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Molecular characterization of *GCD1*, a yeast gene required for general control of amino acid biosynthesis and cell-cycle initiation

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ABSTRACT

The *GCD1* gene product of *Saccharomyces cerevisiae* has been implicated in the coordination of the cell cycle with the general control of amino acid biosynthesis (M. Wolfner et al., J. Mol. Biol. 96:273-290, 1975). Strains containing the *gcd1*-1 allele constitutively express the amino acid biosynthetic genes at the induced levels normally found only during conditions of amino acid starvation. In addition, *gcd1*-1 strains do not grow at high temperatures because under these conditions they are unable to proceed beyond the *START* step of the cell division cycle. We have cloned and sequenced the *GCD1* gene and examined various aspects of cellular metabolism in order to elucidate its role(s) in regulating gene expression and the cell cycle. *GCD1* encodes a 1.7 kb RNA whose expression is not regulated as a function of amino acid starvation. Overexpression of this RNA does not affect the regulation of amino acid biosynthetic genes or cell growth. *GCD1* is an essential gene because cells containing a *gcd1-HIS3* disruption are unable to grow. The essential function of *GCD1* may be involved in protein synthesis because a *gcd1*-1 strain incorporates low levels of ^{35}S-methionine into protein when cells are shifted to the restrictive temperature. *GCD1* encodes a protein of 511 amino acids whose predicted sequence does not exhibit significant homology to any other known proteins and appears too large to be a ribosomal protein. We suggest that *GCD1* encodes a component of the normal protein synthesis machinery that is involved in the translational regulation of GCN4, a protein that coordinately activates the transcription of amino acid biosynthetic genes. *GCD1* may also be part of a sensing mechanism in which cells monitor the protein synthesis capacity prior to initiating a new cell division cycle.

INTRODUCTION

Saccharomyces cerevisiae coordinately regulates the expression of many different amino acid biosynthetic genes in response to starvation for various amino acids (reviewed in 1,2). This cross-pathway regulation, termed general control, also affects the isoleucine tRNA synthetase gene (*ILS1*) and the *ATR1* gene involved in aminotriazole resistance (3). Coordinate regulation occurs at the level of transcription and is mediated by the binding of GCN4 protein to specific sequences found in the promoter regions of coregulated genes (4,5). Saturation mutagenesis of the *HIS3* regulatory site and examination of sequences from coregulated genes has identified the 9-bp dyad symmetric sequence ATGACTCAT as the consensus element and optimal site for GCN4 binding (6).

Expression of the *GCN4* gene is regulated at the level of translation (7,8). During normal conditions, translation of *GCN4* is very low and consequently the transcription of general control

genes occurs at the basal level. Under conditions of amino acid limitation, the translation of *GCN4* is increased as much as 30 fold while *GCN4* mRNA synthsis increases only 2-3 fold. The increase in GCN4 protein directly results in the coordinate induction of the amino acid biosynthetic genes.

Translational regulation of GCN4 appears to involve the four small open reading frames in the 5' non-coding sequences of the *GCN4* mRNA. Under normal circumstances, translation is precluded due to the presence of the four AUG codons that are upstream of the AUG initiation codon for GCN4 protein. After deletion or mutation of all these ORFs, *GCN4* translation is constitutively high even during non-starvation conditions (9,10). Moreover, this segment of GCN4 mRNA confers translational control when fused to heterologous mRNA coding sequences (11). It has been postulated that the upstream AUG codons present in the *GCN4* mRNA leader are somehow bypassed for translational initiation when the cell is starved for amino acids. However, the mechanism of this translational regulation and the various effector molecules involved are unknown.

In addition to *GCN4*, recessive mutations in a number of other genes alter the regulation of the amino acid biosynthetic genes. Mutations in four other *GCN* genes confer an inability to induce the transcription of general control genes during amino acid limitation (12-15). Mutations in five *GCD* genes exhibit constitutive induction of general control genes under all growth conditions (13,16,17). Interestingly, many *gcd* mutations are temperature-sensitive for growth even under non-starvation conditions. The *GCD* genes are postulated to be negative regulators of *GCN4* because *gcd* mutants override the upstream AUG codons and constitutively overexpress GCN4 protein independently of growth conditions. It is the constitutive overexpression of GCN4 that results in the induced transcription of general control genes.

The best characterized *GCD* gene is *GCD1* (originally called *TRA3*), which was initially identified by a single mutation that conferred constitutive induction of the amino acid biosynthetic genes as well as temperature sensitive growth (13). The *gcd1*-1 mutation causes constitutive high level translation of *GCN4* (7,8), and it also confers a cell cycle arrest phenotype on cells. When *gcd1*-1 cells are shifted from 23°C, the permissive growth temperature, to 37°C, the non-permissive temperature, they uniformly arrest at the unbudded stage of the yeast cell cycle. This point of arrest of is coincident with the position of α-factor arrest in haploid strains (13), which is operationally defined as the start of the cell cycle. From the dual phenotype of cell cycle arrest and regulation of general control, it has been suggested that *GCD1* may function to integrate the cellular requirements for amino acids for cell growth with the decision to enter a new cell-division cycle (13). In previous work, we excluded the trivial possibility that *gcd1*-1 artifactually caused amino acid starvation by a failure in tRNA charging (18). In this communication, we have cloned and sequenced the *GCD1* gene and have examined certain aspects of cellular metabolism in *gcd1*-1 strains in order to elucidate the function(s) of *GCD1* in general control and cell cycle regulation.

MATERIALS AND METHODS

Genetic manipulations

The yeast strains used in this work were KY403 (a *gcd1*-1 *ura3*-52 *leu2*-3,112 *cyh2 can1*-k2), KY119 (a/α *ura3*-52 *ade2*-101 *lys2*-801 *trp1*-Δ1 *his3*-Δ200), and KY29 (a *ura3*-52 *trp1*-289). The growth, mating, sporulation, tetrad dissection, and DNA transformation of yeast strains were carried out by standard procedures (19).

Cloning of *GCD1*

A library of yeast DNA segments cloned in YEp13, a vector containing the *LEU2* selectable marker and the 2μ origin of replication, was introduced into spheroplasts of strain KY403. After the treated spheroplasts were plated on minimal medium lacking leucine, they were allowed to grow at 23°C for 18 hours before imposing temperature selection at 37°C. DNA was prepared from the putative yeast transformants and introduced into *E.coli* by selecting for ampicillin resistance, and plasmid DNAs were introduced back into KY403 to confirm the phenotype.

Disruption of the chromosomal *GCD1* gene

The 1.7 kb DNA *Bam*HI fragment encoding the *HIS3* gene, Sc2676, (20), was cloned into the unique *Bgl*II site within the *GCD1* coding region of Sc4010. The resulting 5.5 kb BamHI fragment, Sc4016, now containing *GCD1* disrupted by the *HIS3* gene, was purified and used to transform the diploid strain KY119 to His^+. Transformants containing the *gcd1-HIS3* disruption on one chromosome were identified by genomic hybridization analysis using either *GCD1* or *HIS3* sequences as a probe. The diploid strain was sporulated and then subject to tetrad dissection. In a control experiment, YIp5-Sc4016 was integrated into KY119 without loss of the endogenous *GCD1* allele, and the resulting strain was analyzed by tetrad dissection.

Sequencing of *GCD1*

Various DNA fragments produced by restriction endonuclease cleavage of Sc4010, the 2.4 kb genomic fragment that fully complements *gcd1*-1, were subcloned into M13mp18 and M13mp19 (21). Templates for DNA sequencing were generated by digesting double-stranded DNA with DNase I in the presence of Mn^{2+} to generate double-strand breaks at random positions in the DNA and subsequent circularization with T4 DNA ligase (22). In addition, a *Bal*31 deletion series of Sc4010 was also generated and cloned into M13mp18. The M13-derived clones were sequenced by the dideoxy method (23). Both strands of *GCD1* were sequenced over the entire open reading frame, and the 5' and 3' non-coding ends of Sc4010 were sequenced at least twice on one strand and generally on both strands.

RNA analysis

Yeast strains were grown at 23°C in medium containing amino acid and nucleotide supplements until the culture reached a density of A_{600} = 1.0. Aminotriazole was added to a final concentration of 10 mM to half of the culture, and the cells were permitted to grow for an additional 4 hrs. For the experiments involving quantitation of *GCD1* mRNA, total RNA was isolated from yeast cells, electrophoretically separated in a 1.7% agarose gel containing 6%

formaldehyde and then transferred to a nitrocellulose filter (24). The separated RNAs were hybridized with a mixture of ^{32}P-labeled probes prepared by nick-translation. The hybridization probes were Sc4014 DNA (Fig. 1) for measuring *GCD1* expression and Sc3119 for measuring *HIS3* and *DED1* expression as internal controls (19,24). The 5' ends of *GCD1* mRNA were mapped by the standard primer extension method. Specifically, a synthetic oligonucleotide corresponding to nucleotides +42 to +65 was ^{32}P-labeled at its 5' end with T4 polynucleotide kinase, hybridized to total yeast RNA, and extended with reverse transcriptase. The products of this primer extension were compared to those of di-deoxy sequencing reactions (23) using the identical labeled oligonucleotide as a primer.

Protein synthesis

Overnight cultures of various yeast strains were inoculated into fresh synthetic medium containing 0.3% casamino acids plus uracil and adenine and grown at 23°C. The methionine concentration was determined to be 500 μM based on quantitative amino acid analysis of a 1% casamino acids solution using a Beckman amino acid analyzer. When the cultures reached an A_{600} of approximately 0.5, aliquots were transferred to 37°C or were left at 23°C. For continuous labelling during protein synthesis, ^{35}S-methionine was added to a final specific activity of 10 mCi/mmole five minutes after the cultures were shifted to 37°C. At various times after the addition of label, 1 ml aliquots were withdrawn and mixed with 0.25 ml of 50% trichloroacetic acid (TCA) to rapidly stop protein synthesis. The TCA-treated samples were heated to 90°C for 10 min to deacylate methionyl-tRNA complexes, subjected to 15000 X g centrifugation for 3 min, and then resuspended in 1 ml of 5% TCA. A 200 μl aliquot of each sample, in duplicate, was passed through a Whatman GFA glass fiber filter, and the retained radiolabelled material was quantitated by liquid scintillation counting. For pulse labelling, cultures were divided into 5 ml aliquots and shifted to 37°C. At various times after the shift, ^{35}S-methionine was added to 10 mCi/ mmole and the incubation continued for 5 min at which point unlabelled methionine was added to 10 mM and the incubation continued for 10 min. Aliquots were removed and processed as described above.

RESULTS

Cloning of *GCD1*

The *GCD1* gene was obtained by complementation of the temperature sensitive phenotype of strain KY403 (relevant genotype *gcd1*-1 *leu2*-2,112). A library of *S. cerevisiae* DNA fragments cloned into the YEp13 shuttle vector was introduced into KY403 spheroplasts, and 8 transformants able to grow at 37°C in the absence of leucine were obtained. Restriction endonuclease cleavage of plasmid DNAs obtained from the KY403 transformants identified common fragments that defined two overlapping but non-identical genomic clones (Fig. 1).

Restriction mapping identified two *Bam*HI fragments that encompassed Sc4001 and indicated that Sc4005 contained a *Bam*HI site was fortuitously created during the construction of

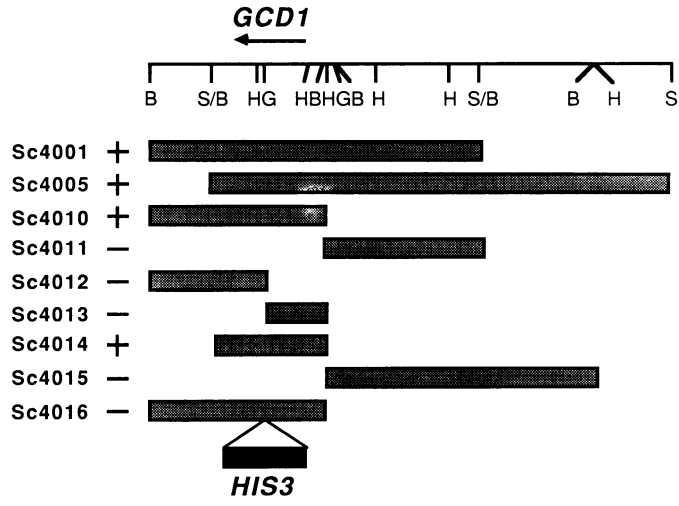

Figure 1: Restriction map and phenotype of *GCD1* DNAs. The upper line shows a composite restriction map of the two overlapping genomic DNAs Sc4001 and Sc4005 which complement *gcd1-1* mutants. The letters below the line refer to the following restriction endonuclease sites: (B) BamHI; (G) BglII; (H) HindIII; (R) EcoRI; (S) Sau3A; (S/B) Sau3A sites that fortuitously regenerate BamHI sites in either Sc4001 or Sc4005, and the arrow above the line indicates the size and direction of the *GCD1* mRNA. The genomic clones, Sc4001 and Sc4005, along with various subclones derived from them are aligned beneath the restriction map with the endpoints corresponding to the BamHI or BglII restriction sites utilized in the subcloning. The + and - symbols refer to the ability of the indicated DNA fragment to complement a *gcd1*-1 mutant when inserted into a multicopy vector YEp13 (Sc4001 and Sc4005), a single copy vector (Sc4010-Sc4015), or an integrating vector (Sc4010, Sc4011, Sc4016). The DNA fragment Sc4016 was constructed by inserting the 1.7 Kb *Bam*HI fragment containing the *HIS3* gene into the unique *Bgl*II site of *GCD1* in plasmid YIp5-Sc4010.

the library (Fig. 1). These *Bam*HI DNA fragments were subcloned into YCp50, a *URA3* centromeric vector, and YIp5, a *URA3* integration vector. Plasmids containing Sc4010 or Sc4014 complemented the *gcd1*-1 mutation, localizing the presumptive *GCD1* gene to a 2.4 kb fragment of yeast DNA. *GCD1* appears to be a single copy gene because a unique 3.8 kb *Bam*HI DNA fragment is seen in genomic hybridization experiments (Fig. 2).

To confirm that the cloned fragments contain the *GCD1* gene, the KY403 transformant containing an integrated copy of YIp5-Sc4010 was mated to an appropriate haploid strain, and the resulting diploid strain was analyzed by tetrad dissection. If the plasmid integrated into the genome by homologous recombination at the *GCD1* locus, then the *URA3* marker on the plasmid should map on chromosome XV roughly 30 centimorgans away from *HIS3*, the normal position of *GCD1*. This result was observed thus directly proving that the DNA sequences which were cloned on the basis of complementation of *gcd1*-1 do in fact encode the *GCD1* gene.

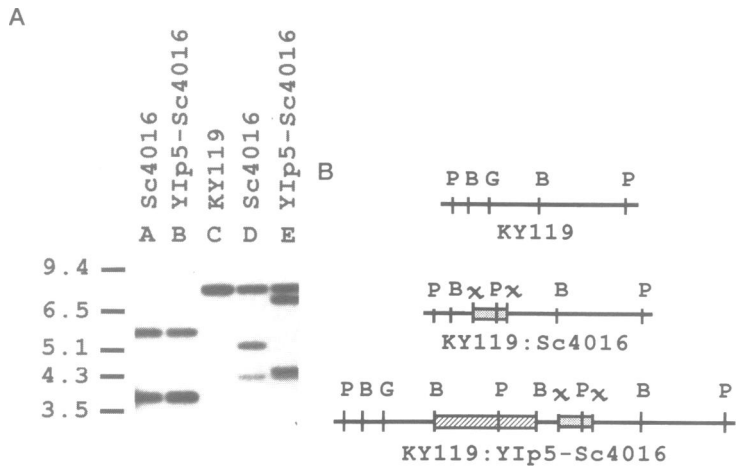

Figure 2: Gene disruption of *GCD1*. Panel A shows the structure of the *GCD1* locus in the KY119 diploid strains transformed by the indicated DNAs. Genomic DNAs were cleaved with *Bam*HI (lanes A-B) or *Pst*I (lanes C-E) and probed with ^{32}-P labeled Sc4010 DNA. Panel B shows the restriction map of the relevant chromosomes (B, *Bam*HI; G, *Bgl*II; P, *Pst*I; X, BamHI/*Bgl*II hybrid site).

GCD1 is an essential gene

The fact that *gcd1-1* confers temperature senstive growth suggests that the *GCD1* gene product is essential for cell viability. To prove that *GCD1* is an essential gene, we performed a gene disruption experiment using the one-step direct gene replacement technique (25). The 5.5 kb *Bam*HI fragment, Sc4016, containing the *GCD1* gene disrupted by the *HIS3* gene, was used to transform the diploid strain KY119 to His$^+$. Genomic hybridization (Fig. 2) confirmed that the His$^+$ transformants of KY119 were the expected *GCD1/gcd1-HIS3* heterozygotes.

If *GCD1* is an essential gene and the *HIS3* insertion abolishes *GCD1* function, each tetrad should contain only two viable His$^-$ spores. Dissection of 31 tetrads yielded 28 that contained two viable His$^-$ spores and two non-viable spores. Microscopic examination of the non-viable spores showed that 45 of 56 spores had failed to germinate and that 11 spores had produced only very tiny buds. Two tetrads yielded one viable His- spore and one tetrad gave three viable His$^-$ spores; presumably, these tetrads are due to gene conversion or random spore inviability. In a control experiment, YIp5-Sc4016 was integrated into KY119 at one *GCD1* locus without loss of the endogenous *GCD1* allele. For all 11 tetrads tested, 4 viable spores were obtained, two being His$^+$Ura$^+$ and two being His$^-$Ura$^-$. These results demonstrate that meiosis in the KY119 transformants occurs normally and is not affected by the presence of the *GCD1* disrupted gene.

DNA and mRNA coding sequence of *GCD1*

To characterize the *GCD1* gene product, we determined the nucleotide sequence of Sc4014, the 2.4 kb fragment that fully complements the *gcd1-1* mutation (Fig. 3). A single open reading

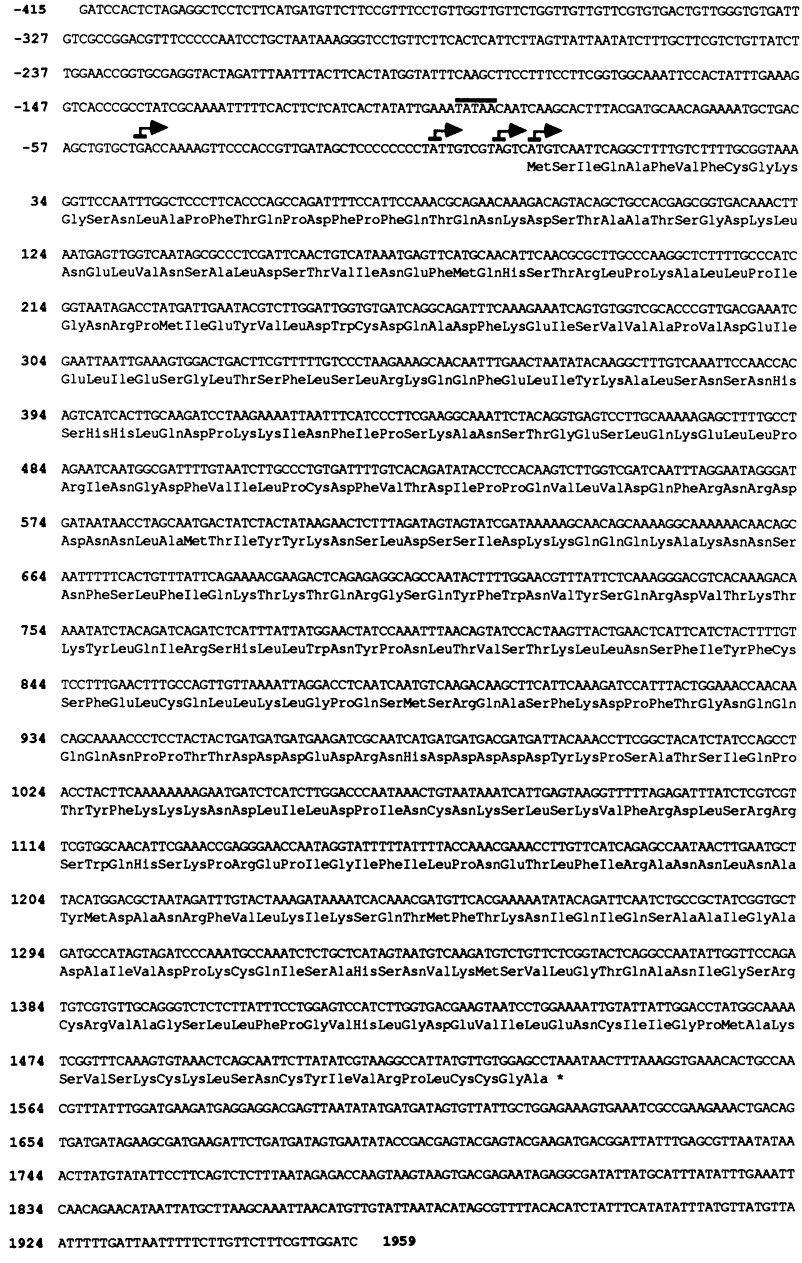

Figure 3: Nucleotide sequence of *GCD1*. The DNA sequence of Sc4014, 2975 bp, is shown with a putative TATA element (double line) and major mRNA initiation sites as determined by S1 mapping (lines with the attached arrows). The sequence is numbered from the first ATG codon observed in the single open reading frame found in Sc4014.

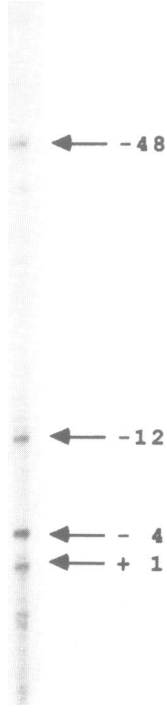

Figure 4: 5' mapping of *GCD1* RNA. The products of primer extension are shown with the major bands indicated; these bands were not observed in the absence of yeast RNA or reverse transcriptase. The location of the 5' ends (defined with respect to the presumptive AUG initiation codon shown in Fig. 3) were determined by comparison with di-deoxy sequencing reactions using the identical primer that were analyzed on the same gel.

frame encoding 511 contiguous amino acids was found. A search of the available protein and DNA data bases did not identify any sequences that are similar to the presumptive *GCD1* coding sequence. No TACTAAC elements for splicing (26,27) were found, suggesting that *GCD1* does not contain introns. The codon usage for the putative GCD1 protein yielded a codon bias index (28) of 0.14, thus suggesting that *GCD1* is not a highly expressed gene. Primer extension analysis revealed four major RNA species whose 5' endpoints are located at positions -48, -12, -4, and +1 with respect to the presumptive AUG initiation codon (Fig. 4). This presumptive initiation codon is the 5'-proximal AUG codon in the three largest *GCD1* RNA species.

The *GCD1* promoter region contains a TATA-like sequence 47 to 95 nucleotides upstream from the mRNA initiation sites, a location that is typical for yeast genes. However, an extended poly(dA-dT) sequence, which acts as an upstream element for constitutive transcription of some yeast genes (29), in not observed. Although *GCD1* is involved in the general control of amino

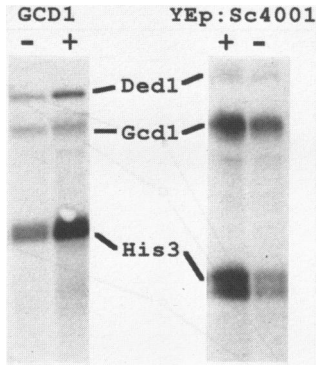

Figure 5: Analysis of *GCD1* RNA levels. Total RNA from either a wild-type (*GCD1*) or a *gcd1-1* strain transformed with the multicopy plasmid YEp13-Sc4001 was prepared from cultures grown in the presence (+) or absence (-) of aminotriazole and probed with ^{32}P-labeled Sc4014 and Sc3119. The positions of the *GCD1*, *HIS3*, and *DED1* transcripts are indicated. In the strain containing multiple copies of *GCD1*, the slight increase in the *GCD1* band intensity upon aminotriazole addition is due to the fact that approximately 50% more RNA was loaded in that lane.

acid biosynthesis, no sequences resembling GCN4 binding sites (6) were observed within 360 base pairs of the mRNA start site.

Transcriptional regulation of *GCD1*

To measure *GCD1* RNA levels, we performed standard hybridization analysis of total yeast RNA using the levels of *HIS3* and *DED1* RNAs as internal controls (Fig. 5). RNA was prepared from cells grown during both non-starvation and amino acid limitation conditions to determine whether *GCD1* mRNA itself was subject to general control. We also examined the effect of *GCD1* copy number on the ability of *HIS3* to be induced during amino acid starvation by assaying a strain containing approximately 10 copies of *GCD1* in the form of YEp13-Sc4001 (as assayed by densitometric scans of genomic DNA probed with Sc4014; data not shown). The amount of *GCD1* RNA appears to be correlated with the copy number of the gene and is independent of growth conditions. Aminotriazole addition results in an induction of *HIS3* RNA that is independent of *GCD1* copy number. Thus, a high gene dosage of *GCD1* does not substantially alter the cell's ability to respond to amino acid starvation nor does the relative expression level of *GCD1* change as a result of amino acid starvation.

Protein synthesis and cell viability of *GCD1* and *gcd1-1* strains

Although the original characterization of the *gcd1-1* mutation suggested a minimal defect in protein synthesis at the non-permissive growth temperature of 37°C (13), we have reexamined this issue using different methods. In one experiment, protein synthesis was measured by the continuous labeling of proteins with ^{35}S-methionine (Fig. 6A). At 23°C, the rate of protein synthesis in strain KY403 is 2 to 4 fold lower than the corresponding rate in a wild type strain.

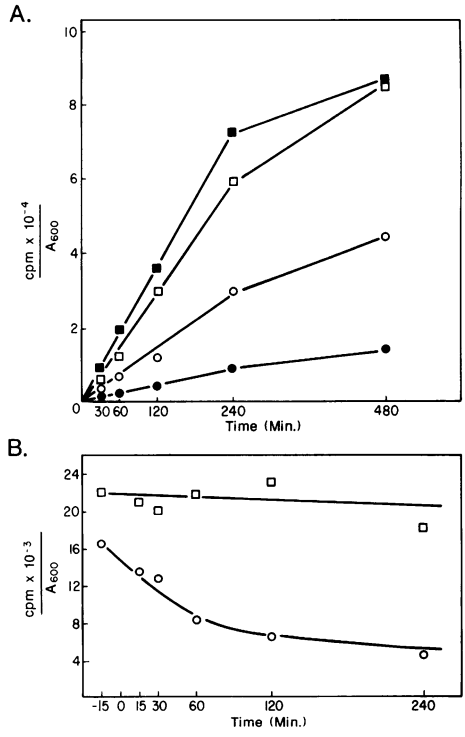

Figure 6: Protein synthesis in *GCD1* and *gcd1-1* strains. Panel A shows the amount of total protein synthesis from continuous labeling of *GCD1* (□,■) or *gcd1*-1 (○,●) cultures incubated at either 23° C (□,○) or 37° C (■,●) for varying times. The cpm of ^{35}S-methionine labelled TCA precipitable material was divided by total cell number as determined by absorbance measurements at 600 nm wavelength to normalize the data to a per cell basis. Panel B shows the amount of protein synthesis from a 15 minute pulse labelling of *GCD1* (□) or *gcd1*-1 (○) cultures after incubation at 37°C for varying times. The -15 time point represents the amount of pulse-labeled material from cells incubated at 23°C.

This reduced rate of protein synthesis correlates with the 2 fold slower growth rate of the *gcd1* strains at 23°C. After shifting a culture to 37°C, the rate of proteins synthesis in the *gcd1* strain is 8 to 10 fold lower than in the wild-type strain.

In another set of experiments (Fig. 6B), yeast cells were pulse-labeled for five minutes with ^{35}S-methionine at various times after shifting cultures from 23°C to 37°C. Within 15 minutes after a shifting a culture to 37°C, a *GCD1* strain is already impaired in its ability to incorporate ^{35}S-methionine into protein whereas the wild type strain is unaffected. (While yeast exhibit a mild heat shock response at 37°C, the cells do not significantly alter their pattern of protein synthesis.) The inability to incorporate ^{35}S-methionine into protein at 37°C in a *gcd1* strain is not due to the inability to take up methionine from the medium because both mutant and wild-type

strains contain similar amounts of radioactive material after harvesting and washing the cells. However, we have not directly excluded the unlikely possibility that the *gcd1* mutation alters the intracellular pool of methionine. These experiments strongly suggest, but do not prove, that *GCD1* performs an important function in general protein synthesis.

Yeast strains carrying the *gcd1-1* allele behave as *START* mutants because they arrest at the start of the cell cycle after shifting the culture to 37°C (13). By incubating KY403 cultures at 37°C for periods from 1 to 72 hours and then returning the culture to 23°C, we observed that nearly 100% of the cells survived a 24 hour incubation and at least 50% of the cells survived a 48 hour incubation. The fact that the *gcd1* mutant can survive long periods at 37°C without a significant loss of viability suggests that the *gcd1* restrictive condition is characterized by an orderly shutdown of overall cellular metabolism.

DISCUSSION

GCD1 was originally defined by a single allele that prevented growth at high temperature and caused constitutive induction of the amino acid biosynthetic genes at lower temperatures (13). In this paper, we obtained two overlapping genomic segments that complemented the temperature-sensitive phenotype caused by the *gcd1-1* allele and mapped genetically to the *GCD1* locus. A 2.4 kb *GCD1* DNA fragment encoding a single open reading frame of 511 amino acids restored normal regulation of *HIS3* and presumably other amino acid biosynthetic genes. In addition, this fragment fully complemented all the phenotypes exhibited by several new *gcd1* mutant alleles (17). Thus, it is likely that a single protein product carries out all known *GCD1* functions.

The temperature-sensitive phenotype of *gcd1-1* suggested that *GCD1* encoded an essential function. This was confirmed by showing that disruption of the *GCD1* locus is a lethal event. This lethal phenotype is probably analogous to the cell-cycle phenotype observed in *gcd1*-1 cells that have been shifted to the restrictive temperature. Although *GCD1* is clearly involved in the general control of amino acid biosynthesis and in the translational control of the GCN4 activator protein, *GCD1* RNA is not regulated as a function of amino acid starvation and the *GCD1* promoter region does not contain a sequence that is recognized by GCN4. Overexpression of *GCD1* mRNA does not affect the regulation of amino acid biosynthesic genes or cell growth.

Pulse labeling of cells with ^{35}S-methionine suggests that protein synthesis in a *gcd1* strain is impaired only 15 minutes after a shift to 37°C. This suggests that the essential function of *GCD1* is involved in protein synthesis and that *GCD1* is an important component of the basic protein synthetic machinery. Although the specific function of *GCD1* in protein synthesis is unknown, several possibilities can be excluded. *GCD1* is unlikely to be a amino acyl-tRNA synthetase because the level of charged amino acyl-tRNA complexes remains normal in a *gcd1* strain even after 4 hr incubation at 37°C (18). It is also unlikely that *GCD1* encodes a ribosomal protein because its predicted length of 511 amino acids is considerably larger than any ribosomal

protein except L3, which is encoded by the *TCM* gene (30). More likely candidates for *GCD1* function are translational initiation, elongation, or termination factors. In this regard, however, the predicted *GCD1* sequence does not exhibit significant homology to any other known proteins nor does it not contain motifs that resemble GTP-binding proteins.

It seems likely that the proposed involvement of *GCD1* in general protein synthesis is related to its role in the translational regulation of GCN4. One possibility is that GCD1 is part of the machinery that determines where translation is initiated. In this view, viable *gcd1* mutants would relax the "first AUG rule" and permit initiation at the (fifth) AUG codon that specifies GCN4. In wild-type cells, *GCD1* might directly or indirectly sense amino acid starvation and possibly undergo a conformational change or bind an effector molecule to generate an altered GCD1 protein with relaxed initiation specificity. One problem with this view is that a mutation of the first AUG codon abolishes translation of *GCN4* mRNA even in a *gcd1*-1 background (9).

An alternative view, which we favor, is that *GCD1* might be involved in translational termination, specifically in the mechanism that prevents reinitiation at downstream AUG codons. In wild-type cells grown in normal conditions, translational termination at the *GCN4* upstream ORFs would occur, ribosomes would be released from the *GCN4* mRNA and GCN4 protein would not be synthesized. During amino acid starvation or in viable *gcd1* strains, the termination event and/or ribosome release at the upstream ORFs of *GCN4* would be prevented, thereby allowing translating ribosomes to initiate translation at the correct AUG of *GCN4*. As mentioned above, GCD1 would presumably exist in two states depending on the state of amino acid biosynthesis. To explain why a mutation of the first AUG codon abolishes translation of *GCN4* mRNA under all circumstances, the alteration in the normal termination machinery that permits reinitiation might require specific initiation at the first AUG codon. This idea is reminiscent of antitermination of transcription by the bacteriophage λ N or Q proteins where specific sequences in the template are necessary to alter the properties of the transcription machinery (31).

Yeast cells initiate new cell cycles only if they have sufficient amino acids to complete the cycle. Auxotrophs for a particular amino acid arrest at the start of the cell cycle upon being switched to medium lacking this amino acid. Interestingly, *gcd1* mutants are viable for days after a shift to 37°C, a situation similar to auxotrophic cells starving for the required amino acid. Although *gcd1* strains retain normal levels of charged tRNAs at the restrictive temperature and hence are not actually starving for amino acids (18), *GCD1* may be part of the sensing mechanism by which cells assess the state of amino acid biosynthesis before initiating a new cycle. As a component of protein synthesis, *GCD1* would be ideally situated to monitor the level of amino acid precursors. During mild starvation conditions, *GCD1* might sense that the reduced levels of charged tRNAs were insufficient to allow completion of the cell cycle. Consequently, *GCD1* would signal the cell to arrest growth unless or until the cell was able to increase its levels of charged tRNAs by increasing *GCN4* translation thereby inducing genes under general control and increasing amino acid levels. By potentially being involved directly in the translational

control mechanism for the synthesis of GCN4 activator protein, *GCD1* may be an important link between the capacity for protein synthesis by controlling the amount of amino acid precursors and the decision to initiate new rounds of cell growth.

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