The Global Transcriptional Activator of *Saccharomyces cerevisiae*, Gcr1p, Mediates the Response to Glucose by Stimulating Protein Synthesis and CLN-Dependent Cell Cycle Progression

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ABSTRACT

Growth of *Saccharomyces cerevisiae* requires coordination of cell cycle events (e.g., new cell wall deposition) with constitutive functions like energy generation and duplication of protein mass. The latter processes are stimulated by the phosphoprotein Gcr1p, a transcriptional activator that operates through two different Rap1p-mediated mechanisms to boost expression of glycolytic and ribosomal protein genes, respectively. Simultaneous disruption of both mechanisms results in a loss of glucose responsiveness and a dramatic drop in translation rate. Since a critical rate of protein synthesis (CRPS) is known to mediate passage through Start and determine cell size by modulating levels of Cln3p, we hypothesized that *GCR1* regulates cell cycle progression by coordinating it with growth. We therefore constructed and analyzed ger1Δ cln3Δ and ger1Δ cln1Δ cln2Δ strains. Both strains are temperature and cold sensitive; interestingly, they exhibit different arrest phenotypes. The ger1Δ cln1Δ strain becomes predominantly unbudded with 1N DNA content (G1 arrest), whereas ger1Δ cln1Δ cln2Δ cells exhibit severe elongation and apparent M phase arrest. Further analysis demonstrated that the Rap1p/Gcr1p complex mediates rapid growth in glucose by stimulating both cellular metabolism and *CLN* transcription.

The budding yeast *Saccharomyces cerevisiae* is an excellent model system to study eukaryotic regulatory networks that control the proliferative response to nutrients. Although the rate of cellular growth varies widely and is known to depend upon the type and availability of nutrients, cell size remains virtually constant. This is accomplished by adjusting the length of the G1 phase of the cell cycle to match the rate of increase in cellular mass. The transition from late G1 into S phase, at a critical point called Start, is therefore linked to cellular growth through regulatory mechanisms that remain to be fully elucidated. Four important observations concerning coordination of the cell and growth cycles are as follows: (1) bud initiation occurs only after a critical rate of protein synthesis (CRPS) is attained (Popolo et al. 1982; Moore 1988; Baroni et al. 1994); (2) the level of the G1 cyclin Cln3p is determined by the rate of protein synthesis through both transcriptional and posttranscriptional mechanisms (Polymenis and Schmidt 1997; Gallego et al. 1997; Hall et al. 1998; Danaie et al. 1999; Anthony et al. 2001); (3) deletion of *GCR1* eliminates the glucose-dependent transcriptional induction of *CLN3* in response to translation rate (Parviz and Heideman 1998); and (4) the normal dramatic increase in growth rate upon addition of glucose is absent in ger1Δ cells, which accumulate in the G1 phase of the cell cycle (*vide infra*).

The *GCR1* product is a component of the Rap1p/Gcr1p/Gcr2p complex, which activates transcription of translational component and glycolytic genes (Santangelo and Tornow 1990; Tornow et al. 1993; Zeng et al. 1997; Deminoff and Santangelo 2001). Activation of each class of target genes occurs via a distinct mechanism. The general mechanism, specific for translational component [including most ribosomal protein (RP)] genes, occurs in the absence of Gcr2p and requires the main homodimerization domain of Gcr1p (1LZ, a leucine zipper; Deminoff et al. 1995). The specialized Gcr1p mechanism, which stimulates transcription of glycolytic genes, is 1LZ independent but requires Gcr2p. Both mechanisms must be disrupted to eliminate the response to glucose. Together, the two classes of Rap1p/Gcr1p/Gcr2p target genes make up a substantial fraction of RNA polymerase II transcription and provide the engine for cellular growth. For example, characterization of the transcriptome by SAGE has demonstrated that 26 of the 30 most highly expressed yeast genes are either translational component or glycolytic genes, each of which generates at least 60 mRNA copies per cell (Veliclescu et al. 1997).

Several observations beyond those mentioned above suggest that the *GCR1*-mediated response to nutrients...
plays a critical role in the decision at Start. For example, deletion of GCR1 (or of GCR2) is lethal in the absence of the cyclin-dependent kinase gene PHO85 (Lenburg and O’Shea 2001; our unpublished data). In conjunction with its cyclin partners Pcl1p, Pcl2p, and Pcl9p, Pho85p makes specific contributions to Start and to other G1 and M phase events (Measday et al. 1994, 1997; Tennyson et al. 1998). Further, the list of Rap1p/Gcr1p/Gcr2p activation contributes to coordination between the separate but tightly linked GCR1 genes or deletion of genes required for passage through Start (Pringle and Hartwell 1981)—the glycolytic gene PYK1 (CDC19) and the translational component gene CDC33 (encoding eIF-4E). Lesions in eIF-4E cause cell cycle arrest at Start due to low protein synthetic activity, and artificial restoration of Cln3p levels suppresses the cdc33 arrest phenotype (Danaie et al. 1999). It therefore seems likely that Rap1p/Gcr1p/Gcr2p activation contributes to coordination between the separate but tightly linked growth and cell cycles.

We further investigated GCRI involvement in cell cycle regulation by constructing and analyzing ger1Δ cdc3Δ and ger1Δ cdc2Δ strains. All ger1Δ strains have a severe defect in translation rate accompanied by a cell cycle delay. This delay predominates in either G1 (ger1Δ or ger1Δ cdc3Δ strains) or M phase (ger1Δ cdc1Δ cdc2Δ strains). Combination of ger1Δ with a deletion of any one of the CLN genes or deletion of GCR1, CLNI, and CLN2 together also leads to temperature sensitivity, conditional cell cycle arrest, and severe cellular elongation and flocculation at the nonpermissive temperature. Quantitation of mRNA demonstrated that deletion of GCRI results in decreased levels of CLN transcripts; we therefore propose that Gcr1p plays a critical role in glucose-dependent stimulation of CLN-dependent processes in the M and G1 phases of the cell cycle.

### MATERIALS AND METHODS

**Strain construction and media:** The strains used in this study are listed in Table 1. Strain KW1474 was constructed by transforming an a/a diploid of BY263 with a 6483-bp SalI-Xhol ger1::URA3 fragment (Deminoff et al. 1995). The resulting diploid (KW1400) was sporulated and tetrads were dissected to obtain a GCR1-disrupted MATa haploid segregant (KW1474). Strain KW1970 was constructed in an analogous manner by generating the diploid KW1900 with a 1539-bp PvuII-NsiI ger2::TRP1 fragment. GMS1203 is a segregant generated by mating BY347 with KW1474 and dissecting tetrads after sporulation of the resulting diploid. GMS3501 and GMS3503 are segregants of the diploid GMS3500, which contains disrupted GCR1 (ger1::HYG1) and CLN3 (cna3::URA3) alleles. GMS4101 was constructed by allelic replacement with a ΔD allele of GCR1 (DEMINOFF et al. 1995) in which TRP1 was inserted into the 3′ flanking region at the KpnI site. The ΔD lesion removes hypomutable region D, which contains the Gcr1p leucine zipper (1LZ).

Cells were grown in YEP containing 2% glucose (YEPD) unless otherwise indicated; semisolid media contained 2% agar. For *in vitro* translation assays, strains were grown to mid-logarithmic phase at 23°C in YNB supplemented with required nutrients (histidine, leucine, lysine, tryptophan, adenine, and uracil).

**In vitro translation rate assay:** Translation rates were measured by adding [3H]glutamate (22.5 Ci/mmol) to YNB cultures to a final concentration of 0.5 μCi/ml; samples were taken every 15 min for an hour. After precipitation with 7.5% trichloroacetic acid and 7.5 μg/ml BSA, the samples were heated in a 100°C water bath for 3 min and then allowed to cool. Each precipitate was collected on a Whatman GF/A circular filter and counts per minute were determined in a Beckman scintillation counter. Translation rate (picomoles per minute

### TABLE 1

#### Yeast strains

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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<tr>
<td>BY263</td>
<td>MATa ade2-107 his3Δ200 leu2Δ1 trp1Δ63 ura3-52 lys2-801</td>
<td>MEASDAY et al. (1994)</td>
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<td>BY347</td>
<td>Same as BY263 and cln1::TRP1 cln2::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>KW1400</td>
<td>Same as BY263 but MATa/MATa GCR1/gcr1::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>KW1474</td>
<td>Same as BY263 but MATa and ger1::URA3</td>
<td>This study</td>
</tr>
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<td>KW1900</td>
<td>Same as BY263 but MATa/MATa GCR2/gcr2::TRP1</td>
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</tr>
<tr>
<td>GMS4101</td>
<td>Same as BY263 but MATa and GCR1Δc1Z-TRP1</td>
<td>This study</td>
</tr>
</tbody>
</table>

* All strains carry the flo8 mutation.
* Segregant of KW1400.
* Segregant of KW1900.
* Segregants of GMS3500.
of [3H]glutamate incorporated) was calculated and normalized to cell number. Overall uptake of precursor was measured by collecting 0.5 ml of cells onto a 0.2-μm nitrocellulose filter 60 min after labeling as described above. Samples were then washed three times to remove any [3H]glutamate adhering nonspecifically to the surface of the cells. Counts per minute were determined as above and normalized to cell number.

**Phenotypic analysis:** For both light microscopy and 4′,6-diamidino-2-phenylindole (DAPI) staining, cells were pelleted in a microcentrifuge and resuspended in 0.25 m EDTA prior to slide preparation. Phase contrast photomicrographs were taken with an Olympus BH2 with a ×40 Plan40L objective. DAPI staining (Sherman et al. 1986) was visualized with an Olympus BX60 with a ×40 UPlanApo oil iris Ph3. Cell size distributions were determined with a Coulter Multisizer 3 (Beckman Coulter, Fullerton, CA). Flow cytometry was done by staining cells with propidium iodide; DNA content was then analyzed as described previously (Measday et al. 1994). Quantitation of transcripts by primer extension was done by using the following primers (CLN1, CLN2, CLN3, and ACT1, respectively): 5′-TCTGCTTTTGCAGTGACAATTAACCCAGT-3′, 5′-AGCATTGATGACGAGTCCCATACGGGGT-3′, 5′-TACCTAAAGACTCCTTTC-3′, and 5′-TAACCAAAGCAGCAACCTCAGAATCCAT-3′. The primer extension reactions and subsequent sample processing were done as described previously (Deminoff and Santangelo 2001).

**Flocculation assay:** The flocculation assay was derived from previously described protocols (Bony et al. 1998; Palecek et al. 2000). Yeast cultures grown in YEPD were pelleted, deflocculated twice by washing with buffer (50 mM sodium citrate, 5 mM EDTA pH 3.0), and resuspended in the same buffer at a density of 10^7 cells/ml. Flocculation was induced by the addition of CaCl_2 at a final concentration of 20 mM. Cells were then incubated on a roller drum (model TC-7; New Brunswick Scientific, Edison, NJ) at full speed for 10 min, after which 0.2 ml was removed from just below the meniscus and added to 1 ml of 0.25 mM EDTA. OD_600 was measured in a Beckman DU-65 spectrophotometer (Beckman Coulter, Fullerton, CA) and this was defined as time zero (t_0). Flocculation levels are expressed as the t_0/t_10 ratio, and are normalized to wild-type BY263 at 23°C.

**RESULTS**

Cells lacking GCRI fail to respond to glucose and accumulate in G_1: It has long been known that glucose is the preferred carbon source of *S. cerevisiae* (Polakis and Bartley 1966). We have found that addition of glucose (between 0.2 and 2%) to cells in rich medium fails to stimulate strains lacking the transcriptional activator Gcr1p. In the absence of glucose, wild-type and *gcr1Delta* strains have indistinguishable growth rates (Figure 1). A significant accumulation of *gcr1Delta* cells in the G_1 phase of the cell cycle accompanies this defect in glucose responsiveness (Figure 2). In contrast, impairment of either the specialized Gcr1p mechanism that activates

**Figure 1.—** Cells deleted for GCRI fail to respond to glucose. Isogenic strains were grown at 23°C in YEP to 5 × 10^5 cells/ml. Glucose was added (to the percentage indicated in the key) at time zero, and growth was measured by using a Beckman-Coulter Multisizer 3 to count cells. Strains used were BY263 (WT; solid symbols) and KW1474 (*gcr1Delta*; open symbols). The average of two determinations (error ±10%) is shown for each time point; one of four essentially identical repetitions of this experiment is shown.

**Figure 2.—** G_1 delay in *gcr1Delta* cells. DNA content was measured by flow cytometry of cells of strains BY263 (wild type) and KW1474 (*gcr1Delta*) growing logarithmically at 23°C.

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**Figure 1**

<table>
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<tr>
<th>Glucose</th>
<th>WT</th>
<th>gcr1∆</th>
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<tbody>
<tr>
<td>0.0%</td>
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<td>--o--</td>
</tr>
<tr>
<td>0.2%</td>
<td>--o--</td>
<td>--o--</td>
</tr>
<tr>
<td>0.7%</td>
<td>--o--</td>
<td>--o--</td>
</tr>
<tr>
<td>2.0%</td>
<td>--o--</td>
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<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Cells per ml (×10^9)</th>
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<tbody>
<tr>
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<tr>
<td>5</td>
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<td>10</td>
<td>90</td>
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<tr>
<td>15</td>
<td>100</td>
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<tr>
<td>20</td>
<td>100</td>
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**Figure 2**

**Fluorescence / cell**

<table>
<thead>
<tr>
<th>Number of cells</th>
</tr>
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<tbody>
<tr>
<td>wild type</td>
</tr>
<tr>
<td>gcr1∆</td>
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glycolytic genes (by deletion of GCR2) or the general mechanism that activates RP genes (by deletion of 1LZ) causes only a slight decrease in the stimulation of growth by glucose (Deminoff and Santangelo 2001; unpublished data). Thus a severe synthetic defect in glucose responsiveness occurs when both mechanisms are eliminated. It is important to note that addition of glucose to ger1Δ cultures has no negative effect; these cells simply fail to exhibit the normal increase in growth rate observed for wild-type cells (Figure 1).

**GCR1 is required to maintain wild-type translation rates**: Through its two mechanisms of activation (specialized and general), Gcr1p has a large impact on the cell’s potential for growth via energy generation and production of ribosomes (Deminoff and Santangelo 2001). In turn, the translation rate of cells, which can be thought of as a combination of consumable energy and available ribosomes, is a critical determinant of the decisions executed at Start and in G2/M (Moore 1988; Burke and Church 1991). We therefore measured the translation rate for cells with lesions in GCR1 or GCR2. The rate of protein synthesis in a ger1Δ strain is severely reduced relative to GCR1 strains; this reduction is not caused by a change in uptake of precursor (Table 2). Neither the ger2Δ nor the GCR1Δ1LZ lesion alone resulted in more than a minor alteration in the rate of protein synthesis. However, the combined loss of glycolytic gene transcription (the ger2Δ defect) and RP gene transcription (the GCR1Δ1LZ defect) eliminates the upshift in translational capacity normally observed in the presence of glucose (Deminoff and Santangelo 2001; K. E. Barbara and G. M. Santangelo, unpublished data).

**Deletion of CLN genes in a ger1Δ background causes specific defects in cell cycle progression**: The accumulation of ger1Δ cells in G1, the impact of GCR1 deletion on translation rate (a known determinant of passage through Start), and results obtained elsewhere (see Introduction), are all suggestive of a regulatory connection between GCR1 and cell cycle progression. To investigate this relationship further, we measured the percentage of unbudded cells in ger1Δ and in related mutant strains. As expected from the flow cytometry data (Figure 2), there is a two- to threefold increase in the fraction of unbudded ger1Δ cells in rich medium relative to wild-type, ger2Δ, or GCR1Δ1LZ strains (Figure 3). In our strain background, consistent with previous reports (Cross 1988), the cln3Δ mutation also resulted in an accumulation of unbudded cells (Figure 3).

We next constructed two new strains to allow further analysis of the role of Gcr1p in cell cycle regulation (ger1Δ cln3Δ and ger1Δ cln1Δ cln2Δ). The budding index of these two strains was surprisingly disparate. Compared to the ger1Δ mutation alone, accumulation of unbudded cells (the G1 delay) was exacerbated in the ger1Δ cln3Δ strain, but greatly decreased in the ger1Δ cln1Δ cln2Δ strain (Figure 3). Since the latter strain nevertheless retains the characteristic slow growth of all ger1Δ strains, a delay outside of G1 phase would appear to predominate in ger1Δ cln1Δ cln2Δ cells (see below). Interestingly, the ger1Δ cln1Δ cln2Δ strain has an increased percentage of large budded cells (data not shown). Although this does not by itself distinguish between defects in G2 vs. M phase, the defined role of CLN1 and CLN2 (Lew et al. 1997 and see below) support an M phase interpretation. The ger1Δ cln3Δ and ger1Δ cln1Δ cln2Δ strains therefore appear to harbor distinct but temporally connected defects in cell cycle progression.

**Combination of GCR1 and CLN mutations results in conditional lethality**: We next screened for conditional phenotypes resulting from the combined loss of GCR1 and CLN genes. We found that combination of ger1Δ with a deletion of any one of the CLN genes or deletion of GCR1, CLN1, and CLN2 together leads to both temperature sensitivity at 37°C (Figure 4 and data not shown) and cold sensitivity at 16°C (data not shown). To study this conditional lethality further, we grew ger1Δ cln1Δ cln2Δ and ger1Δ cln3Δ cells under permissive conditions (23°C) and then shifted to the nonpermissive temperature (37°C) to look for an arrest phenotype. We found that, although both ger1Δ cln1Δ cln2Δ and ger1Δ cln3Δ strains failed to grow at 37°C (Figure 5A), the morphology of arrest differed. Cells of both strains increasingly developed elongated structures over the first 8 hr at 37°C. At this point, the percentage of elongated cells in ger1Δ cln3Δ reached a plateau, while in ger1Δ cln1Δ cln2Δ this

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**Table 2**

| Strain* | Rate of [3H]glutamate incorporation | % WT incorporation | Uptake |\
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<tr>
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<tbody>
<tr>
<td>WT</td>
<td>1.36 ± 0.29</td>
<td>100 ± 21</td>
<td>258.1 ± 54.1</td>
</tr>
<tr>
<td>ger2Δ</td>
<td>0.97 ± 0.09</td>
<td>71 ± 7</td>
<td>128.3 ± 38.7</td>
</tr>
<tr>
<td>GCR1Δ1LZ</td>
<td>2.41 ± 0.37</td>
<td>177 ± 27</td>
<td>250.5 ± 12.1</td>
</tr>
<tr>
<td>ger1Δ</td>
<td>0.20 ± 0.12</td>
<td>15 ± 9</td>
<td>250.2 ± 26.1</td>
</tr>
</tbody>
</table>

* All strains carry the flo8Δ mutation.
* Translation rate (pmol/min/10⁷ cells).
* Total amount of [3H]glutamate (pmol/10⁷ cells) taken up from the media.
Figure 3.—Elimination of \textit{GCR1} function results in specific cell cycle impairment. Budding index indicates a G\(_1\) delay in \textit{gcr1} strains. Isogenic strains were grown in YEPD at 23°C to midlogarithmic phase; the fraction of un budded cells was determined with a hemacytometer. Strains are as follows: BY263 (WT), KW1970 (\textit{gcr2}), GMS4101 (\textit{GCR1}1LZ), BY347 (\textit{cln1} cln2), GMS3503 (\textit{cln3}), KW1474 (\textit{gcr1}), GMS3501 (\textit{gcr1} cln3), and GMS1203 (\textit{gcr1} cln1 cln2). The \textit{GCR1}1LZ allele contains a small deletion that removes the primary leucine zipper in Gcr1p (Deminoff et al. 1995; Deminoff and Santangelo 2001).

percentage continued to increase beyond 29 hr after the shift to 37°C, eventually reaching >70% of the total (Figures 5B and 6). The trend for each strain shown in Figure 5A persisted for over 5 days; by 126 hr, \textit{gcr1} cultures reached an average density of 4 × 10\(^6\) cells/ml, while \textit{gcr1} cln3 and \textit{gcr1} cln1 cln2 strains remained at 1 × 10\(^6\) cells/ml. To test whether or not slow growth alone (comparable to that of a \textit{gcr1} mutant) would produce this temperature sensitivity, we repeated the experiment shown in Figure 5A, but grew the cells in YEP rather than in YEPD. Under these conditions, both \textit{GCR1} and \textit{gcr1} strains grow very slowly, phenocopying \textit{gcr1} (see Figure 1). None of the control strains (\textit{GCR1}, cln3, cln1 cln2, and \textit{gcr1}) were temperature sensitive when grown in YEP (data not shown). The synthetic \textit{gcr1} cln3 and \textit{gcr1} cln1 cln2 arrest is therefore a specific consequence of \textit{GCR1} deletion and not a general effect that results from a slow growth phenotype.

Accumulation of arrested \textit{gcr1} cln3 and \textit{gcr1} cln1 cln2 cells in G\(_1\) and M phase, respectively: For both \textit{gcr1} cln3 and \textit{gcr1} cln1 cln2 strains, virtually all morphologically normal cells were un budded. Thus the vast majority of \textit{gcr1} cln3 cells become un budded at the nonpermissive temperature, suggestive of a G\(_1\) arrest phenotype. To test this idea and to determine whether elongated cells had completed S phase, we DAPI-stained arrested \textit{gcr1} cln3 and \textit{gcr1} cln1 cln2 cells and looked at nuclear morphology. We found that elongated cells in both strains appeared to contain two nuclei (Figure 7, arrows). As expected, all un budded cells contained a single nucleus. Thus all elongated cells (a group including most \textit{gcr1} cln1 cln2 cells) appeared to accumulate postanaphase, and most \textit{gcr1} cln3 cells were un budded and contained a single nucleus, i.e., they appeared to accumulate in G\(_1\). These observations are consistent with the cell cycle delays suggested by measurement of budding index at the permissive temperature (Figure 3).

Note the swollen appearance of all \textit{gcr1} strains in Figure 7; this is another well-known feature of the prototypical G\(_1\) delay associated with \textit{CLN3} deletion (Cross 1988; Linskens et al. 1993; Polymenis and Schmidt 1997; MacKay et al. 2001). We quantified this phenotype by measuring cell size distributions at 23°C (Figure 8); deletion of \textit{GCR1} results in an average cell volume comparable to cln3 cells and twice that measured for the wild type strain. The size distribution of cln1 cln2 cells was virtually identical to wild type (Figure 8A). Both the \textit{gcr1} cln3 and \textit{gcr1} cln1 cln2 strains (at
the permissive temperature) exhibited a further increase in cell volume relative to those that contained the ger1Δ or cln3Δ lesion alone (Figure 8B). Thus removal of Cln1p and Cln2p does appear to affect cell volume, although this takes place only in the ger1Δ background.

Finally, we did flow cytometry after a shift to the nonpermissive temperature to analyze the DNA content of arrested cells directly. Unfortunately, the extremely irregular morphology of ger1Δ cln1Δ cln2Δ cells (Figure 6) interfered to the extent that an unambiguous determination of DNA content in those arrested cultures was not possible. However, flow cytometry with the ger1Δ cln3Δ strain was less problematic and yielded the result shown in Figure 9. Most of the cells were 1N after 20 hr at 37°C, consistent with G1 arrest of the 80% unbudded cells observed in previous experiments (Figures 5, 6, and 7).

Enhanced flocculation of ger1Δ cln1Δ cln2Δ and ger1Δ cln3Δ cells: Combined deletion of GCR1 and CLN genes also resulted in dramatic flocculation at the nonpermissive temperature; we used a standard assay to measure this morphological alteration in ger1Δ cln3Δ and ger1Δ cln1Δ cln2Δ strains (Table 3). We first did PCR and DNA sequence analysis to confirm that our strain background contains the flo8Δ mutation (Liu et al. 1996). This allele, which is indeed present in all of the strains analyzed here (data not shown), completely eliminates the function of FLO8. Flo8p activates transcription of FLO1, a gene encoding an important cell surface flocculation protein (Bony et al. 1998; Kobayashi et al. 1999). Flo8p was not required for the high level of flocculation of either ger1Δ cln1Δ cln2Δ or ger1Δ cln3Δ cells relative to all other strains (>4-fold over wild type), a phenotype that was exacerbated by incubation at the nonpermissive temperature (>13-fold over wild type; Table 3).

GCR1 deletion leads to decreased expression of the G1 cyclins CLN1, CLN2, and CLN3: We next sought direct evidence that GCR1 function is linked to CLN activity in cell cycle progression. Previous work by others (Parviz and Heideman 1998) suggested the possibility that removal of GCR1 might result in reduced and/or misregulated CLN transcription. If this is true, the cell cycle phenotypes described above might derive from a relatively simple synthetic CLN defect: reduction in CLN activity upon deletion of one or more CLN genes, combined with the perturbation in expression of the remaining CLN(s) by eliminating Gcr1p. Taken together, the data would then also suggest that GCR1 plays an important role in stimulating cell cycle progression in wild-type cells. We tested this idea by doing primer extension analysis to quantitate CLN transcripts in asynchronous cultures (Figure 10). The extension products shown in Figure 10, A and C, were those predicted by previously published maps of transcriptional start sites in CLN1, CLN2, and CLN3 (Ogas et al. 1991; Polymenis and Schmidt 1997).

First, CLN3 mRNA was measured in wild-type (WT), ger1Δ, and ger1Δ cln1Δ cln2Δ cells (Figure 10). Gcr1p was indeed required for normal CLN3 transcription; further removal of CLN1 and CLN2 caused only a minor reduction in CLN3 mRNA levels beyond that caused by GCR1 deletion. The latter result was not surprising, since
**CLN Response to Glucose Requires GCR1**

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**DISCUSSION**

We have discovered that combination of *ger1Δ* with a deletion of any one of the *CLN* genes or deletion of *GCR1, CLN1*, and *CLN2* together leads to temperature sensitivity (Figure 4). At the permissive temperature, *ger1Δ cln3Δ* and *ger1Δ cln1Δ cln2Δ* cells appear to accumulate at distinct but temporally connected phases of the cell cycle—the former in G1 (unbudded) and the latter in M (postanaphase). This phenomenon is exacer-

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**CLN1** and **CLN2** are not thought to influence **CLN3** expression (Nasmyth and Dirick 1991). Second, **CLN1** and **CLN2** mRNA levels were measured in wild-type, *cln3Δ, ger1Δ, and ger1Δ cln3Δ* cells (Figure 10). The data indicate that, consistent with previous reports (Tyers et al. 1993; Dirick et al. 1995; Stuart and Wittenberg 1995), maximal induction of **CLN1** and **CLN2** transcription requires a functional **CLN3** gene. Further, **GCR1** deletion causes a *cln3Δ*-like (two- to threefold) reduction in the steady-state levels of **CLN1** and **CLN2** mRNAs. Interestingly, combined deletion of **GCR1** and **CLN3** resulted in a severe synthetic loss of **CLN1** and **CLN2** transcription.

The **CLN** transcript levels in these strains correlate well with their budding indices during logarithmic growth (Figure 3); *cln3Δ* and *ger1Δ* display a similar increase in the percentage of unbudded cells relative to isogenic wild-type cultures, while the *ger1Δ cln3Δ* double knockout exhibits a further synthetic increase in the fraction of cells in G1 phase of the cell cycle. The underlying defect in *ger1Δ cln3Δ* cells is apparently severe enough to result in G1 arrest at the nonpermissive temperature (Figures 4–7 and 9).

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**Figure 5.**—Temperature shift causes growth arrest and elongated morphology of *ger1Δ cln3Δ* and *ger1Δ cln1Δ cln2Δ* cells. Isogenic strains were grown in YEPD at 23°C to midlogarithmic phase (10^6 cells/ml) and then shifted to 37°C at time zero. Error bars represent the standard error of the mean (SEM). (A) After the temperature shift, the density of each culture in cells per milliliter was determined with a hemacytometer. (B) The fraction of cells exhibiting an elongated morphology was monitored in each culture from A; a cell was scored as elongated if the ratio of its length to its width was ≥3. Strains used are as follows: BY263 (WT; solid circles), GMS3503 (*cln3Δ*; solid triangles), BY347 (*cln1Δ cln2Δ*; solid squares), KW1474 (*ger1Δ*; open circles), GMS3501 (*ger1Δ cln3Δ*; open triangles), and GMS1203 (*ger1Δ cln1Δ cln2Δ*; open squares). Similar results were obtained with several other independent *ger1Δ, ger1Δ cln3Δ,* and *ger1Δ cln1Δ cln2Δ* segregants (not shown).
bated upon a shift to the nonpermissive temperature: most \textit{gcr1\textDelta cln3\Delta} cells appear to arrest in G\textsubscript{1}, whereas arrested \textit{gcr1\textDelta cln1\Delta cln2\Delta} cells have a predominantly M phase appearance (Figures 6 and 7).

This phenotypic difference between \textit{gcr1\textDelta cln3\Delta} and \textit{gcr1\textDelta cln1\Delta cln2\Delta} strains may reflect the asymmetry that characterizes \textit{CLN3} vs. \textit{CLN1-CLN2} function. Our current understanding of \textit{CLN3} suggests that its role is limited to G\textsubscript{1} progression and the related functions of cell size determination and \textit{CLN1-CLN2} activation. In contrast, \textit{CLN1} and/or \textit{CLN2} appear to play more specific roles in (for example) bud emergence (Benton et al. 1993; Cvrckova and Nasmyth 1993), inhibition of B cyclin degradation (Amon et al. 1994), and cytokinesis.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Distinct arrest phenotypes of \textit{gcr1\textDelta cln3\Delta} and \textit{gcr1\textDelta cln1\Delta cln2\Delta} cells. Isogenic strains GMS3501 (\textit{gcr1\textDelta cln3\Delta}; left) and GMS1203 (\textit{gcr1\textDelta cln1\Delta cln2\Delta}; right) were grown in YEPD at 23\textdegree\ to midlogarithmic phase (10\textsuperscript{6} cells/ml) and then shifted to 37\textdegree\ at time zero. Phase contrast photomicrographs (\times400 magnification) were taken just before (23\textdegree) and 17 hr after (37\textdegree) the temperature shift. Bar, 10 \textmu m. The criterion of Palecek et al. (2000) was used to score elongated cells. Cells were counted as elongated if the ratio of their major to minor axes exceeded 3. The average of this ratio for \textit{dia} mutants described by Palecek et al. (2000) was 1.4 ± 0.24.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{Nuclear morphology of arrested \textit{gcr1\textDelta cln3\Delta} and \textit{gcr1\textDelta cln1\Delta cln2\Delta} cells. Seventeen hours after arrest at 37\textdegree, fluorescence microscopy of DAPI-stained cells was done at \times400 magnification. (Right) Arrows point to putative nuclei that have migrated into elongated structures in GMS3501 (\textit{gcr1\textDelta cln3\Delta}) and GMS1203 (\textit{gcr1\textDelta cln1\Delta cln2\Delta}). (Left) BY263 (WT) and KW1474 (\textit{gcr1\textDelta}) are shown as controls. Bar, 10 \textmu m.}
\end{figure}
The data we present here provide an explanation for the swollen appearance of ger1Δ cells, since loss of CLN3 function is known to increase cell size (Figure 8). There are two nonexclusive possibilities through which Gcr1p deletion could stimulate CLN3 expression, since GCR1 deletion both reduces CLN3 transcription (Figure 10) and leads to a severely deficient translation rate (Table 2). The

**TABLE 3**

Severe flocculation phenotype of ger1Δ čln3Δ and ger1Δ čln1Δ čln2Δ cells at the nonpermissive temperature

<table>
<thead>
<tr>
<th>Strain*</th>
<th>23°C</th>
<th>37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>čln3Δ</td>
<td>2.2</td>
<td>4.1</td>
</tr>
<tr>
<td>čln1Δ čln2Δ</td>
<td>1.6</td>
<td>3.5</td>
</tr>
<tr>
<td>ger1Δ</td>
<td>1.9</td>
<td>3.1</td>
</tr>
<tr>
<td>ger1Δ čln3Δ</td>
<td>4.3</td>
<td>13.5</td>
</tr>
<tr>
<td>ger1Δ čln1Δ čln2Δ</td>
<td>4.5</td>
<td>19.4</td>
</tr>
</tbody>
</table>

*Strains: BY263 (WT), GMS3503 (čln3Δ), BY347 (čln1Δ čln2Δ), KW1474 (ger1Δ), GMS3501 (ger1Δ čln3Δ), and GMS1203 (ger1Δ čln1Δ čln2Δ). This isogenic series of strains is flo8am and thus lacks Flo8p.

A standard flocculation assay was done on midlogarithmic YEPD cultures grown at 23°C or 17 hr after shifting to 37°C. Each mean value (SD <10%) was normalized to the wild-type level at 23°C, which was assigned the value 1.0.

(Cvrčkova et al. 1995). A defect in one or more of these CLN1-CLN2-specific functions may therefore be responsible for the more complex temperature-induced terminal phenotype in ger1Δ čln1Δ čln2Δ cells. Indeed, Benton et al. (1993) isolated mutants in a čln1Δ čln2Δ background that arrest as tubular, multinucleate cells, whose phenotypes were exacerbated at higher temperature. While perhaps in some cases attributable to indirect effects, the post-Start contributions of the CLNs are an important feature of cell cycle control that requires further investigation.

**Figure 8.**—Increased cell size caused by deletion of GCR1. Cell size distribution was determined for isogenic strains grown to midlogarithmic phase in YEPD at 23°C. (A) Analysis of BY263 (WT), KW1474 (ger1Δ), BY347 (čln1Δ čln2Δ), and GMS3503 (čln3Δ). (B) Analysis of GMS1203 (ger1Δ čln1Δ čln2Δ) and GMS3501 (ger1Δ čln3Δ); BY263 (WT) and KW1474 (ger1Δ) from A are included as references.

**Figure 9.**—Predominant G1 arrest of ger1Δ čln3Δ cells at the nonpermissive temperature. Flow cytometry was done as in Figure 2 for strain GMS3501 (ger1Δ čln3Δ) after shifting from 23°C to 37°C and incubating for the number of hours indicated.
Figure 10.—Deletion of GCR1 reduces transcription of all three CLN genes. Ten micrograms of poly(A)⁺ RNA was used in each primer extension reaction to quantitate CLN1, CLN2, CLN3, and ACT1 (actin control) transcript levels. For each gene shown, the analysis was repeated at least three times; freshly extracted poly(A)⁺ RNA was used in each experiment. (A) Representative analysis of CLN3 and ACT1 transcripts in WT, gcr1Δ, and gcr1Δ cln1Δ cln2Δ strains. (B) The results of densitometric quantitation of CLN3 transcript levels (normalized to ACT1 levels) are plotted relative to the corresponding value for wild type, which was designated as 100%; error bars indicate SEM. (C) Representative analysis of CLN1, CLN2, and ACT1 transcripts in WT, cln3Δ, gcr1Δ, and gcr1Δ cln3Δ strains. (D) Densitometric quantitation of CLN1 and CLN2 transcript levels as described for CLN3 levels in B.

latter defect should cause a protracted delay in reaching the CRPS, which in turn results in defective Cln3p accumulation (Polymenis and Schmidt 1997). From this it might be expected that combination of the gcr1Δ and cln3Δ alleles would result in a G₁ delay and sizing defect no worse than that of either alone. This does not appear to be the case, however; gcr1Δ cln3Δ cells are more swollen on average than either gcr1Δ or cln3Δ cells,

Figure 11.—Growth stimulation by Gcr1p in response to glucose. Yeast cells grow rapidly in the presence of glucose, a response that is eliminated in the absence of the transcriptional activator and phosphoprotein Gcr1p (see Figure 1). The dashed line indicates the expected involvement of currently unidentified signal transduction components that are required for transmission of the glucose signal to Gcr1p, which uses distinct mechanisms of transcriptional activation to induce expression of glycolytic genes and translational component genes (Deminoff and Santangelo 2001). Gcr1p also increases the level of CLN3 mRNA and may augment Cln3p expression further through its stimulation of translation rate; in doing so it likely induces CLN1 and CLN2 transcription indirectly. However, Gcr1p also appears to increase CLN1/CLN2 expression in a Cln3p-independent manner; CLN1/CLN2 transcripts are barely detectable in gcr1Δ cln3Δ cells.
and a larger fraction of ger1Δ cln3Δ cultures remain unbudded during logarithmic growth (Figure 3). Thus, Ger1p seems to contribute to G1 progression beyond its induction of Cln3p. This may be explained by the apparent Cln3p-independent induction of CLN1 and CLN2 by GCR1; steady-state levels of CLN1 and CLN2 transcripts drop synthetically (indeed they become nearly undetectable) upon removal of both CLN3 and GCR1 (Figure 10).

It is worth noting that GCR1 transcription has a cell cycle-regulated peak in G2 (Spellman et al. 1998; Shedden and Cooper 2002). Transcription of RAP1, which recruits Gcr1p to the promoters of its target genes, also peaks in G2 (Spellman et al. 1998; Shedden and Cooper 2002). This is not surprising since Rap1p and Gcr1p colocalize to nuclear foci for the majority of the cell cycle. Remarkably, toward the end of M phase, Rap1p is partially released from these foci (Laroche et al. 2000; G. M. Santangelo, unpublished data). Subnuclear localization may therefore have an impact on Rap1p/Gcr1p function at the M/G transition, which might help to explain the disparate arrest phenotypes of ger1Δ cln1Δ cln2Δ cells (apparent M phase) and ger1Δ cln2Δ cells (G1 phase).

The terminal phenotype in both ger1Δ cln3Δ and ger1Δ cln1Δ cln2Δ strains included severe flocculation and the development of elongated structures (particularly in the latter, where >70% of the cells became dramatically elongated; Figures 5 and 6). All cells with elongated structures appeared to contain two separate nuclei, although we were unsuccessful in using flow cytometry to rule out the possibility that some of the DAPI foci in ger1Δ cln1Δ cln2Δ cells (Figure 7) represented nuclear fragments or mitochondria. The flocculation response (Table 3) was Flo8p independent, since the strain background used in this study contains the flo8Δ allele. Flocculation therefore also appears to be Flo1p independent, since FLO1 transcription is undetectable in the absence of Flo8p (Kobayashi et al. 1999; S. J. Deminoff and G. M. Santangelo, unpublished data). Given that the flocculation phenotype was strongly correlated with the synthetic ger1/cln cell cycle arrest, our data suggest a potential (and thus far unexplored) relationship between flocculation and cell cycle regulation. Several studies have implicated glucose and other fermentable sugars as inhibitors of flocculation (Masy et al. 1992; Soares and Mota 1996); our results suggest the possibility that GCR1 is required for this repression.

Interestingly, glucose also inhibits filamentation and suppresses the hyperelongation and invasiveness caused by hsl7Δ and RAS2p79 mutations (Cullen and Sprague 2000). It is therefore particularly intriguing that the ger1Δ lesion both eliminates growth stimulation in response to glucose and, in combination with cln mutations, causes cellular elongation and flocculation despite the presence of glucose. A screen for dia (digs into agar) mutants also implicated GCR1 and at least one of its target genes (ADH1) in repression of agar invasion (Palecek et al. 2000). Indeed, fusel alcohols like butanol may promote cellular elongation by inhibiting protein synthesis (Ashe et al. 2001), a mechanism that could prove correct for GCR1 deletion.

A model depicting the essential role for Ger1p in glucose-mediated growth stimulation is shown in Figure 11. Information concerning the two known mechanisms of Ger1p transcriptional activation is omitted from the model but has been described in detail elsewhere (Deminoff and Santangelo 2001; for review see Deminoff et al. 2005). Briefly, the Rap1p/Gcr1p complex binds upstream from glycolytic genes (specialized activation) and translation component genes (general activation); the latter can be distinguished by its requirement for TAF1 (previously known as TAF130; Shen and Green 1997; Mencia et al. 2002). In TAF1-independent specialized activation, a dramatic increase in expression occurs only if the Rap1p/Gcr1p complex also contains Gcr2p. If either of these mechanisms (general or specialized activation) is eliminated, translation rate and cell cycle progression are only slightly perturbed, and a mild growth defect ensues (Deminoff and Santangelo 2001). If both mechanisms are impaired (e.g., in ger1Δ cells), translation rate is reduced fivefold, cells become swollen and accumulate in G1 as unbudded forms, and stimulation of growth by glucose is virtually eliminated. The G1 delay and increase in cell size appear to be exacerbated by removal of CLN3. Although the growth rates of ger1Δ and ger1Δ cln3Δ cells are comparable, the latter are temperature sensitive and arrest in G1 at the nonpermissive temperature. In contrast, although ger1Δ cln1Δ cln2Δ cells are also temperature sensitive, they appear to arrest in M phase just prior to cytokinesis. The latter phenotype may be explained by perturbations in Cdc1 expression or in other CLN1/CLN2-specific functions; further work is necessary to decide among these alternatives. Answering this and related questions should yield insights regarding several important phenomena, including the divergent roles of CLN3 and CLN1-CLN2 at Start and the regulatory impact of growth on cell cycle progression.

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