities and similarities are indicated by solid lines or dots, respectively. Large bold letters indicate residues conserved between the GATA and Glo protein families, and smaller letters refer to conserved residues within each family. X designates any amino acid.

shows some resemblance to the type of Zn-finger motif found in an extended family of well-characterized DNA binding proteins (see Figure 6B). This family of structurally related proteins includes the GATA transcription factors that are implicated in the developmental control of gene expression in hematopoietic cells (see Orkin, 1992), as well as Gln3p and Dal80p from yeast, AreA protein from *Aspergillus* and Nit2 protein from *Neurospora*, all regulators of nitrogen metabolism (reviewed in Orkin, 1992). Many of these GATA transcription factors are sequence-specific DNA binding proteins (Ko and Engel, 1993; Merika and Orkin, 1993); a Zn-finger domain from the mammalian GATA-1 protein has sequence-specific DNA binding activity *in vitro* (Omichinski *et al.*, 1993). The GATA proteins found in vertebrate cells contain two Zn-finger domains, while fungal versions of the GATA protein, like Gcs1p, contain only a single Zn-finger domain. All of these Zn-finger domains are followed by a sequence enriched in basic amino acids, which has been shown to be necessary for specific DNA binding activity *in vitro* (Omichinski *et al.*, 1993). Although the Gcs1p Zn-finger domain is only distantly related to any of those of the GATA family, with minimal similarity in the basic region (Figure 6B), the consensus sequence for the Zn-finger region within the Gcs1p yeast family of proteins is similar to the GATA Zn-finger domain (Figure 6). This similarity suggests that the Gcs1 polypeptide, through this Zn-finger domain, may have DNA binding ability.

Bypassing the need for Gcs1p activity

The *gcs1-6* null mutation removes all of the *GCS1* coding sequence, yet these null mutant cells have no discernible growth phenotype at 29°C, either in the resumption of cell proliferation or in the maintenance of ongoing proliferation. On the other hand, preliminary experiments show significant transcriptional alterations due to the *gcs1-6* null mutation, even at 29°C (unpublished observations). These transcriptional effects show that the wild-type Gcs1p is active even at the permissive temperature of 29°C, but the null phenotype suggests that this activity is inconsequential to the cell at this temperature. Apparently these Gcs1p effects only become important at low temperatures, such as the restrictive temperature of 15°C. At higher temperatures the cell may have additional mechanisms to generate important effects that result from Gcs1p activity, thus bypassing the need for Gcs1p.

The ability of a null allele to cause a conditional phenotype is not without precedent. Indeed, many yeast genes can be deleted or disrupted to cause heat sensitivity. Among these are *CDC26* (Araki *et al.*, 1992; Kawakami *et al.*, 1992), *DPR1/SGP2* (Nakayama *et al.*, 1988), *HIT1* (Kawakami *et al.*, 1992; Reijo *et al.*, 1993), the *PET18* locus (Toh-e and Sahashi, 1985; Kawakami *et al.*, 1992), *SSN6* (Schultz and Carlson, 1987), *SWI4* (Ogas *et al.*, 1991), *VPS33* (Banta *et al.*, 1990), *VPS34* (Herman and Emr, 1990) and the *SSA1*, *SSA2* gene pair (Craig and Jacobsen, 1983). Members of this collection of genes encode polypeptides involved in a wide variety of processes, including transcriptional activation and repression, signal transduction, protein folding and prenylation and vacuolar protein sorting. Remarkably, deletion of *SRB2* or *SRB5*, which encode proteins that interact with RNA polymerase II, or deletion of *RPB4*, encoding a subunit of RNA polymerase II itself, allows growth at intermediate temperatures but causes both heat sensitivity and cold sensitivity (Woychik and Young, 1989; Koleske *et al.*, 1992; Thompson *et al.*, 1993). Analogous sensitivity to temperature extremes is conferred by the disruption of *STI1*, a gene of unknown function (Nicolet and Craig, 1989). Yet other genes can be inactivated to cause cold sensitivity. For example, disruption of the *SSB1*, *SSB2* gene pair, whose hsp70 protein products associate with nascent polypeptides at the translating ribosome (Nelson *et al.*, 1992), causes cold sensitivity (Craig and Jacobsen, 1985). Similarly, the *SAC1* gene, identified by a suppressor mutation that reverses the temperature-sensitive actin mutation *act1-1*, is needed for growth only at low temperatures (Novick *et al.*, 1989); these authors suggest that some process, perhaps associated with the actin cytoskeleton, may be intrinsically cold-sensitive and therefore requires Sac1p to facilitate growth in the cold. Similarly, Gcs1p may play an essential role in the resumption of cell proliferation from stationary phase at 15°C because of some needed activity that is inherently inefficient in the cold.

Bypass activity at the low temperature of 15°C differs from that manifested at 29°C, where cells may be able to activate mechanisms that bypass the need for Gcs1p. However, the re-entry mutant phenotype of *gcs1-6* null mutant cells (Table I, phenotype B) shows that activation of these bypass functions is complex, and depends on growth phase as well as growth temperature. In these *gcs1* mutant cells, bypass activity is not achieved at 15°C until stationary-phase cells resume cell proliferation, which accounts for the inability of mutant cells to resume proliferation at 15°C (no bypass activity yet) and the ability to maintain cell proliferation at 15°C (bypass active). In these same mutant cells at 29°C, however, bypass activity must be achieved promptly upon the transfer of stationary-phase cells to fresh growth medium, for at this temperature Gcs1p is dispensable even for the resumption of cell proliferation from stationary phase.

Materials and methods

Strains and plasmids

The yeast strains W303-1A (*MAT***a** *leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*), IIIXD (MATα *cdc4-6 his[1,5,7]*) and 21R (*MAT***a** *leu2-3,112 ura3-52 ade1*) have been described previously (Johnston and Hopper, 1982; Singer *et al.*, 1984; Archambault *et al.*, 1992). Strain MD025-5 (*MAT*α *gcs1-1 leu2-3,112 ura3-52 his6 ade1*) was derived by mating strain 21R with strain MDG3-QS39 (*MAT*α *gcs1-1 his6 ura1*; Drebot *et al.*, 1987). Strain MDG1-3X (*MAT***a** *gcs1-1 leu2-3,112 ura3-1 his3-11,15 trp1-1 ade2-1*) was derived by repeated backcrosses of the *gcs1-1* mutation from strain MD025-5 into strain W303-1A. Strains harboring the *gcs1-6* null allele were constructed by replacing the *GCS1* ORF with the *URA3* gene. Replacement of the *GCS1* ORF was accomplished by first transferring a *Bam*HI–*Nde*I fragment containing the *GCS1* gene and flanking sequences (Figure 1A) into the *Sma*I site of plasmid pUC19. The *Bsa*AI–*Bst*EII fragment of this plasmid containing the *GCS1* ORF was then replaced with a *Sma*I–*Cla*I fragment carrying the *URA3* gene, to create plasmid pLI-1. Diploid cells homozygous for *ura3-1* were transformed to uracil prototrophy using a *Bam*HI–*Eco*RI fragment of pLI-1. Replacement of the *GCS1* chromosomal locus with the *gcs1-6* null allele was confirmed by genetic linkage and Southern analysis. To produce strains harboring the *gcs1-2* truncation allele encoding a Gcs1 polypeptide that lacks the C-terminal 125 codons, a 487 bp *Bgl*II–*Pst*I fragment internal to the *GCS1* ORF was subcloned into the *URA3*-based integrative vector YIp352 (Hill *et al.*, 1986); the resulting plasmid was linearized by treatment with *Xba*I to direct integration at the *GCS1* locus

and produce the truncated form of the *GCS1* gene. Integrative disruption of the *GCS1* locus after transformation with this plasmid was confirmed by Southern blot analysis. The *gcs1-2* allele encodes additional amino acids from out-of-frame *lacZ* sequences. We therefore also truncated Gcs1p in a different way, by filling in the unique *Xba*I site to place in-frame a UAG stop codon at amino acid 200, thus creating the *gcs1-7* allele. The resultant translation replaces the arginine at position 199 with serine (see Figure 1B). The *gcs1-7* sequence was confirmed by restriction analysis and direct nucleotide sequencing.

A plasmid-borne *gcs1-1* allele was produced by gap repair using plasmid pBN316ΔH, a derivative of pRS316 (Sikorski and Hieter, 1989) harboring a version of the *GCS1* gene deleted for the *Hin*dIII fragment (Figure 1A). Strain MDG1-3X was transformed with pBN316ΔH, and plasmids derived from Ura^+ transformants were transferred to *E.coli* cells for analysis. Plasmids containing the expected *Hin*dIII fragment within insert sequences were verified by double-stranded sequencing using oligonucleotide primers.

Genetic mapping

Standard yeast genetic manipulations were as described (Guthrie and Fink, 1991). Chromosomal assignment of the cloned *GCS1* gene was determined by hybridization to a blot of separated yeast chromosomes.

Culture conditions and assessment of cellular parameters

Cells were grown in YM1 complex liquid medium (Hartwell, 1967) or in YNB defined medium (Johnston *et al.*, 1977) supplemented with 2% glucose, amino acids (40 μg/ml) and nucleotide bases (20 μg/ml) as required to satisfy auxotrophies. Cultures were incubated in gyratory shaking water baths; for the maintenance of 15°C cultures, the water bath was equipped with a refrigeration unit. Cell concentration was determined using an electronic particle counter (Coulter Electronics Inc.), and cell morphology was assessed by direct microscopic examination; before assessment, cells were fixed with formalin and sonicated briefly to separate cells. Viable counts were determined by incubating replicate samples of serial dilutions (in phosphate-buffered saline) on YEPD solid complex medium (Hartwell, 1967). To measure RNA and protein accumulation, stationary-phase cells were stimulated in medium containing [^{3}H]uracil or [^{35}S]methionine, respectively (Drebot *et al.*, 1990b). Total DNA and RNA contents were determined as described by Storms *et al.* (1984) and Rubin (1975), respectively. Thermotolerance was assessed as described (Drebot *et al.*, 1990a). Nuclei were visualized using the DNA stain 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.). Cells were photographed using a Nikon microphot FX equipped with epi-fluorescence.

RNA blot analysis

Cells were harvested at concentrations of $2–5\times10^6$ cells/ml, and total RNA was extracted as described (Penn *et al.*, 1984). Equal amounts of RNA (20 μg/lane) were resolved electrophoretically through formaldehyde-agarose gels, transferred to nylon membranes (NEN Research Products) and cross-linked using a UV cross-linker (Stratagene). Hybridization to visualize transcripts was performed using restriction fragments (Rowley *et al.*, 1991). The *ACT1* probe was a 1 kbp *Hin*dIII−*Xho*I fragment from pRS208 (a gift from R.Storms), the *LEU2* probe was a 1.7 kbp *Hpa*I−*Acc*I fragment from YEp351 (Hill *et al.*, 1986), the *SSA3* probe was a 750 bp *Rsa*I fragment from pUC9-SSA3, and the *UBI4* probe was an ~2.3 kbp *Eco*RI fragment from pUB200 (provided by D.Finlay).

Gene isolation and directed integration

The wild-type *GCS1* gene was cloned by transformation of cold-sensitive *gcs1-1* mutant haploid cells with a YCp50-based recombinant yeast DNA library (obtained from D.Botstein) and selection for cold-resistant transformants. Directed integration of plasmid DNA to the homologous chromosomal location (Rothstein, 1991) was used to show that the 6 kbp insert of plasmid p6d-3, one of the plasmids conferring cold resistance, was derived from the *GCS1* genomic locus. For this directed integration, the p6d-3 genomic insert was transferred into the integrating *URA3* vector YIp5, and the resulting plasmid was linearized at a unique *Kpn*I site within the insert (Figure 1A) and transformed into *gcs1-1 ura3-52* recipient cells. Southern analysis of transformants confirmed that the linearized plasmid had integrated at the chromosomal locus homologous to insert DNA. A cold-resistant haploid transformant was then mated to a wild-type tester strain, the resultant diploid was sporulated and 33 complete tetrads were assessed for cold resistance. All meiotic segregants were cold-resistant, indicating that the integrated plasmid sequence conferring cold resistance cosegregated with the *gcs1-1* mutation. This tight genetic linkage between the *gcs1-1* mutation and the integrated plasmid sequences indicated that the wild-type *GCS1* gene is located on the p6d-3 insert. Several subclones of p6d-3, constructed by *Sau*3A partial digestion of the entire p6d-3 insert and religation of 2–3 kbp size-fractionated DNA fragments into YCp50, were tested for complementation of the *gcs1-1* cold sensitivity to localize the *GCS1* gene (Figure 1A).

Isolation of the gcs1-1 mutant gene

The *gcs1-1* lesion was localized to the 5′ end of the *gcs1-1* allele by the integrative transformation of *gcs1-1* mutant cells with a plasmid containing the 5′ two-thirds of the wild-type *GCS1* gene. Homologous recombination of this plasmid at the *gcs1-1* locus was directed (Rothstein, 1991) by linearizing plasmid DNA at a unique *Xba*I site within the *GCS1* nucleotide sequence. Transformed cells harboring this chimeric *GCS1* gene were able to grow at 15°C.

The wild-type *GCS1* gene from strain IIIXD, the parent strain mutagenized to create the *gcs1-1* mutation (Drebot *et al.*, 1987), and the *gcs1-1* mutant allele introduced into strain W303-1A were isolated by PCR (35 cycles) using a Coy thermocycler (Coy Lab Products, Inc.) and primers centered at positions −278 and +1051 (see Figure 1B).

Nucleotide sequence determination

Overlapping fragments of the *GCS1* gene were cloned into M13 um20 and um21 using the cyclone method (International Biotechnologies, Inc.). The nucleotide sequences of both strands were determined by Sanger dideoxy chain termination DNA sequencing reactions using T7 polymerase as provided by the Sequenase Version 2.0 kit (US Biochemical). The *GLO3* gene was sequenced by ligating fragments (produced by sonication) into M13mp19. Sequence ambiguities were resolved by double-strand sequencing using specific oligonucleotides as primers. DNA sequence data were analyzed using the University of Wisconsin Genetics Computer Group sequence analysis software (Devereux *et al.*, 1984).

Nucleotide sequence accession number

The GenBank accession numbers for the *GCS1* and *GLO3* nucleotide sequences are L24125 and X79514, respectively.

Primer extensions

Extension reactions were carried out using M-MLV reverse transcriptase, with [γ-^{32}P]ATP-labeled oligonucleotides complementary to nucleotides −1 to +19 and +12 to +32 (see Figure 1B) as primers and total RNA as template. Reaction products were treated with RNase A and resolved by electrophoresis through a polyacrylamide−urea sequencing gel.

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References

Araki,H., Awane,K., Ogawa,N. and Oshima,Y. (1992) *Mol. Gen. Genet.*, **231**, 329–331.

Archambault,J., Drebot,M.A., Stone,J.C. and Friesen,J.D. (1992) *Mol. Gen. Genet.*, **232**, 408–414.

Banta,L.M., Vida,T.A., Herman,P.K. and Emr,S.D. (1990) *Mol. Cell. Biol.*, **10**, 4638–4649.

Boorstein,W.R. and Craig,E.A. (1990) *Mol. Cell. Biol.*, **10**, 3262–3267.

Coe,J.G.S., Murray,L.E. and Dawes,I.W. (1994) *Mol. Gen. Genet.*, in press.

Craig,E.A. and Jacobsen,K. (1983) *Cell*, **38**, 841–849.
Craig,E.A. and Jacobsen,K. (1985) *Mol. Cell. Biol.*, **5**, 3517–3524.
Devereux,J., Haeberli,P. and Smithies,O. (1984) *Nucleic Acids Res.*, **12**, 387–395.
Dingwall,C. and Laskey,R.A. (1991) *Trends Biochem. Sci.*, **16**, 478–481.
Drebot,M.A., Johnston,G.C. and Singer,R.A. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 7948–7952.
Drebot,M.A., Barnes,C.A., Singer,R.A. and Johnston,G.C. (1990a) *J. Bacteriol.*, **172**, 3584–3589.
Drebot,M.A., Veinot-Drebot,L.M., Singer,R.A. and Johnston,G.C. (1990b) *Mol. Cell. Biol.*, **10**, 6356–6361.
Filipak,M., Drebot,M.A., Ireland,L.S., Singer,R.A. and Johnston,G.C. (1992) *Curr. Genet.*, **22**, 471–477.
Finley,D., Ozaynak,E. and Varshavsky,A. (1987) *Cell*, **48**, 1035–1046.
Guthrie,C. and Fink,G.R. (eds) (1991) *Methods Enzymol.*, **194**.
Hartwell,L.H. (1967) *J. Bacteriol.*, **93**, 1662–1670.
Hartwell,L.H. (1974) *Bacteriol. Rev.*, **38**, 164–198.
Herman,P.K. and Emr,S.D. (1990) *Mol. Cell. Biol.*, **10**, 6742–6754.
Hill,J.E., Myers,A.M., Koerner,T.J. and Tzagoloff,A. (1986) *Yeast*, **2**, 163–167.
Hinnebusch,A.G. (1990) *Trends Biochem. Sci.*, **15**, 148–152.
Jensen,R., Sprague,G.F. and Herskowitz,I. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 3035–3039.
Johnston,S.A. and Hopper,J.E. (1982) *Proc. Natl Acad. Sci. USA*, **79**, 6971–6975.
Johnston,G.C., Pringle,J.R. and Hartwell,L.H. (1977) *Exp. Cell Res.*, **105**, 79–98.
Kawakami,K., Shafer,B.K., Garfinkel,D.J., Strathern,J.N. and Nakamura,Y. (1992) *Genetics*, **131**, 821–832.
Ko,L.J. and Engel,J.D. (1993) *Mol. Cell. Biol.*, **13**, 4011–4022.
Koleske,A.J., Buratowski,S., Nonet,M. and Young,R.A. (1992) *Cell*, **69**, 883–894.
Merika,M. and Orkin,S.H. (1993) *Mol. Cell. Biol.*, **13**, 3999–4010.
Nakayama,N., Arai,K.-I. and Matsumoto,K. (1988) *Mol. Cell. Biol.*, **8**, 5410–5416.
Nelson,R.J., Ziegelhoffer,T., Nicolet,C., Werner-Washburne,M. and Craig,E.A. (1992) *Cell*, **71**, 97–105.
Nicolet,C.M. and Craig,E.A. (1989) *Mol. Cell. Biol.*, **9**, 3638–3646.
Novick,P., Osmond,B.C. and Beach,D. (1989) *Genetics*, **121**, 659–674.
Ogas,J., Andrews,B.J. and Herskowitz,I. (1991) *Cell*, **66**, 1015–1026.
Omichinski,J.G., Trainor,C., Evans,T., Gronenborn,A.M., Clore,G.M. and Felsenfeld,G. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 1676–1680.
Orkin,S.H. (1992) *Blood*, **80**, 575–581.
Penn,M.D., Thireos,G. and Greer,H. (1984) *Mol. Cell. Biol.*, **4**, 520–528.
Reijo,R.A., Cho,D.-S. and Huffaker,T.C. (1993) *Genetics*, **135**, 955–962.
Riles,L., Dutchik,J.E., Baktha,A., McCauley,B.K., Thayer,E.C., Leckie,M.P., Braden,V.V., Depke,J.E. and Olson,M.V. (1993) *Genetics*, **134**, 81–150.
Rothstein,R. (1991) *Methods Enzymol.*, **194**, 281–301.
Rowley,A., Singer,R.A. and Johnston,G.C. (1991) *Mol. Cell. Biol.*, **11**, 5718–5726.
Rubin,G.M. (1975) *Methods Cell Biol.*, **12**, 45–64.
Schultz,J. and Carlson,M. (1987) *Mol. Cell. Biol.*, **7**, 3637–3645.
Sikorski,R.S. and Hieter,P. (1989) *Genetics*, **122**, 19–27.
Singer,R.A., Bedard,D.P. and Johnston,G.C. (1984) *J. Cell Biol.*, **98**, 678–684.
Slater,M.R. and Craig,E.A. (1989) *Nucleic Acids Res.*, **17**, 4891.
Storms,R.K., Ord,R.W., Greenwod,M.T., Mirdamadi,B., Chu,F.K. and Belfort,M. (1984) *Mol. Cell. Biol.*, **4**, 2858–2864.
Thompson,C.M., Koleske,A.J., Chao,D.M. and Young,R.A. (1993) *Cell*, **73**, 1361–1375.
Toh-e,A. and Sahashi,Y. (1985) *Yeast*, **1**, 159–171.
Werner-Washburne,M., Braun,E., Johnston,G.C. and Singer,R.A. (1993) *Microbiol. Rev.*, **57**, 383–401.
Woychik,N.A. and Young,R.A. (1989) *Mol. Cell. Biol.*, **9**, 2854–2859.

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