MOLECULAR AND CELLULAR BIOLOGY, Dec. 1990, p. 6482–6490
0270-7306/90/126482-09$02.00/0

Vol. 10, No. 12

Cell Cycle Arrest Caused by *CLN* Gene Deficiency in *Saccharomyces cerevisiae* Resembles START-I Arrest and Is Independent of the Mating-Pheromone Signalling Pathway

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Received 13 June 1990/Accepted 13 September 1990

Null mutations in three genes encoding cyclin-like proteins (*CLN1*, *CLN2*, and *CLN3*) in *Saccharomyces cerevisiae* cause cell cycle arrest in G1 (*cln* arrest). In *cln1 cln2 cln3* strains bearing plasmids containing the *CLN3* (also called *WHI1* or *DAF1*) coding sequence under the transcriptional control of a galactose-regulated promoter, shift from galactose to glucose medium (shutting off synthesis of *CLN3* mRNA) allowed completion of cell cycles in progress but caused arrest in the ensuing unbudded G1 phase. Cell growth was not inhibited in arrested cells. Cell division occurred in glucose medium even if cells were arrested in S phase during the initial 2 h of glucose treatment, suggesting that *CLN* function may not be required in the cell cycle after S phase. However, when the coding sequence of the hyperactive C-terminal truncation allele *CLN3-2* (formerly *DAF1-1*) was placed under *GAL* control, cells went through multiple cycles before arresting after a shift from galactose to glucose. These results suggest that the C terminus of the wild-type protein confers functional instability. *cln*-arrested cells are mating competent. However, *cln* arrest is distinct from constitutive activation of the mating-factor signalling pathway because *cln*-arrested cells were dependent on the addition of pheromone both for mating and for induction of an α-factor-induced transcript, *FUS1*, and because *MAT*a/*MAT*α (pheromone-nonresponsive) strains were capable of *cln* arrest in G1 (although a residual capacity for cell division before arrest was observed in *MAT*a/*MAT*α strains). These results are consistent with a specific *CLN* requirement for START transit.

Following mitosis in the *Saccharomyces cerevisiae* cell cycle, there is a G1 period of varying length before commitment to a new cell division cycle (53). START, the point of commitment, is also the point in the cell cycle at which size control is exerted: small cells have a lower probability of passing START than larger cells (25, 27, 29, 70; for reviews, see references 13 and 53). It is likely that most of G1 in *S. cerevisiae* is taken up by achieving this size requirement (62, 63). A number of gene products are required for exit from the pre-START G1 phase. Some of these are also required for cell growth (the accumulation of cell mass independent of cell cycle progression). Others may be more specifically required for cell cycle progression, since cell growth continues in the absence of functional gene product (2, 29, 52, 54). The former class includes mutations affecting the cyclic AMP pathway (reviewed in reference 68); the latter class includes mutations in the central cell cycle regulatory component *CDC28* (reviewed in reference 13). The relationship between the growth control system and the cell cycle control system is unclear at present (reviewed in references 13, 53, and 68). Arrests at START concomitant with growth arrest have been called START-II arrests; arrests at which cell growth continues have been called START-I arrests (54). These two arrests can also be distinguished by mating competence of START-I-arrested but not START-II-arrested cells (2, 3, 52–54).

Mating pheromones also cause START-I arrest as a part of a program of preparation for mating (reviewed in reference 12). Some mutations resulting in START-I arrest do so because the mutations are in genes that may be negative regulators of the pheromone response pathway; the mutations activate the pheromone response pathway in the absence of pheromone. *cdc36*, *cdc39* (11, 16, 45, 54, 61), and *scg1* (17, 28, 37, 43) mutations are in this category; only pheromone-responsive *MAT*a or *MAT*α cells are arrested at START by the mutations, while non-pheromone-responsive cells such as *MAT*a/*MAT*α diploids are not arrested at START. (Curiously, *cdc36* and *cdc39* mutations are still lethal in non-pheromone-responsive cells, but the lethality is not associated with specific cell cycle arrest.) *cdc28* and *cdc37* mutations result in START-I arrest by a mechanism independent of the pheromone response pathway (11, 28).

Three genes (*CLN1*, *CLN2*, and *CLN3*; *CLN3* was also named *WHI1* or *DAF1*) have been characterized whose activity may be limiting for START transit (9, 14, 15, 22, 44, 56, 66). The encoded proteins share homology with cyclins (22, 44), which are required for the G2/M transition in many systems (7, 20, 23, 36, 39, 40, 65, 67). Dominant activating mutations in or overexpression of any of these three genes results in transit of START at a reduced cell size and a shortened or absent G1 period. Reduced expression results in transit of START at a larger size, and cells with null mutations in all three genes arrest in G1 (14, 15, 22, 44, 56). In this study, I have examined the kinetics of G1 arrest in *cln1,2*-deficient strains on cessation of synthesis of either *CLN3* or *CLN3-2* (formerly *DAF1-1*), and I have characterized the arrest as likely to correspond to specific START-I arrest on the basis of continued cell growth and mating competence.

MATERIALS AND METHODS

Yeast strains. Strains were derived from strain YFC110 (*MAT*a/*MAT*α *cln1*::*TRP1*/*CLN1 cln2*::*LEU2*/*CLN2 cln3*::*URA3*/*CLN3 ade1 his2 leu2 trp1 ura3* [56]), which is congenic with strain BF264-15D (71). Standard yeast genetic methods (60) were used to construct the strains used here.

Plasmid and strain nomenclature. All plasmids used in this study were derivatives of YCp50, a low-copy-number cen-

tromere-containing vector (48). p204 was the wild-type *CLN3* gene cloned into YCp50 (*Sal*I-*Bgl*II fragment containing *CLN3* [14] cloned between the *Sal*I and *Bam*HI sites of YCp50). p205 was the C-terminal truncation allele *CLN3-2* (*DAF1-1*) cloned into YCp50 (identical to p204 but containing the *DAF-1-1* mutation [14]). pW16 was a *GAL-CLN3* (galactose-regulated *CLN3* coding sequence) gene cloned into YCP50 (construction described below). pD4 was *GAL-CLN3-2* (galactose-regulated *CLN3-2* coding sequence) cloned into YCp50 (construction described below).

Strains carrying the three chromosomal mutations *cln1*::*TRP1 cln2*::*LEU2* Δ*cln3* (56; also see below) will be referred to as *cln*$^-$. *cln*$^-$ strains carrying p204, p205, pW16, or pD4 are referred to as *cln*$^-$ [*CLN3*], *cln*$^-$ [*CLN3-2*], *cln*$^-$ [*GAL-CLN3*], and *cln*$^-$ [*GAL-CLN3-2*], respectively.

Construction of a complete *cln3* deletion. A plasmid containing *CLN3* was digested with *Xho*I and *Hpa*I (the *Xho*I site is 5′ to the *CLN3* initiation codon, and the *Hpa*I site is downstream of the termination codon [14]), the ends were blunted with DNA polymerase I, and the plasmid was closed with DNA ligase and recovered by *Escherichia coli* transformation (34). A fragment encompassing the deletion was excised from the resulting plasmid and used together with an episomal *TRP1* plasmid to cotransform a *CLN1 cln2*::*LEU2 cln3*::*URA3* strain. One-step gene replacement (58) of the *cln3*::*URA3* gene in the strain by the *Xho*I-*Hpa*I-deleted fragment deletes the entire *CLN3* coding sequence and simultaneously removes the *URA3* gene, so the desired cotransformants could be selected on 5-fluoro-orotic acid plates (5-fluoro-orotic acid kills *URA3* strains [4]). The structure of the deletion was confirmed by Southern blotting (data not shown). This strain was mated to a *cln1*::*TRP1 cln2 cln3*::*URA3* strain. Mitotic recombination in this diploid resulting in homozygosis for the allele completely deleted of all *CLN3* sequence was selected for on 5-fluoro-orotic acid plates (4) to yield strain 487-1: *MAT***a**/*MAT*α *cln1*::*TRP1*/*CLN1 cln2*::*LEU2*/*CLN2* Δ*cln3*/Δ*cln3 ura3*/*ura3*.

Construction of galactose-dependent *CLN3* alleles. Oligonucleotides were designed with 5′ *Bam*HI sites and 3′ homology to the 5′ or (complementary) 3′ *CLN3* coding sequence. The polymerase chain reaction with Taq DNA polymerase (Cetus) was performed by using these oligonucleotides and *CLN3* or *CLN3-2* DNA as template to precisely amplify the coding sequence and to add *Bam*HI sites to either end (59). The reaction products were digested with *Bam*HI and ligated into pBM272 (YCp50 [*CEN-ARS-URA3*]) containing the *GAL1* promoter upstream of a *Bam*HI cloning site (30; kindly provided by M. Johnston). Clones obtained were characterized by restriction mapping and some sequencing of terminal regions. These plasmids (pW16; *GAL-CLN3*; pD4, *GAL-CLN3-2*) were transformed into strain 487-1 (see above). Transformants were sporulated, and tetrads were dissected and germinated on YEP-galactose medium (60).

Assay of cell cycle parameters. Cultures grown in Yc-ura plus 3% galactose (Yc-ura+gal) or in YEP plus 3% galactose (60) at 30°C were either sonicated and directly plated on glucose-containing solid media or were centrifuged and transferred to glucose-containing liquid media for variable intervals. Cultures on plates were assayed microscopically for the number of cells plus buds per microcolony at various times; liquid cultures were assayed for budding index, cell number, electronic cell volume, and turbidity as described previously (14). The electronic cell volumes (from Coulter Channelyzer analysis) were plotted as the relative volume at the peak position for the population. The distributions for the samples tested showed only one clear peak.

Mating assay. *MAT***a** *cln*$^-$ [*CLN3*] or *cln*$^-$ [*GAL-CLN3*] strains or congenic *CLN1, 2, 3* controls were mated to *MAT*α strain W303-1B (*MAT*α *leu2 ura3 trp1 his3*) or a strain isogenic to W303-1B but lacking the structural genes for α-factor (31; strains provided by C. Jackson). Zygotes were assayed by selection on Yc-his medium. The *cln*$^-$ [*GAL-CLN3*] strain was arrested by transfer of a log-phase YEP-galactose culture to YEP-glucose (YEPD) for 4 h (see Results) and was then mated to a W303-1B lawn on a YEPD plate by adding spots of serial dilutions of the *MAT***a** strain to the lawn. After 4 h at 30°C, plates were replica plated to Yc-his or Yc-ura+gal medium to estimate the number of *MAT***a**/*MAT*α zygotes (colonies on Yc-his) compared with the number of viable unmated *MAT***a** + *MAT***a**/*MAT*α cells (colonies on Yc-ura+gal). (The viability of the *cln*-arrested parent through the mating procedure was determined to be 50 to 80% [data not shown].) This replica plating assay gave reproducible qualitative results. For quantitation of mating efficiency, a lawn of W303-1B or α-factor-deficient W303-1B mixed with a minority of *cln*-arrested *MAT***a** cells on a YEPD plate was incubated for 4 h and then resuspended from the plate, sonicated, and plated as serial dilutions on Yc-his or Yc-ura+gal medium. The mating efficiency is the number of diploids produced divided by the number of viable *MAT***a** cells at the start of the mating procedure.

RNA analysis. Analysis of α-factor induction of the *FUS1* mRNA (35, 69) was performed as described previously (14).

RESULTS

Characterization of *GAL-CLN3* alleles. Triple deficiency for *cln1*, *cln2*, and *cln3* results in arrest in G1 (56). The coding sequence of *CLN3* or *CLN3-2* (*DAF1-1* [14]) was placed under control of the galactose-regulated *GAL1* promoter (30; see Materials and Methods). Centromere-containing plasmids containing *GAL-CLN3*, *GAL-CLN3-2*, or the vector were transformed into a *cln1*::*TRP1*/+ *cln2*::*LEU2*/+ *cln3*/*cln3* diploid. The diploid was sporulated and tetrads were dissected on galactose medium. Trp$^+$ Leu$^+$ (i.e., chromosomal *cln1,2,3*-deficient or *cln*$^-$) progeny were obtained only when the *GAL-CLN3* or *GAL-CLN3-2* plasmid was also inherited; vector was ineffective. These strains were viable on galactose but not on glucose medium, consistent with the *GAL-CLN3* plasmids providing galactose-dependent *CLN3* expression.

Congenic *cln*$^-$ [*CLN3*] and *cln*$^-$ [*CLN3-2*] strains were also constructed as controls for the galactose-dependent strains. These strains were able to grow on glucose or galactose as expected, since the *CLN3* coding sequences were under control of their original promoter in these plasmids (see Materials and Methods).

The *cln*$^-$ [*GAL-CLN3*] and *cln*$^-$ [*GAL-CLN3-2*] strains had slower growth rates on galactose medium than *cln*$^-$ [*CLN3*] or *cln*$^-$ [*CLN3-2*] strains. In one experiment the doubling times for such strains were as follows: *cln*$^-$ [*CLN3*], 2.4 h; *cln*$^-$ [*CLN3-2*], 2.4 h; *cln*$^-$ [*GAL-CLN3*], 2.8 h; *cln*$^-$ [*GAL-CLN3-2*], 3.2 h. The *cln*$^-$ [*GAL-CLN3*] cells were also slightly larger in volume than *cln*$^-$[*CLN3*] cells. The *cln*$^-$[*GAL-CLN3-2*] cells were roughly similar in volume to *cln*$^-$ [*CLN3*] cells and larger than *cln*$^-$ [*CLN3-2*] cells. The basis for these effects of *CLN3* expression from the *GAL* promoter is unclear at present.

Kinetics of cell cycle arrest on cessation of Cln3 synthesis. The *GAL-CLN3* allele results in the production of a high level of a novel *CLN3* transcript detected by RNA blot analysis, and this transcript is reduced to low or undetect-

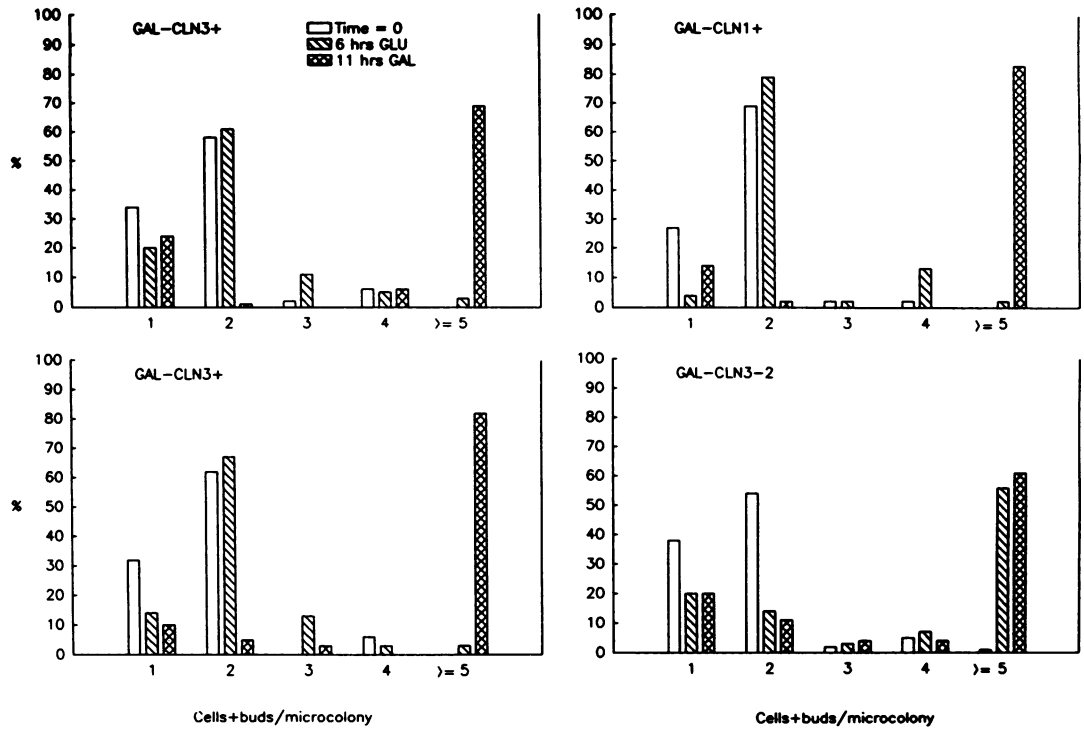

FIG. 1. Residual cell divisions on transfer of strains bearing *GAL-CLN* alleles to glucose medium. *cln*$^-$ [*GAL-CLN3*] (two strains), *cln*$^-$ [*GAL-CLN3-2*], or *cln*$^-$ [*GAL-CLN1*] strains were grown in Yc-ura medium containing 3% galactose. Log-phase cultures were sonicated to disperse clumps and plated on solid Yc-ura medium with glucose (▧) or galactose (▩). The percentage of cells with one, two, three, four or five or more (>=) cell bodies per buds per microcolony was determined microscopically at various times after plating. Also, the distribution of unbudded (1) or budded (2) cells in the initial inoculum was determined (□). The distribution after 6 h on glucose medium and after 11 h on galactose medium is shown. This is because at 6 h on galactose medium, some cells had failed to move into the ">=5" category that did so later; in contrast, the distribution on glucose was unchanged between 6 and 11 h, except that significant cell lysis had begun to occur by 11 h on glucose.

able levels by a shift to glucose medium (data not shown). Similar results have been obtained for the *CLN1* RNA produced by *GAL-CLN1* (S. I. Reed, personal communication). I cannot rule out the possibility that a low level of functional *CLN3* RNA may be produced in *GAL-CLN3* strains in glucose medium, but this level must be much less than the level produced by the wild-type *CLN3* gene (data not shown).

When galactose-grown *cln*$^-$ [*GAL-CLN1*] strains were shifted to glucose-containing medium (shutting off synthesis of *CLN1* RNA), the cells arrested within one cell cycle time of shift in the unbudded G1 phase of the cell cycle, suggesting that an early cell cycle event had a requirement for continued synthesis of Cln1 (56). I have extended these observations to *GAL-CLN3*. When *cln*$^-$ [*GAL-CLN3*] cultures grown in galactose were plated on glucose medium, almost none of the cells which were budded at the time of plating budded again. Of the unbudded cells, some were inviable (remaining as single cells after 11 h on galactose medium), and many of the viable unbudded cells apparently budded once. The results were very similar to results with a *cln*$^-$ [*GAL-CLN1*] strain analyzed in parallel (Fig. 1). Despite failing to bud, the cells continued to increase in size, until a significant level of cell lysis began to occur at about 12 h of incubation.

In contrast, the *cln*$^-$ [*GAL-CLN3-2*] strains exhibited multiple rounds of bud emergence under these conditions (Fig. 1). The microcolonies were not capable of unlimited proliferation, however, and after 24 h consisted of a limited number of very large elongated cells (similar to the G1-arrested shmoo morphology [53, 56; data not shown]).

I followed the kinetics of accumulation of cells, budding, cell volume, and culture turbidity of a *cln*$^-$ [*GAL-CLN3*] strain (glucose sensitive) and a *cln*$^-$ [*CLN3*] strain (glucose insensitive) in liquid medium (Fig. 2). After shift to glucose medium, the *cln*$^-$ [*GAL-CLN3*] strain produced new cells in parallel with the *cln*$^-$ [*CLN3*] strain for about 2 h; cell division then halted. This halt occurred when the cell number had increased by less than a factor of two, indicating that cells early in the cycle at the time of shift failed to divide, whereas cells later in the cycle divided before arresting. The arrested cells were almost all unbudded. After cell cycle arrest, the cells increased almost exponentially in volume throughout the course of the experiment. The cells had the characteristic elongated or pear-shaped shmoo morphology of G1 arrest (53, 56; data not shown), as well as some projections at later incubation times.

In contrast, a *cln*$^-$ [*GAL-CLN3-2*] strain continued cell division after glucose addition for at least 6 h (Fig. 2). This strain slowly arrested over the ensuing 6 to 12 h, eventually accumulating greater than 95% unbudded shmoo-shaped cells in the culture (data not shown).

These results imply that shutoff of *CLN3* mRNA synthesis early in the cell cycle or late in the preceding cycle will prevent a new round of bud emergence but will not prevent division of cells that have already budded. In contrast, the *CLN3-2* mutation allows multiple cell division cycles under the same conditions. These results indicate that the *CLN3*

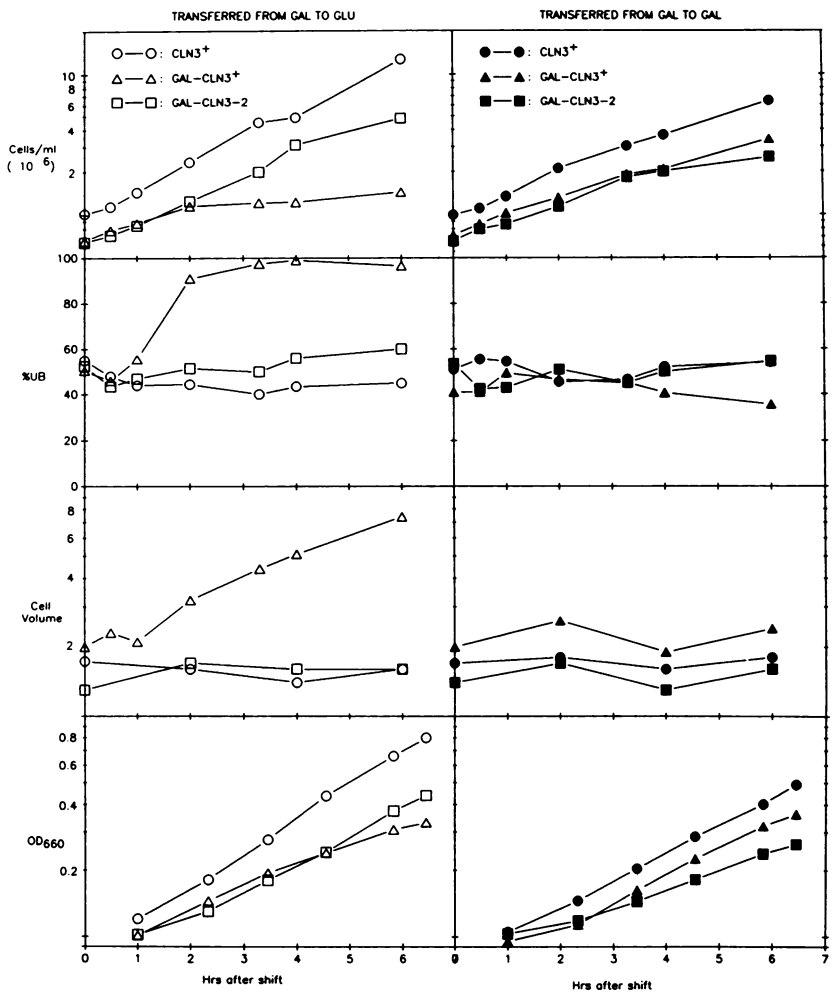

FIG. 2. Kinetics of arrest on transfer of strains bearing *GAL-CLN3* alleles to liquid glucose medium. A *cln*⁻ [*GAL-CLN3*] strain (triangles), a *cln*⁻ [*CLN3*] strain (circles), and a *cln*⁻ [*GAL-CLN3-2*] strain (squares) were grown to log phase in Yc-ura medium with 3% galactose. Cells were collected by centrifugation and resuspended in the same medium (filled symbols) or in Yc-ura medium with 2% glucose (open symbols). At various times after reinoculation, samples were sonicated and analyzed for cell number (by using a hemacytometer), the percentage of unbudded cells (by phase-contrast microscopy), the peak cell volume (by using a Coulter Channelyzer), or the optical density at 660 nm (by using a spectrophotometer). All scales are logarithmic except the budding index (%UB). The cell volumes are plotted in arbitrary units on a logarithmic scale.

product is functionally unstable, and that this instability may be mediated by the C terminus. Consistent with this idea, after loss of an unstable plasmid (42) containing *CLN3*, *cln*⁻ cells arrest either without dividing again or after dividing once, whereas cells that lost a similar plasmid containing *CLN3-2* went through a variable number of cell divisions before arrest (data not shown).

Functional instability of the *CLN3* product would be most simply interpreted as instability of Cln3 protein. This idea might be consistent with the dependence of this instability on the C terminus of the protein, which is rich in potential PEST sequences associated with protein instability (44, 57). However, other kinds of instability (e.g., posttranslational modification inactivating the protein) cannot be ruled out by these genetic data. It is also possible that the Cln3 protein is stable but only functions at a critical threshold level and is quickly diluted below this level upon shutoff of synthesis; this threshold might be lowered by the *CLN3-2* truncation. Preliminary analysis with anti-Cln3 antibodies suggests that the Cln3 protein is very unstable at its normal level of expression and the Cln3-2 protein is more stable; however, the *GAL*-overexpressed Cln3 gives a complex protein pattern with many bands, only some of which appear unstable (F. Cross and C. Blake, unpublished data). Therefore, some caution may be indicated in interpreting the functional instability implied by the genetic experiments above.

Is there a *CLN* requirement for late cell cycle events? The observation that shutoff of *CLN* mRNA synthesis did not prevent cells in the budded phase from completing cell division (see above) could be interpreted as showing that cells have no post-G1 requirement for *CLN* function. Another interpretation is that *CLN* function is also required later in the cycle but that the budded cells exposed to glucose in the last two experiments divided before the *CLN* protein decayed sufficiently to prevent mitosis.

To test this second interpretation, *cln*⁻ [*GAL-CLN1*] or *cln*⁻ [*GAL-CLN3*] cultures in log phase in galactose medium were arrested in S phase (early in the budded phase of the cycle [53]) with 2 h of hydroxyurea treatment (24, 64; Fig. 3). The hydroxyurea block observed under these conditions was not quantitative in that a significant population of cells remained unbudded for unknown reasons. However, the

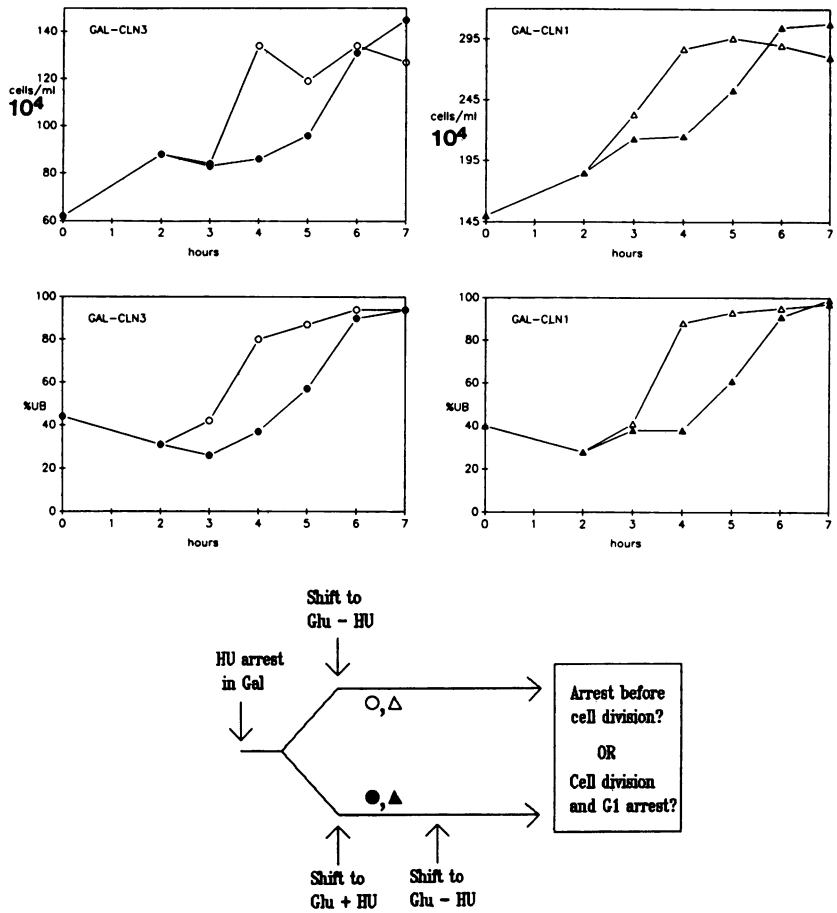

FIG. 3. Test for *CLN* requirement for late cell cycle events. A *cln*⁻ [*GAL-CLN3*] strain (left panels) and a *cln*⁻ [*GAL-CLN1*] strain (right panels) growing in YEP-galactose were shifted to YEP-galactose plus 200 mM hydroxyurea at zero time. After 2 h, the culture was split; one half was shifted into YEP-glucose without hydroxyurea (open symbols), and one half was shifted into YEP-glucose plus 200 mM hydroxyurea (closed symbols). After a further 2 h, all cultures were shifted into YEP-glucose (the culture already in this medium was shifted in parallel into the same medium). At intervals, cell samples were taken and fixed with formaldehyde. Cell number and budding index were determined as described in the legend to Fig. 2. A diagram outlining the protocol is shown at the bottom of the figure.

budded cells observed after hydroxyurea treatment mostly had the large-budded morphology characteristic of S-phase arrest (24, 64). The hydroxyurea-treated cultures were divided in half. One half of the cultures were switched to glucose medium lacking hydroxyurea (Fig. 3, control cultures), and one half were switched to hydroxyurea plus glucose medium (Fig. 3, experimental cultures). The experimental cultures were transferred to glucose medium lacking hydroxyurea 2 h later. Division of a budded cell after release from the hydroxyurea block should produce two unbudded cells that then arrest as a result of glucose shutoff of *CLN* transcription (as in Fig. 2). Division occurred in both the control and experimental cultures, resulting in an increase in cell number and conversion of the culture to almost 100% unbudded cells. The only difference between the control and the experimental cultures was that division in the experimental culture was delayed by about 2 h (the time of excess hydroxyurea treatment). Thus, even when glucose treatment in S-phase cells is prolonged by 2 h, the cells are able to divide on schedule once S phase is completed. The simplest interpretation of this result is that there is no *CLN* requirement for cell cycle events after S phase. (However, as was noted above, it cannot be ruled out that a very low level of GAL-CLN expression occurs in glucose medium, and it is possible if unlikely that such expression could account for the apparent lack of a *CLN* requirement for later cell cycle events in this experiment.)

Mating competence and pheromone response at the *cln* arrest point. Strains arrested at START by a *cdc28* mutation (allowing continued cell growth) are mating competent, whereas cells arrested at other cell cycle phases, or in G1 in a growth-arrested state, are mating incompetent (55). Mutations in four genes (*cdc28,36,37,39*) were selected by requiring mating-competent G1 arrest (START-I; see the introduction) (54). As shown above (Fig. 2), the *cln* arrest is associated with a large increase in cell volume, consistent with a lack of growth arrest. I assayed mating competence of a *cln*⁻ [*GAL-CLN3*] strain after 4 h of incubation in glucose medium (as in Fig. 2). Mating was performed on glucose-containing medium (YEPD plate agar). Prearrest of *cln*⁻ [*GAL-CLN3*] cells had little effect on their mating efficiency. Quantitation of the mating of *cln*⁻ [*GAL-CLN3*] cells after a 4-h glucose arrest gave a mating efficiency of 41%, assuming one doubling of the zygotes produced during the 4 h of incubation. The zygotes produced were diploid as expected, since *cln*-arrested cells are in G1 (56). Therefore, *cln* arrest does not strongly inhibit mating.

Some mutations resulting in START-I arrest can bypass

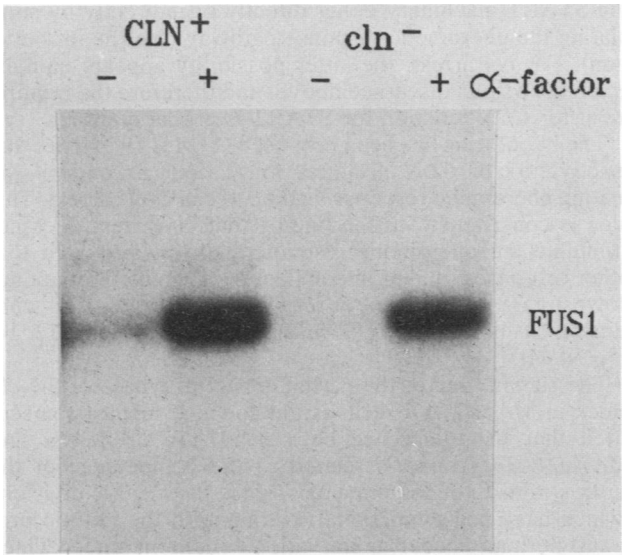

FIG. 4. *FUS1* mRNA induction at the *cln* arrest point. A *cln*⁻ [*CLN3*] strain and a *cln*⁻ [*GAL-CLN3*] strain were grown to log phase in YEP-galactose medium and then shifted to YEP-glucose medium for 4 h. At this time, RNA was isolated from half the culture and α-factor (Sigma) was added to 1 μM to the other half. After 30 min, RNA was again isolated. The RNA was analyzed by Northern hybridization by using a *FUS1* probe (33) after formaldehyde-agarose gel electrophoresis and transfer to GeneScreen membrane. The loading was normalized by ethidium bromide staining of the RNA samples (data not shown). Abbreviations: *cln*⁻, the *cln*⁻ [*GAL-CLN3*] culture; *CLN*⁺, the *cln*⁻ [*CLN3*] culture.

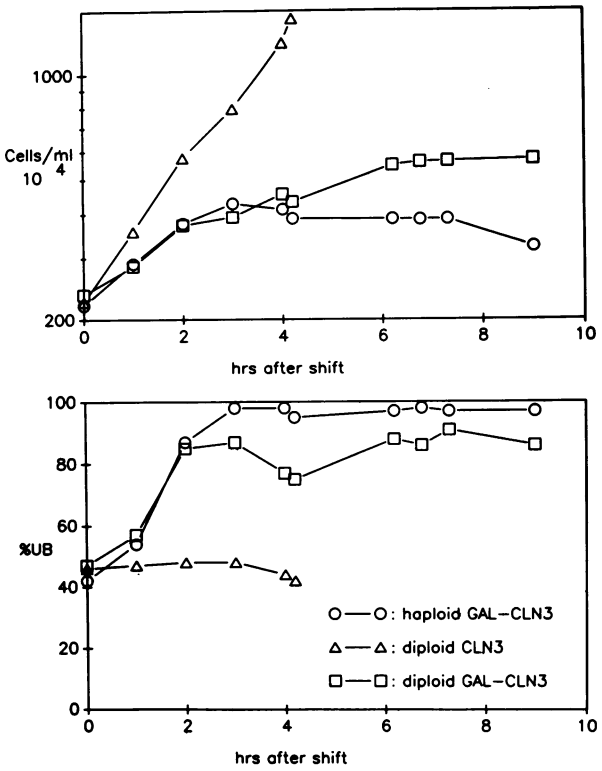

FIG. 5. *cln* arrest in the *MAT***a**/*MAT*α cell type. A *MAT***a**/*MAT*α *cln*⁻ [*GAL-CLN3*] diploid strain, a *MAT***a**/*MAT*α *cln*⁻ [*CLN3*] diploid strain, and a *MAT***a** *cln*⁻ [*GAL-CLN3*] haploid strain were shifted from galactose- to glucose-containing medium. Samples were sonicated and analyzed for the percentage of budded cells and the number of cells per milliliter. The figure contains pooled data from two experiments.

the requirement for receptor-pheromone interaction for mating (16, 28, 37, 45). This is presumed to occur by constitutive activation of the pheromone response pathway, which includes START-I arrest (see the introduction). *MAT***a** *cln*⁻ [*GAL-CLN3*] cells arrested by 4 h of glucose treatment were tested for mating to a *MAT*α strain deficient in the structural genes for α-factor (31; provided by C. Jackson). No mating was observed ($<10^{-6}$ of the mating observed in parallel with the α-factor-producing strain). This result might simply follow from the lack of spatial cues provided by localized synthesis of α-factor by potential mating partners (12, 26). However, inclusion of α-factor in the plate agar used for mating largely suppressed this mating defect (to approximately 50% of the mating observed to the α-factor-producing strain in the absence of added α-factor, in a series of semiquantitative experiments), both with the *cln*⁻ [*GAL-CLN3*] strain and with a *CLN1, 2, 3* (wild-type) strain (data not shown). Thus, *cln* arrest is consistent with mating ability but is not coincident with full induction of the pheromone response. (The rescue of mating to the α-factor-deficient strain by exogenous α-factor observed in these experiments is much better than was observed previously [31]. This may be a result of the low density of *MAT***a** cells on the mating plate since the spot-mating assay was used for this experiment [see Materials and Methods]. This may reduce degradation of the exogenous α-factor by the *BAR1* protease synthesized by *MAT***a** cells; *BAR1* activity greatly reduces the ability of *MAT***a** cells to mate with α-factor-deficient *MAT*α cells upon addition of exogenous α-factor [12, 31].)

Cells arrested by *scg1*, *cdc36*, or *cdc39* mutations resemble pheromone-arrested cells in being induced for *FUS1*, a gene normally induced by pheromone (16, 28, 35, 45, 69). I tested *cln*-arrested cells for their level of *FUS1* and for the inducibility of *FUS1* by α-factor. *FUS1* was not induced by the arrest but could be induced in arrested cells by added α-factor (Fig. 4). (The level of *FUS1* RNA induced by α-factor addition was somewhat lower than the level induced in *cln*⁻ [*CLN3*] cells treated in parallel.) *cdc28* arrest was also found not to induce *FUS1* but to allow *FUS1* induction by α-factor (28; data not shown). These results confirm the conclusion that, like *cdc28* arrest, *cln* arrest is a START-I cell cycle block that does not occur by activation of the mating pheromone signalling pathway.

cln* arrest in *MAT*a/*MAT*α cells.** It has been observed that some START-I arrests are dependent on the mutant cell being pheromone responsive (unlike *MATa**/*MAT*α cells [reviewed in reference 12]). In contrast, inclusion of a *MAT*α plasmid in *MAT***a** spores derived from a diploid segregating triple *cln* deficiency did not rescue viability, and the lethal phenotype appeared similar (56). To extend this finding, I constructed *MAT***a**/*MAT*α *cln*⁻ [*GAL-CLN*] diploid strains.

*MAT***a**/*MAT*α *cln*⁻ [*GAL-CLN3*] strains were viable on galactose but not on glucose. In galactose-to-glucose shift experiments, the *MAT***a**/*MAT*α diploid strain arrested in the first cycle as did the *MAT***a** and *MAT*α haploid strains in this and previous experiments; however, this arrest was not quantitative. After a period of arrest of about 2 h, the number of budded cells increased slightly, and between 2 and 9 h after glucose addition, the cell number increased by a factor of about 1.5 (Fig. 5). As with *MAT***a** or *MAT*α haploids, *cln*⁻ [*GAL-CLN3-2*] *MAT***a**/*MAT*α cells failed to arrest initially on shift to glucose and showed an exponential

TABLE 1. Test of cell division in spores of genotype *cln1 clin2 cln3 MATa/MATα*[a]

Plasmid in parent	*MAT* genotype of *cln*⁻ spore	No. of shmoos produced by spore:		
		1	2	3
Vector	*MATa*	26	0	0
	MATα	16	0	0
YCp *MATα*	*MATa*	14	0	0
	MATa/MATα	2	3	2
	MATα	24	0	0

[a] The genotype of the inviable spores at *CLN1* and *CLN2* was deduced by assuming 2:2 segregation of the disrupted loci and by scoring the viable members of the tetrad. The *MAT* genotype was scored in the same way by assuming 2:2 segregation of *MATa*:*MATα*. The presence of the *URA3-MATα* plasmid was scored in a similar way—the plasmid contained a centromere, and eight of nine four-viable-spore tetrads exhibited either 0:4, 2:2, or 4:0 segregation, presumably depending on whether the parent cell before meiosis contained zero, one, or two copies of the plasmid (10). Therefore the pattern of the Ura⁺ phenotype in viable spores of a tetrad containing a *cln*⁻ spore could be used to deduce with reasonable certainty the presence or absence of the plasmid in the *cln*⁻ spore. Spores were assigned the genotype *MATa/MATα* if they were deduced to be chromosomally *MATa* and to contain the *MATα* plasmid. Only spores whose genotypes were completely determined in this way are included in the table. Spores that failed to germinate (~5% of total) are also excluded.

increase in cell number for more than 6 h after the shift (data not shown). However, none of the *MATa/MATα cln*⁻ [*GAL-CLN*] strains constructed, whether *cln*⁻ [*GAL-CLN1*], *cln*⁻ [*GAL-CLN3*], or *cln*⁻ [*GAL-CLN3-2*], were able to form colonies on glucose medium.

Because of these findings, I reexamined the fate of *MATa/MATα* spores born with triple *cln* deficiency. A diploid of genotype *cln1*::*TRP1*/+ *cln2*::*LEU2*/+ Δ*cln3 MATa/MATα* was transformed with a centromere-containing *URA3-MATα* plasmid or with a control vector plasmid, transformants were sporulated, and tetrads were dissected (55 and 50 tetrads dissected, respectively). After germination, *cln*-arrested cells (about one fourth of the progeny) were observed. Some of these cells had a contorted morphology suggestive of budding or cell division. These cells were manipulated with a microdissecting needle to see if more than one cell could be separated out. Some *MATa/MATα cln*-deficient spores did produce one to two additional cells before terminal arrest (Table 1). These cells had the characteristic shmoo morphology (53, 56). The multiple cells produced by these spores arrested as shmoos within 20 h of dissection, and no further cell division was observed by 48 h. (One apparent division in a *cln*⁻ spore derived from the vector transformant is excluded from Table 1 because only one of the division products was capable of shmooing.) Cell division has been observed only rarely in *MATa* or *MATα cln*⁻ spores (Table 1; data not shown). Micromanipulation was needed to reliably distinguish between multiple cells and a single contorted cell with projections, and this may account for the failure to detect these extra cell divisions previously (56).

Despite this residual capacity for cell division of *cln*-arrested *MATa/MATα* strains, the terminal arrest phenotype is similar to that in haploids and is distinct from the complete viability of *scg1 MATa/MATα* strains or the lethality without significant cell cycle-specific arrest in *cdc36* or *cdc39 MATa/MATα* strains (see above).

DISCUSSION

***cln* arrest and the pheromone response pathway.** A mutation causing START-I arrest might do so by interfering with the START machinery either directly or indirectly by stimulating the pheromone response pathway (see the introduction). For *cln* arrest, the latter possibility appears unlikely given the results discussed above, and therefore the requirement for *CLN* function for START is probably direct.

The hypothesis has been proposed (14, 15, 19, 44, 56) that inactivation of *CLN* products may occur as part of the mating pheromone response leading to cell cycle arrest. This idea is consistent with the finding that *cln* arrest does not stimulate the pheromone response pathway provided that other responses to pheromone (like *FUS1* induction) are not downstream of cell cycle arrest in the pathway. This conclusion has been drawn previously on other grounds (12, 14, 15, 19, 44).

The significance of the partial apparent bypass of the *cln* block in *MATa/MATα* cells is unknown. It might be speculated that this difference between *MATa* or *MATα* and *MATa/MATα* strains is related to the competence of the latter strains to undergo meiosis, since the meiotic divisions (which have some functional overlap with the mitotic program) normally occur in starvation conditions under which *MATa* or *MATα* strains are arrested at START (53).

Is the functional instability of CLN products conferred by PEST sequences? First-cycle arrest caused by stopping Cln synthesis was shown previously for *CLN1* (56) and in this study for *CLN3*. These observations imply functional instability of the Cln products. The dominant activated alleles of *CLN2* and *CLN3* described previously differ from the wild type in the removal of a sequence at the C terminus (14, 22, 44) that is rich in apparent PEST sequences which have been implicated in protein turnover in other systems (57). Removal of this sequence from Cln3 prevents first-cycle arrest on cessation of new synthesis (see above), suggesting that this sequence may be a target for proteolysis. Preliminary antibody analysis suggests instability of the Cln3 protein at its normal level of expression and increased stability of the C-terminally truncated product (F. Cross and C. Blake, unpublished data), as might be predicted from the genetic results (although as noted above, the analysis of the *GAL*-overexpressed Cln3 protein may not be as straightforward). The abundance of Cln2 protein is cell cycle regulated, and the protein is highly unstable; however, unlike the *CLN3* mRNA, the *CLN1* and *CLN2* mRNAs are also cell cycle regulated (71). The effect of the *CLN2* putative PEST sequences on Cln2 protein stability has not yet been addressed.

Relationship of *CLN* START function to cyclin function in other systems. The phenotypes of dominant hyperactive mutations in *CLN* genes, as well as of single, double, and triple null mutations in *CLN* genes, all suggest that these genes are involved in START control (9, 14, 15, 22, 44, 66). The results reported here indicate that *cln*-deficient arrest shares physiological features with START-I arrest (54), i.e., G1 arrest with continued cell growth and mating competence. In cells artificially delayed in S phase, mitosis can occur 2 to 3 h after shutoff of *CLN* RNA synthesis (see above), so if there is a *CLN* requirement for mitosis, it is much less stringent than the START requirement with respect to *CLN* protein levels. A simple interpretation of these results is that the *CLN* requirement is specific for START.

This conclusion is somewhat surprising in that the *CLN* genes share weak homology with cyclins, which act specifically at the G2/M boundary, probably by activating *cdc2* protein kinase (6–8, 18, 20, 21, 32, 36, 38, 40, 50, 51, 67). *cdc2* in *Schizosaccharomyces pombe* and its homolog

CDC28 in *S. cerevisiae* (1, 5, 49, 54a) may act at both START and mitosis, although this has not been clear from previous results with most temperature-sensitive alleles of *cdc28* (33, 46, 47, 53, 54; reviewed in reference 13). One model (13, 39–41, 56, 71) for the *CLN* products is that they associate with Cdc2/Cdc28 to specifically activate START; other (as yet unidentified) *S. cerevisiae* cyclin species might activate Cdc2/Cdc28 for phosphorylation of mitosis-specific substrates at the G2/M boundary. It is not excluded that START in *S. cerevisiae* represents a commitment point for mitosis as well as for the G1/S transition (46), and it is possible that the *CLN* products provide the function of G2/M cyclins in other systems. However, the finding of additional cyclin homologs in *S. cerevisiae* more closely homologous to G2/M cyclins and possibly functioning in mitosis (S. I. Reed, personal communication; A. B. Futcher, personal communication) may make this idea less likely.

Cyclin B from sea urchin is stabilized against degradation in mitosis by deletion of an N-terminal sequence lacking PEST sequences (41), whereas deletion of a C-terminal PEST-rich region by the *CLN3-2* truncation functionally stabilizes Cln3. The failure to degrade cyclin B may result in cell cycle arrest in mitosis (41), whereas the potentially stabilized version of Cln3 produced by the *CLN3-2* truncation allows multiple cell cycles in the absence of new synthesis (Fig. 1 and 2). Thus, for G2/M cyclins, degradation in M phase may be obligatory for cell cycle progression into the next interphase, perhaps because inactivation of the mitotic *cdc2/CDC28* protein kinase activity is required for exit for M phase (41). This may not be the case for *CLN3*, which could pose a paradox for the alternating specificity model for *cdc2/CDC28* (13, 39–41, 56, 71). The significance of these differences will not be clear until the mechanism of action of Cln proteins is defined.

ACKNOWLEDGMENTS

I thank C. Epstein, H. Weintraub, and D. Wilson for comments on the manuscript, and I. Herskowitz, S. Reed, and C. Wittenberg for communicating results before publication. I also thank H. Weintraub for lab space, funding, and materials for initial studies.

I am a Lucille P. Markey Scholar and received support from the Rockefeller University. Funding for initial studies was from U.S. Public Health Service grant GM26176 from the National Institutes of Health to H. Weintraub.

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