Comparison of the *Saccharomyces cerevisiae* G₁ cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins

Mike Tyers², George Tokiwa¹ and Bruce Futcher³

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724 and
¹Graduate Program in Genetics, State University of New York,
Stony Brook, NY 11792, USA
²Present address: Banting and Best Department of Medical Research,
University of Toronto, 112 College Street, Toronto, Ontario, Canada
³Corresponding author

Communicated by K. Nasmyth

In the budding yeast *Saccharomyces cerevisiae*, the G₁ cyclins Cln1, Cln2 and Cln3 regulate entry into the cell cycle (Start) by activating the Cdc28 protein kinase. We find that Cln3 is a much rarer protein than Cln1 or Cln2 and has a much weaker associated histone H1 kinase activity. Unlike Cln1 and Cln2, Cln3 is not significantly cell cycle regulated, nor is it down-regulated by mating pheromone-induced G₁ arrest. An artificial burst of CLN3 expression early in G₁ phase accelerates Start and rapidly induces at least five other cyclin genes (CLN1, CLN2, HCS26, ORFD and CLB5) and the cell cycle-specific transcription factor SWI4. In similar experiments, CLN1 is less efficient than CLN3 at activating Start. Strikingly, expression of HCS26, ORFD and CLB5 is dependent on CLN3 in a cln1 cln2 strain, possibly explaining why CLN3 is essential in the absence of CLN1 and CLN2. To explain the potent ability of Cln3 to activate Start, despite its apparently weak biochemical activity, we propose that Cln3 may be an upstream activator of the G₁ cyclins which directly catalyze Start. Given the large number of known cyclins, such cyclin cascades may be a common theme in cell cycle control.

Key words: Cdc28/cell cycle/Cln/G₁ cyclin/Start

Introduction

The cell cycle of *Saccharomyces cerevisiae* is controlled mainly in G₁ phase at a point called Start. At Start environmental signals such as nutrient status are monitored until under appropriate conditions cells commit to division (reviewed by Cross et al., 1989). Passage through Start depends on CDC28, which encodes a protein kinase catalytic subunit, and on CLN1, CLN2 and CLN3 (reviewed by Reed, 1991). The Clns are distinctly related to the mitotic cyclins, which regulate the Cdc2/Cdk28 protein kinase at mitosis (reviewed by Nurse, 1990). Biochemical similarities between the Clns and the mitotic cyclins have been established (Wittenberg et al., 1990; Tyers et al., 1992). For these reasons the Clns have been called ‘G₁ cyclins’.

CLN1 and CLN2 were isolated as high copy number suppressors of the cdc28-4 mutation (Hadwiger et al., 1989). CLN3 (originally known as WHI1, and also later called DAF1) was identified by two dominant alleles, WHI1-1 (= CLN3-1) and DAF1-1 (= CLN3-2), that confer small cell size and resistance to the G₁ arrest caused by mating pheromone (Sudbery et al., 1980; Cross, 1988). The CLN3-1 and CLN3-2 mutations remove the C-terminal third of the protein which is rich in PEST residues (Rogers et al., 1986) and destabilizes Cln3 (Tyers et al., 1992). All of the possible single and double cln deletions are viable, but the cln1 cln2 cln3 triple deletion strain is inviable and arrests in G₁ with a Start defect (Richardson et al., 1989; Cross, 1990). This genetic result suggested that the three CLNs comprise a redundant family with overlapping functions. As we show below, this interpretation may be an oversimplification.

CLN1 and CLN2 are in some respects quite dissimilar to CLN3. Cln1 and Cln2 are ~75% identical to each other but only 20–25% identical to Cln3 (Hadwiger et al., 1989). The CLN1 and CLN2 mRNAs fluctuate periodically in the cell cycle, peaking in G₁ phase, whereas CLN3 mRNA is constant through the cell cycle (Nash et al., 1988; Wittenberg et al., 1990). Transcription of the CLN1, CLN2 pair depends on the transcription factors encoded by the SWI4 and SWI6 genes and on CDC28 (Nasmyth and Dirick, 1991; Ogas et al., 1991). Together these elements form a positive feedback loop that helps account for the strong G₁ phase periodicity of CLN1, CLN2 expression (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Nasmyth and Dirick, 1991). In contrast, CLN3 expression is independent of this feedback loop (Nasmyth and Dirick, 1991). CLN3 is also regulated differently by α-factor since the mating pheromone abolishes CLN1 and CLN2 transcription (Wittenberg et al., 1990), but actually induces CLN3 expression (Nash et al., 1988). In this paper we present a quantitative and qualitative comparison of Cln1, Cln2 and Cln3 and their associated kinase activities. We find that Cln3 is much less abundant than and is regulated quite differently from Cln1 and Cln2. The biochemical properties of the three Clns suggested experiments that showed Cln3 is a potent, and in some situations essential, activator of expression of other cyclin genes.

Results

Reagents for comparing Cln1, Cln2 and Cln3

We analyzed the Clns by epitope addition (Field et al., 1988). Versions of Cln3 tagged with three tandem copies of the HA1 epitope have been described (Tyers et al., 1992). At least for Cln3, triplication of the epitope is essential for detection of the protein. The CLN1 and CLN2 reading frames were also tagged at their 3’ ends with the triple epitope; these alleles are called CLN1C and CLN2C. The tagged genes did not have any mutant phenotype in wild-type strains or in CLN1C cln2 cln3 or cln1 CLN2C cln3 strains (where viability depends on the tagged Cln) with respect to cell size, α-factor resistance or growth rate (data not shown). Cell size depends on the activity of Cln3 (Nash et al., 1988; Tyers et al., 1991), and since tagged CLN3 still gives wild-type size, it seems likely that the tag does not significantly affect function.
1956

**Fig. 2.** Cln abundance and kinase activities in chemically arrested cultures. **CLN1C (GT100-23c), CLN2C (GT102), CLN3C (MT236)** and **CLN3C cdc34 (MT212)** strains were grown at room temperature to log phase and then arrested with α-factor (αF), hydroxyurea (HU) or nocodazole (N). (A) Lysates were immunoprecipitated and 75% used for immunoblot analysis. Lanes 9–13 were exposed for 15 times longer than lanes 1–8. In lanes 9–12, Cln3 is visible just above the IgG heavy chain. (B) Kinase reactions performed with the remaining 25% of the immunoprecipitates used in panel A. The same exposure was used for all the kinase assays (20 h at room temperature). The H1 kinase activity in the Cln3 lanes is largely non-specific; the apparent extra activity at the nocodazole arrest is probably non-specific activity, possibly due to the high levels of Cln–Cdc28 activity at this arrest point. The additional panel shows Cln3-associated kinase activity in extracts from a **CLN3C cdc34-2** strain (MT212) where activity is elevated ~20-fold. Lane 13 in this panel is an assay from cells treated with 50 μg/ml cycloheximide for 10 min, which eliminates Cln3 (Tyers *et al.*, 1992).

**Fig. 1.** Comparison of Cln1, Cln2 and Cln3. (A) Direct immunoblot analysis of each tagged Cln. Two different extraction methods are compared. Samples in lanes 1–4 were isolated by the glass bead method (>95% breakage) in a non-denaturing buffer (this method is used in all other experiments described in this paper); samples in lanes 5–8 were the same as in lanes 1–4 except that the broken cell–glass bead suspension was boiled in SDS sample buffer and re-vortexed before spinning out cell debris. The strains are isogenic (GT104, MT263, GT108, K699, respectively). The blot shown is a maximum length exposure. (B) Cln3 abundance is not increased in a **cln1 cln2** background. The strains used in lanes 1–4 are isogenic (GT104, MT263, GT108, K699), as are the strains in lanes 5–8 (MT137, MT238, BF21 CW3, BF305-15d). The same immunoblot was reprobed with an anti-β-tubulin antibody to show equal loading. (C) Immunoprecipitations of the same extracts used in panel B followed by immunoblot analysis. By comparing immunoblots of Cln immunoprecipitations to direct immunoblots, we estimate that the immunoprecipitation is 10–50% efficient for each Cln. (D) Histone H1 kinase activity associated with anti-Cln immunoprecipitates from lysates used in panel C. The histone H1 band is indicated; other bands are due to co-precipitated endogenous substrates which include Far1 (p116) and the Clns themselves (data not shown). Exposure time was 6 h at room temperature.

(Tyers *et al.*, 1991). Most experiments described below were done with strains bearing one tagged and two wild type **CLN** genes (i.e. **CLN1C CLN2 CLN3, CLN1 CLN2C CLN3** or **CLN1 CLN2 CLN3C**). Thus, each protein was tagged in the same place with the same tag and was immunoprecipitated with the same monoclonal antibody.

**Cln3 is less abundant than Cln1 or Cln2 and has a weaker associated histone H1 kinase activity in vitro**. Cln1 and Cln2 were easily visualized by direct immunoblot analysis using an enhanced chemiluminescence (ECL) detection system (Figure 1A and B). In contrast, visualization of Cln3 required at least 100-fold longer exposure times or prior concentration by immunoprecipitation (Figure 1C).

It is possible that Cln3 is more difficult to extract from cells than Cln1 or Cln2. However, even harsh denaturing extraction conditions, such as boiling in SDS after cell breakage, did not increase the Cln3 signal (see Figure 1A). Thus, Cln3 is probably a much rarer protein than Cln1 or Cln2.

Each Cln is phosphorylated (data not shown); this was reflected in their considerable size heterogeneity on SDS–PAGE (Figure 1A and B). In contrast to Cln1 and Cln2, Cln3 was heavily phosphorylated only in cdc34 mutant strains, which have a defective ubiquitin conjugating enzyme (Tyers *et al.*, 1991, 1992).

A histone H1 kinase activity co-precipitated with each Cln (Figures 1D and 2B). Immunoprecipitation of the kinase activity was blocked by excess epitope peptide (Tyers *et al.*, 1992; data not shown) and required a tagged Cln (Figure 1), showing specificity. The kinase activity associated with anti-Cln3 immunoprecipitates from wild type strains was so weak that it was not clearly distinguishable from background kinase activity (Figure 1D). However, the Cln3-specific kinase activity could be demonstrated either by over-expressing **CLN3** from the **GAL1** promoter or by extracting the Cln3-associated kinase from a **cdc34-2** strain (Figure 2B; Tyers *et al.*, 1991, 1992). Each of these conditions increases the Cln3-specific activity 10- to 20-fold. We have shown that anti-Cln3 immunoprecipitates contain Cdc28 and that most of the histone H1 kinase activity associated with Cln3
Table 1. Phenotypic comparison of various CLN genotypes

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Mode cell size (in fl)</th>
<th>% unbounded cells</th>
<th>Arrest by α-factor</th>
<th>Cln-associated kinase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>42</td>
<td>44</td>
<td>3</td>
<td>No tag</td>
</tr>
<tr>
<td>cln1</td>
<td>47</td>
<td>45</td>
<td>3</td>
<td>CLN1/C</td>
</tr>
<tr>
<td>cln2</td>
<td>53</td>
<td>44</td>
<td>3</td>
<td>CLN2/C</td>
</tr>
<tr>
<td>cln3</td>
<td>64</td>
<td>64</td>
<td>1</td>
<td>CLN3/C</td>
</tr>
</tbody>
</table>

aAll strains were isogenic with K699.
bAt least 300 cells from a low density log phase culture were scored.
cThe inhibitory concentration of α-factor (in μM) was determined in a grid assay as in Figures 5 and 7.
dThe histone H1 kinase activity associated with each Cln was determined in strains carrying the relevant wild type, tagged CLN gene (wild type cells did not have a tagged CLN gene so only non-specific kinase activity was precipitated from this strain). Values are the means of three and eight independent determinations and were normalized to the Cln3-associated kinase activity (e.g. Cln1 has 210 times more associated H1 kinase activity than Cln3).

is CDC28-dependent (Tyers et al., 1992). Cdc28 also co-precipitated with Cln1 and Cln2 (data not shown) confirming a similar observation made by Wittenberg et al. (1990) for Cln2. Much more Cdc28 co-precipitated with Cln1 and Cln2 than with Cln3. Thus, for each Cln there was an associated histone H1 kinase activity and Cdc28 probably the catalytic subunit for most of this activity.

Each Cln complex also had an array of specifically co-precipitated in vitro substrates (see Figure 1D), which included the Cln itself and other specific proteins (M. Tyers and B. Fuchter, submitted).

In cells expressing wild type amounts of each Cln, the histone H1 kinase activity co-precipitated with Cln1 and Cln2 was ~200-fold greater than that co-precipitated with Cln3 (Figure 1D; Table I). This was consistent with the relatively small amount of Cln3 protein and with the small amount of co-precipitating Cdc28. These results were surprising in the view of the viability of cln1 cln2 CLN3 strains, a result that had suggested CLN3 could function in place of CLN1 and CLN2. Furthermore, cln3 null mutations have a larger effect on the critical size requirement for Start than do cln1 or cln2 mutations (Table I) showing that CLN3 is relatively potent genetically despite its apparently low biochemical activity. It may be that Cln3 has a different role in the cell than Cln1 or Cln2, a notion consistent with the low degree of sequence similarity between Cln3 and the Cln1, Cln2 pair.

We examined several of the most obvious explanations for the low Cln3-associated kinase activity. We ruled out the possibility that CLN3 expression is up-regulated in cln1 cln2 mutants since there was no increase in Cln3 abundance or associated with kinase activity in such backgrounds (Figure 1B, C and D, lanes 5–8). The efficiency of immunoprecipitation was the same for each Cln (see Figure 1 legend). Of many reaction conditions tested, those presented were best for Cln3-associated histone H1 kinase activity in vitro. We have surveyed several exogenous substrates, including histone H1, myelin basic protein, casein and an N-terminal fragment of Far1 (data not shown). Of these, histone H1 was the best substrate and none of the tested substrates altered the relative kinase activities of the different Cln complexes. The similarities in substrate preference are consistent with the fact that all three Cln kinase complexes have Cdc28 as a catalytic subunit. Thus, we cannot find any obvious explanation for the unexpectedly small amount of histone H1 kinase activity associated with Cln3 in vitro. The most obvious remaining explanations are: first, there is some special in vivo substrate against which the Cln3 complex has an unusually high activity; or secondly the Cln3 complex actually has little kinase activity, but is functionally poten because it promotes Start in a different way than the Cln1 and Cln2 complexes.

Cln1 and Cln2-associated histone H1 kinase activities oscillate through the cell cycle; the Cln3-associated activity shows little (or no) oscillation

It has previously been shown that CLN1 and CLN2 mRNAs and the Cln2 protein oscillate through the cell cycle with a peak in late G1 phase (Wittenberg et al., 1990). In contrast, CLN3 mRNA does not oscillate (Nash et al., 1988; Wittenberg et al., 1990). We used the epitope-tagged Clns to see if the Cln-associated kinase activities oscillated through the cell cycle, since such an oscillation has never been demonstrated. Because Cln3 is rare and difficult to assay, we have repeated each type of experiment several times; representative experiments are shown, but our conclusions are based on all of the experiments.

Chemical arrests. Cells were arrested at Start with α-factor, in S phase with hydroxyurea or in mitosis with nocodazole. As anticipated from the fact that Cln2 oscillates through the cell cycle with a peak in G1 (Wittenberg et al., 1990), the abundance of Cln1 and Cln2 was greatly diminished in the α-factor- and nocodazole-arrested cultures. Furthermore, the associated kinase activities were greatly diminished in all three arrested cultures (Figure 2, lanes 1–8).

In contrast, Cln3 abundance in these arrested cultures was about the same as in asynchronous cells (Figure 2A). The Cln3-associated histone H1 kinase activity in this CDC34 strain was very weak. The experiment was repeated in a cdc34-2 background, in which Cln3-specific activity could be observed (Tyers et al., 1992), and activity did not appear to vary amongst the arrested cultures (Figure 2B, lowest panel). Similar experiments were also done with strains with readily detectable Cln3-associated kinase activities [GAL1–CLN3 and CLN3-1C in wild type and cdc34 backgrounds (Tyers et al., 1992)] and again there was no significant change in the level of Cln3 or associated kinase activity at the different arrest points (data not shown).

There are three other points of interest. First, in the hydroxyurea arrest, the loss of Cln1 and Cln2-associated kinase activity seemed to be relatively greater than the loss of Cln protein, hinting that inactivation of the kinase complex may occur before loss of Cln. Secondly, in cells arrested with α-factor, levels of Cln3 and its associated histone H1 kinase activity were as high as in asynchronous cells (see also Figures 3 and 6). Thus, presence of the Cln3-associated histone H1 kinase activity is not a sufficient condition for progress through Start, though of course some qualitative change in the kinase activity (its location, its substrate preference, its access to substrates) may have occurred. Thirdly, in the presence but not the absence of α-factor, a Cln3-associated protein of 116 kDa became phosphorylated in vitro. A protein of the same molecular weight was also a co-precipitated substrate of Cln1 and Cln2 from untreated, asynchronous cells (Figures 1D and 2B). The ability of the Cln2-associated kinase to phosphorylate p116 was barely reduced by arresting the cells with α-factor, even though
Fig. 3. Cell cycle regulation of CLN activity after release from G₁ arrest induced by mating pheromone. Cells were synchronized at Start with α-factor, released into fresh medium and analyzed for percentage budded cells (%B), mRNA (CLN2, CLN3 and ACT1), protein and Cln-associated in vitro kinase activity at the indicated time-points. In each experiment, synchrony was confirmed by budding and by oscillation of CLN2 mRNA; these were essentially identical for each culture. The RNA blots shown are for the CLN3C culture. For each Cln immunoblot equal loading was shown by immunoreactivity with an anti-β-tubulin antibody. (A) CLN1C strain (GT100-23c); (B) CLN2C strain (GT102); (C) CLN3C strain (MT236).
had points Cln2-associated histone paralleled protein in some clearly demonstrated in G1 (Herskowitz, 1990).

block complex. of removal Cln2-associated a kinase associated identified p synchronous progression a budded cells percentage content 4. CLN3 In contrast to CLN3C strain (Figure 3). Abundance of Cln1 and Cln2 mRNA and protein paralleled each other within the resolution of the time-points in these experiments (data not shown). Interestingly, in some experiments there was a substantial amount of Cln2-associated histone H1 and p16 kinase activity in the α-factor-arrested cells, even though most of the Cln2 protein had disappeared. A brief but substantial drop in the Cln2-associated kinase activity then occurred when the α-factor was removed. The surprising observation that removal of α-factor initially reduces kinase activity may indicate that recovery from mating pheromone arrest is complex.

In contrast to Cln1 and Cln2, Cln3 abundance and associated kinase activity did not obviously oscillate, despite synchronous progression through the cell cycle and normal oscillation of CLN2 mRNA (Figure 3). Although in this particular experiment there was a slightly increased Cln3 signal at the 100 and 120 min time-points (when the cells were in mitosis), there was no peak at 60 min, where Cln1 and Cln2 peaked. In this (as in other types of block and release experiments, data not shown), a gradual decrease in Cln3 abundance was observed, suggesting Cln3 is regulated under some conditions. Although the corresponding Cln3-associated kinase assays are shown for the sake of completeness, much of this activity was not Cln3-specific. Similar experiments were done using cdc15 block and release experiments, and again Cln1 and Cln2 and associated kinase activity oscillated, while Cln3 did not (data not shown).

Elutriation. In order to perturb cell growth as little as possible, we used the method of centrifugal elutriation to analyze Cln3 through the cell cycle. Small G1 cells were obtained by elutriation and reinoculated into fresh medium and followed through two synchronous cell cycles. Direct immunoblot analysis failed to reveal any convincing variation in Cln3 levels (Figure 4). In the particular experiment shown, there was no peak at Start, but there was a slight peak in Cln3 abundance at about the time of mitosis (120 and 140 min); the significance of this is uncertain. The first sample has very low levels of Cln protein; however, this was not
that Cln3 would have a major peak activity levels protein anticipated from the cell major find any Cln2-associated histone kinase activities go through a major cell cycle oscillation with a peak near Start, as anticipated from previous work on the oscillation of Cln2 protein levels (Wittenberg et al., 1990). However, we cannot find any convincing evidence that either Cln3 or its associated kinase activity undergo a major oscillation through the cell cycle. Since the system is experimentally difficult, there may in fact be some small oscillation that we have failed to observe (perhaps with a peak at mitosis), but any such undulation would have to be minor compared with that of Cln1 and Cln2. The lack of a major oscillation again suggests that Cln3 works differently from Cln1 and Cln2.

**High Cln3-associated kinase confers α-factor resistance**

We wondered why α-factor arrested wild type cells failed to pass Start even though they contained Cln3 and its associated histone H1 kinase activity (Figures 2 and 3). Is the type of Cln3 activity in an α-factor-treated cell qualitatively unsuitable for Start? Or is it simply quantitatively insufficient? To address this question, we constructed a GALI – CLN3 cdc34 strain, which contains ~200 relative units of Cln3-associated histone H1 kinase activity (Tyers et al., 1992), an amount similar to that of the Cln1 and Cln2 associated activity (Table I). Such cells were completely resistant to α-factor arrest (Figure 5A). The α-factor resistance was reversed by a plasmid carrying CDC34 and depended on induction of the GALI – CLN3 construct (Figure 5A). This is consistent with the idea that the Cln3-associated kinase is qualitatively capable of activating Start even in the presence of α-factor, but is normally present at levels quan-

---

**Fig. 5.** Effects of CLN3 dosage on mating pheromone resistance. 3000 cells of the indicated genotypes were spotted on a grid of medium containing the indicated amounts of α-factor. (A) Overexpression of CLN3 in a cdc34-2 strain confers resistance to α-factor. Absence or presence of a high copy CDC34 plasmid is indicated by − or +, respectively. The medium used was YEP + raf + gal (to turn on GALI – CLN3) except in row 5, where 2% glucose was added to turn the GALI promoter off. The strains used were: MT212 (rows 1 and 2), BF310-7c (rows 3, 4 and 5); BF338-4b #43p (row 6), BF338-4b #43 (row 7). (B) The α-factor resistance of CLN3-1C strains depends in part on CLN1 and CLN2. Strains used were: BF305-15d (row 1); BF338-3b #23p (row 2); strain BF #21 CW3 (row 3); strain BF #21 MW12-1 (row 4); and strain BF #21 CM3 (row 5). All strains were isogenic to BF305-15d except for BF338-3b #23p, which is a related strain.
the role of Farl

Interestingly, levels.

distinctively insufficient for this task. This genetic interaction between \( \text{GAL1} - \text{CLN3} \) and \( \text{cdc34-2} \) suggests that the increased Cln3-associated kinase activity seen \textit{in vitro} from such strains (Tyers et al., 1992) reflects an increase in the \textit{in vivo} activity of Cln3.

\textbf{Involvement of CLN1 and CLN2 in the \( \alpha \) factor resistance of CLN3-1C}

It has been found that \( \text{CLN1} \) and \( \text{CLN2} \) transcription is not permanently repressed by \( \alpha \)-factor in \( \text{CLN3-1C} \) or \( \text{CLN3-2} \) cells (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). In contrast, \( \text{GAL1} - \text{CLN3} \), which does not relieve \( \alpha \)-factor arrest (Figure 5A), does not relieve the \( \alpha \)-factor repression of \( \text{CLN1} \) and \( \text{CLN2} \) transcription (data not shown). To determine whether transcription of \( \text{CLN1} \) and \( \text{CLN2} \) is critical for \( \alpha \)-factor resistance, we constructed strains carrying various alleles of \( \text{CLN3} \), and lacking \( \text{CLN1} \) and \( \text{CLN2} \). We found that \( \text{cln1 cln2 CLN3-1C} \) strains were more sensitive to \( \alpha \)-factor than \( \text{CLN1 CLN2 CLN3-1C} \) strains (Figure 5B); a similar result has been obtained by Dirick and Nasmyth (1991). The \( \alpha \)-factor resistance of \( \text{CLN3-1C} \) cells therefore depends in part on \( \text{CLN1} \) and \( \text{CLN2} \). This \textit{in vivo} result is supported by Figure 6, which shows the Cln-associated kinase activity at different times after application of \( \alpha \)-factor. For \( \text{Cln1} \) and \( \text{Cln2} \), \( \alpha \)-factor drastically decreased the amount of protein (Figure 6A) and also the associated kinase activity (Figure 6B). For Cln3 and Cln3-1, \( \alpha \)-factor modestly increased the amount of protein (Figure 6A), though the associated kinase activity was, as usual, difficult to distinguish from background. Strikingly, when tagged Cln2 was examined in a \( \text{CLN3-1} \) background, Cln2 and its associated kinase activity dipped when \( \alpha \)-factor was first applied, but by 3 h had recovered to relatively high levels. Interestingly, in this strain p116/Far1 was heavily phosphorylated \textit{in vitro} by Cln2-associated kinase activity; the role of Far1 phosphorylation in recovery from \( \alpha \)-factor-induced arrest will be considered in detail elsewhere (M. Tyers and B. Futcher, submitted). The presence of Cln3-1 allowed a rapid recovery of Cln2-associated kinase activity and Figure 5 suggests that Cln2- and presumably, Cln1-associated activity was important for Start. This result is consistent with the idea that \( \text{CLN3-1C} \) functions as an activator of \( \text{CLN1} \) and \( \text{CLN2} \). We do not know why \( \text{CLN3-1C} \) should be able to activate \( \text{CLN1} \) and \( \text{CLN2} \) in the presence of \( \alpha \)-factor when an equivalent amount of \( \text{CLN3} \) expressed from the \( \text{GAL1} \) promoter cannot.

\textbf{Heterologous cyclins can suppress \( \text{cln1 cln2 cln3} \) lethality without causing \( \alpha \)-factor resistance}

Several heterologous cyclins expressed at high levels can suppress the lethality of a \( \text{cln1 cln2 cln3} \) triple deletion (Xiong et al., 1991). Among the complementing cyclins are three truncated B-type cyclins of \textit{Schizosaccharomyces pombe} (including \( \text{cdc13} \), a known mitotic cyclin) and truncated versions of human cyclins B1 and B2 (T. Connolly, Y. Xiong, M. Caligiuri, B. Futcher and D. Beach, unpublished results). These B-type cyclins are presumed to operate in mitosis and even \textit{S. cerevisiae} mitotic cyclins are not inactivated by \( \alpha \)-factor, since \( \alpha \)-factor does not inhibit mitosis. Furthermore, the \( \text{ADH1} \) promoter from which the heterologous B-cyclins are expressed is not repressed by \( \alpha \)-factor. Therefore, these heterologous cyclins should be able to activate Start in the presence or absence of \( \alpha \)-factor. However, we have found that this is not true. Two \textit{cln}-suppressing B-type human cyclins still allowed wild type levels of \( \alpha \)-factor sensitivity (Figure 7). Similarly, it has been reported that the \textit{Drosophila} cyclin \( \text{C} \) gene suppresses the \( \text{cln} \) deficiency, but does not confer resistance to \( \alpha \)-factor (Leopold and O’Farrell, 1991). One B-type cyclin from \( \text{S. pombe} \) (a truncated form of \( \text{cig1}^{+} \)) did confer resistance to \( \alpha \)-factor, while two others (truncated forms of \( \text{cdc13}^{+} \) and a previously unidentified B-type cyclin) did not (data not shown). It was striking that while one heterologous cyclin gave apparently complete resistance, the other five had no detectable effect at all. The \( \alpha \)-factor sensitivity of the cells living on a heterologous B cyclin suggests there is an unidentified \( \alpha \)-factor-sensitive requirement for Start.

\textbf{A model for CLN3 function}

Many of the results described above did not easily fit the idea that the three Clns have similar and redundant functions.
We therefore considered an alternative model of the role of Cln3 (Tyers et al., 1991). The essence of this model is that CLN3 is an upstream activator of other G₁ cyclins, which include but are not limited to CLN1 and CLN2. These downstream G₁ cyclins provide the catalytic function required for Start and are the targets of α-factor inactivation. In this model, cln1 cln2 double mutants are not living on CLN3 alone; rather, they are living on other G₁ cyclins as activated by CLN3.

Recently, three new *S. cerevisiae* cyclin homologs have been found. HCS26 was isolated as a high copy number suppressor of an allele of SWI4 (Ogas et al., 1991). It was then found (T. Hunt, personal communication) that *S. cerevisiae* contains a previously sequenced HCS26 homolog called ORFD (Frohlich et al., 1991). Finally, CLB5 was recently cloned as a suppressor of cln1 cln2 cln3 lethality (Epstein and Cross, 1992). Thus, there are at least three cyclin homologs that might be the missing G₁ cyclins we have predicted (Tyers et al., 1991).

The model predicts that bursts of Cln3 activity would induce activity of the other G₁ cyclins. It also makes a strong prediction that in the absence of CLN1 and CLN2, activity (and perhaps transcription) of the other putative G₁ cyclins would be dependent on CLN3. As described below, both of these predictions are correct.

**CLN3 efficiently activates transcription of five other cyclins and also of SWI4**

Cross and Tinkelenberg (1991) have shown that CLN3 can induce transcription of CLN1 and CLN2. We used a GAL1–CLN3 strain to ask whether CLN3 could regulate other cyclins and to find out more about the relationship between CLN-induced CLN transcription and Start. Three different methods were used to assay Start: percentage of budded cells, FACS analysis (to show how many cells have gone through S phase) and an α-factor execution point assay. Analysis of budded cells and FACS analysis both assay events that happen shortly after Start. However, the α-factor execution point assay measures Start directly. In this assay, a large excess of α-factor is added to a sample of cells, and after a suitable incubation period, the percentage of cells that have budded is measured. These, by definition (Hartwell et al., 1974), are past Start. When the percentage of cells able to bud in an excess of α-factor reaches 50%, we define this as Start for the culture.

**GAL1–CLN3 cells were grown in raffinose medium (i.e. CLN3 off) and very small G₁ cells were obtained by elutriation. These cells were reinoculated into raffinose medium and then (i) left in raffinose (Figure 8A) or (ii) induced with galactose for the duration of the experiment (Figure 8B), or (iii) induced with galactose for 15 min,**
followed by removal of galactose (Figure 8C). When the \textit{GALI} – CLN3 construct was not induced the G1 cells did not pass Start for >2 h, despite increasing their cell volume to a size well beyond that for cells bearing a wild type \textit{CLN3} allele. Thus, \textit{CLN3} is essential for setting the critical size threshold and allowing normal passage through Start in G1 daughter cells. [This result and the result shown in Figure 11E are at odds with experiments reported by Lew et al. (1992); it now seems that these previous results were in error (M.Linksens, M.Tyers and B.Futcher, submitted; D.J.Lew, personal communication).] In the absence of \textit{CLN3}, levels of the various cyclin mRNAs remained at a low, fairly constant level. Induction of \textit{GALI} – CLN3 by galactose caused rapid accumulation of \textit{CLN1}, \textit{CLN2}, \textit{HCS26}, \textit{ORFD} and \textit{CLB5} mRNAs, with peak accumulation at \(\sim 45\) min after addition of galactose (Figure 8B). This treatment caused cells to pass Start and bud >40 min before uninduced cultures. Since the only difference between the two cultures was the induction of \textit{CLN3}, \textit{CLN3} must be able to activate transcription of these other cyclins.

Once Start had occurred, the levels of \textit{CLN1}, \textit{CLN2}, \textit{HCS26}, \textit{ORFD} and \textit{CLB5} fell despite the continued high levels of \textit{CLN3} mRNA (Figures 8B and 9B). Thus, these genes become insensitive to \textit{CLN3} induction after Start. This insensitivity implies loss of the positive feedback loop after Start. This is probably an important part of the mechanism for down-regulating the G1 cyclins after Start.

The premature Start induced in the experiment described above could have been directly due to the high levels of \textit{CLN3}, or it could have been due to the high levels of the other cyclins induced by \textit{CLN3}. To distinguish these possibilities, we pulsed a \textit{GALI} – \textit{CLN3} culture with galactose then either washed out the galactose with pre-conditioned raffinose medium (Figure 8C) or repressed the \textit{GALI} promoter with glucose (Figure 9D). In both cases, Start was accelerated with exactly the same kinetics as in the culture where \textit{CLN3} was induced permanently. Importantly, the \textit{CLN3} mRNA was lost before the peak in the other G1 cyclins and well before Start. Since the Cln3 protein is very unstable (Tyers et al., 1992), this suggests that Cln3 may not be required at the time of Start. Importantly, this result also shows that the positive feedback loop operates in small cells undergoing a normal G1 phase and not just in large, arrested cells as in previous investigations.

Is \textit{CLN3} uniquely well suited to carry out this induction or would any G1 cyclin work? To find out, we repeated the experiment above using \textit{GALI} – \textit{CLN1} instead of \textit{GALI} – \textit{CLN3}. When \textit{GALI} – \textit{CLN1} was left off, Start occurred rather late in the time-course (Figure 9A), though not as late as in the cln3 strain (Figure 8A). When \textit{GALI} – \textit{CLN1} was induced for the whole time-course, Start was accelerated; this was accompanied by induction of the other cyclin transcripts (Figure 9B). Thus, \textit{CLN1} was able to influence the timing of Start. Finally, when \textit{GALI} – \textit{CLN1} was pulsed for 20 min, there was a slight induction of other cyclins, but budding occurred in only about one-third of the cells, after which the other cyclin mRNAs decayed back to baseline levels when \textit{GALI} – \textit{CLN1} was turned off (Figure 9C). Start eventually occurred in the remaining cells at the same time it had in the raffinose (uninduced) culture. When an identical experiment was done with \textit{GALI} – \textit{CLN3}, a short pulse of \textit{CLN3} expression resulted in persistent activation of the other cyclins and acceleration of Start in essentially all of the cells (Figure 9D). The pulse of \textit{CLN3} expression (but not a pulse of \textit{CLN1}) also accelerated DNA replication as shown by FACS analysis (Figure 9E). Thus, it appears that \textit{CLN3} can provide relatively long-lasting activation of the transcriptional positive feedback loop in the pre-Start G1 phase of the cell cycle by some mechanism not available to \textit{CLN1}.

It is quite clear that pulses of \textit{CLN3} induce the other cyclins before Start. In Figure 9D, for instance, \textit{CLN1}, \textit{HCS26} and \textit{ORFD} levels were high by 30 min, when only 1% of the cells had budded. In this experiment, it was not until 60 min that 50% of the cells passed through Start by the \(\alpha\)-factor execution point assay. Thus, the peak in \textit{CLN1}, \textit{HCS26} and \textit{ORFD} transcripts is not a consequence of Start.

Since \textit{GALI} – \textit{CLN1} is off but chromosomal \textit{CLN1} is still present in Figure 9A, this panel shows what happens when wild type cells grow through G1 phase to Start. There seems to be a fairly gradual, linear increase in the abundance
of CLN1, HCS26 and ORFD mRNAs. Also, comparison of Figure 9A with E (the FACS profiles for the same cells) suggests that these mRNAs are not eliminated immediately after Start, but rather persist into S phase, consistent with the chemical block experiments (Figure 2).

SWI4 is required for the CLN positive feedback loop (Nasmyth and Dirick, 1991) and the SWI4 transcript oscillates through the cell cycle (Breeden and Mikesell, 1991) and under some circumstances is essential for Start (Fernandez-Sarabia et al., 1992). Since nothing was known about the mechanism of SWI4 oscillation, we asked if SWI4 is controlled by CLN activity. We found that the SWI4 transcript itself seems to be subject to the positive feedback loop and can be induced by CLN3 and, to a lesser extent, by CLN1 (Figures 9 and 10). This result shows that Swi4, like Cln1 and Cln2, is an autoregulatory component of the positive feedback loop.

In a cln1 cln2 mutant, transcription of HCS26, ORFD and CLB5 depends on CLN3

Since cln1 cln2 strains depend on CLN3 for viability, we wished to see if the expression of other G1 cyclins depended on CLN3 in this situation. When a cln1 cln2 GAL1–CLN3 strain, which depends on induction of the galactose promoter

Fig. 9. CLN1 is less effective than CLN3 at activating the positive feedback loop. A similar experiment to that shown in Figure 8 was carried out with isogenic GAL1–CLN1 (MT335; GAL1–CLN1 CLN1 CLN2 CLN3) and GAL1–CLN3 (MT313; GAL1–CLN3 CLN1 CLN2 cln3) strains. Small GAL1–CLN1 cells were reinoculated into either (A) YEP + Raf (GAL1–CLN1 off), (B) YEP + Raf + gal (GAL1–CLN1 on for the entire time-course) or (C) YEP + Raf + gal for 20 min, then GAL1–CLN1 expression was repressed to a low level by addition of glucose to 2% (GAL1–CLN1 on for 20 min). In a parallel experiment, small G0 GAL1–CLN3 cells were reinoculated into YEP + Raf + gal for 20 min, then CLN3 expression was repressed by addition of glucose to 2% (CLN3 on for 20 min). In all four experiments, CLN2 and CLB5 mRNAs behaved like the other cyclin mRNAs (not shown). Passage through Start was assessed by the percentage of budded cells (%B) and directly by testing sensitivity to α-factor. Cells from each time-point were incubated with α-factor for 3 h; the point at which 50% of the cells were able to divide in the presence of α-factor is indicated by an asterisk. (E) The cell cycle position of samples from panels A, C, D and a parallel GAL1–CLN3 YEP + Raf culture was assayed by FACS analysis. Cell volume (f) is also shown. Note that the GAL1–CLN3 YEP + Raf culture is effectively a cln3 deletion strain and progresses through Start at a much larger critical size than the GAL1–CLN1 YEP + Raf culture, which is wild type for both CLN1 and CLN3 when grown in raffinose since the GAL1–CLN1 construct is carried on a centromeric vector and does not replace the wild type CLN1 locus.

Fig. 10. CLN3 is required for normal levels of HCS26, ORFD and CLB5 mRNAs in a cln1 cln2 strain. A control CLN1 CLN2 CLN3 strain (BF305-15d, wt) and a cln1 cln2 GAL1–CLN3 strain (BF411-2c ΔΔ, all other lanes) were grown to mid-log phase in YEP + Raf + gal. At t = 0, the cln1 cln2 GAL1–CLN3 strain was harvested, washed twice with YEP and reinoculated into YEP + Raf. After 300 min, >95% of the cells were unbudded and large. Galactose was added to 0.5% at 303 min and glucose added to 1% at 333 min. The first buds appeared at 360 min. Lanes 1 and 3 are identical, except that the sample shown in lane 1 was held on ice for an hour before processing, to show that mRNA yield is not significantly affected by minor changes in processing time.
for viability, was shifted to raffinose medium, the \( CLN3 \) transcript disappeared almost immediately (Figure 10). The \( HCS26, ORFD \) and \( CLB5 \) transcripts also dropped drastically in abundance. After 300 min, all of the cells were arrested at Start. Despite being at exactly the point in the cell cycle where a \( G_1 \) cyclin would be maximally transcribed, the \( HCS26, ORFD \) and \( CLB5 \) transcripts were barely visible, and were present at much lower levels than in a control (\( CLN1 \) \( CLN2 \) \( CLN3 \)) culture of asynchronous cells, where only a small fraction of the cells were in late \( G_1 \). When \( CLN3 \) transcription in the arrested culture was induced briefly with galactose, \( HCS26, ORFD, CLB5 \) and \( SWI4 \) were rapidly induced, peaking at \( \sim 10 \) min after the \( CLN3 \) transcript (Figure 10) and \( \sim 10 \) min before Start (not shown). \( SWI4 \) transcription was also dependent on \( CLN3 \), but less so than the other \( G_1 \) cyclins. We conclude that in the absence of \( CLN1 \) and \( CLN2 \), \( HCS26, ORFD, CLB5 \) transcription becomes absolutely dependent on \( CLN3 \).

Discussion

\( Cln3 \) behaves differently than \( Cln1 \) and \( Cln2 \)

\( Cln3 \) is quite different from \( Cln1 \) and \( Cln2 \). Unlike \( Cln1 \) and \( Cln2 \), \( Cln3 \) and its associated histone H1 kinase activity do not oscillate through the cell cycle, and are not diminished by \( \alpha \)-factor. \( Cln3 \) is much less abundant than \( Cln1 \) or \( Cln2 \) and has a much weaker associated histone kinase activity \textit{in vitro}. Of course, we do not know whether histone H1 is an appropriate substrate; nevertheless, the large \textit{in vitro} differences between \( Cln3 \) on the one hand, and \( Cln1, Cln2 \) on the other make it worth considering whether their \textit{in vivo} roles are different.

Paradoxes

We have been working with an implicit model in which Start occurs when \( Cln \)-associated kinase activity increases to a critical threshold. The viability of all \( cln \) double mutants suggested that any one \( Cln \) could generate the critical level of activity. \( \alpha \)-factor was presumed to arrest cells at Start by inhibiting each \( Cln \)-associated kinase activity. Both the \( Wei \) phenotype and the \( \alpha \)-factor resistance of \( CLN3 \)-I cells were interpreted as consequences of constitutively high \( Cln3 \)-1-associated kinase activity. Heterologous cyclins that suppressed lethality of the \( cln1 cln2 cln3 \) triple deletion were presumed to produce the necessary critical level of \( Cln \)-like kinase activity and induce Start directly. The ability of many different structural classes of cyclin to do this (reviewed by Hunter and Pines, 1991) implied that different cyclins produced kinase activities that were qualitatively similar or overlapping.

However, there are now several results that conflict with this model. First, compared with \( CLN1 \) and \( CLN2 \), \( CLN3 \) is genetically potent (Nash \textit{et al.}, 1988; Richardson \textit{et al.}, 1989; Table I) but biochemically weak, at least as judged by abundance and associated histone H1 kinase activity. Secondly, cells arrested with \( \alpha \)-factor still contain about the same amount of precipitable \( Cln3 \)-associated histone H1 kinase activity as cycling cells, suggesting that this \( Cln3 \) activity is not sufficient for Start. Thirdly, Nasmyth and co-workers have found that \( swi4 swi6 \) double mutants (or \( swi4/swi4-ts1 \) strain; Ogas \textit{et al.}, 1991) are lethal because \( CLN1 \) and \( CLN2 \) are not transcribed (Nasmyth and Dirick, 1991). The arrest is due to lack of \( CLN \) activity, since the lethality can be suppressed by a \( CLN \) expressed from a heterologous promoter (Nasmyth and Dirick, 1991), and yet the \( CLN3 \) transcript is still present at wild type levels. Like the result from \( \alpha \)-factor-arrested cells, this suggests that \( CLN3 \) is not sufficient for Start. Fourthly, most heterologous cyclins that complement the \( cln1 cln2 cln3 \) triple deletion fail to make cells resistant to \( \alpha \)-factor. That is, in the presence of \( \alpha \)-factor they cannot activate Start. The second, third and fourth paradoxes suggest that Start is influenced by some activity other than \( Cln1 \), \( Cln2 \) and \( Cln3 \), and that this other activity is \( \alpha \)-factor-sensitive, and \( SWI4 \) and \( SWI6 \) dependent. The fact that \( CLN3 \)-I or \( cdc34 GAL1 \) - \( CLN3 \) can cause Start in the presence of \( \alpha \)-factor and the fact that \( swi4 swi6 \) lethality can be suppressed by \( CLN2 \) expressed from a heterologous promoter, show that this other activity can be replaced by a \( Cln \), suggesting that it is \( Cln \)-like. That is, it may be another \( G_1 \) cyclin.

Resolution of paradoxes: a model

To resolve these paradoxes, we invoke the finding that \( Cln \) activity depends on a positive feedback loop (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Nasmyth and Dirick, 1991; Figure 11). \( Cln-\text{Cdc28} \) activity acts through the \( Swi4 \) and \( Swi6 \) transcription factors to increase \( CLN1 \) and \( CLN2 \) transcription, which leads to an increase in \( Cln-\text{Cdc28} \) activity and so on. Thus, \( Cln \) activity begets more \( Cln \) activity. \( CLN3 \) transcription is independent of this feedback loop (Nasmyth and Dirick, 1991). In addition, we propose that there are other \( G_1 \) cyclins contributing to Start. These other \( G_1 \) cyclins probably include, but are not necessarily limited to, Hcs26, OrfD and Clb5. We imagine that \( Cln1, Cln2, Hcs26, OrfD \) and \( Clb5 \) are 'downstream' \( G_1 \) cyclins that depend on the \( Swi4/Swi6 \) positive feedback loop for transcription and are sensitive to \( \alpha \)-factor inactivation, while the 'upstream' cyclin \( Cln3 \), is not dependent on the feedback loop and is not sensitive to \( \alpha \)-factor.

The model is as follows: \( Cln1, Cln2 \) and the other downstream \( G_1 \) cyclins directly activate Start, perhaps by reaching a critical level of kinase activity via a positive
feedback loop. In contrast, Cln3 does not and cannot activate Start directly, simply because its associated kinase activity is too weak. Rather, Cln3 functions near the top of a cascade to help activate the downstream G1 cyclins. Since CLN3 is not essential, it cannot be the only way to activate CLN1 and CLN2; perhaps CLN1 and CLN2 have some constitutive activity, perhaps from their Cdc28-independent level of transcription (Dirick and Nasmyth, 1991). However, in the absence of CLN1 and CLN2, CLN3 is necessary to activate the remaining G1 cyclins.

We have demonstrated that CLN3 can activate transcription of at least five other cyclins; this may be partly due to activation of the Swi4 and Swi6 transcription factors and partly due to increased SWI4 transcription. In addition, some activation of other Clns by Cln3 might occur directly by phosphorylation of the Cln kinase complexes. We note that the Cln3 kinase complex (Tyers et al., 1992) and also the Cln1 and Cln2 kinase complexes (data not shown) require phosphorylation for activity.

The model resolves the paradoxes as follows. (i) cln1 cln2 mutants are not living on CLN3 alone; rather, they are living on HCS26, ORFD, CLB5 and perhaps other downstream cyclins as activated by CLN3. (ii) In cells treated with α-factor, essential downstream cyclins are inactivated. The Cln3 activity remains, but is insufficient for Start and so the cells arrest. When Cln3 activity is increased (e.g. in GAL1—CLN3 cdc34-2 strains), it promotes Start directly even in the presence of α-factor. (iii) Similarly, cells lacking the Swi4 and Swi6 transcription factors lose the downstream cyclins. Cln3 remains, but is insufficient for Start. (iv) In most cases, overexpressed heterologous cyclins provide only a trace of Cln-like function. This is sufficient to complement the ctn3 defect and activate downstream cyclins, which promote Start. α-factor inactivates the downstream cyclins as usual and the cells arrest, despite continued presence and activity of the heterologous cyclin. This explains why truncated members of each of the six known structural classes of cyclins, A, B, Cln, C, D and E, are able to complement the triple ctn deletion in S.cerevisiae (Koff et al., 1991; Lew et al., 1991; Xiong et al., 1991). We now believe that heterologous cyclins overexpressed in S.cerevisiae seldom activate Start directly; instead, they meet a less stringent requirement—they have just enough Cln3-like activity to activate the downstream cyclins. In cases where the heterologous cyclins are truly Cln-like, they activate Start directly and make cells α-factor resistant.

We cannot rule out the possibility that Cln3 gives Cdc28 a preference for some ‘special’ substrate, allowing Cln3 to activate Start directly. However, this model does not help explain other puzzling results: the lack of Cln3 oscillation,

Table II. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Parental strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF305-15d</td>
<td>MATa leu2 his3 ura3 trpl adel arg5,6 met14</td>
</tr>
<tr>
<td>BF305-15d #21</td>
<td>MATa cln3::URA3—GAL1—CLN2 cln1::HIS3 cln2::TRP1 leu2 his3 ura3 trpl adel arg5,6 met14</td>
</tr>
<tr>
<td>BF338-3b</td>
<td>MATa ura3 his3 adel</td>
</tr>
<tr>
<td>BF338-4b</td>
<td>MATa ura3 his3 CLN3-1</td>
</tr>
<tr>
<td>BF410-7c</td>
<td>MATa cdc34-2 cln3::URA3—GAL1—CLN3 ura2 leu2</td>
</tr>
<tr>
<td>BF411-2cF</td>
<td>MATa cln3::ura3—GAL1 CLN3 cln2::TRP1 ura3 trpl his2 adel arg5,6 met14</td>
</tr>
<tr>
<td>GT100-23c</td>
<td>MATa CLN1C leu2 his3 ura3 trpl adel arg5,6 met14</td>
</tr>
<tr>
<td>GT102</td>
<td>MATa CLN2C leu2 his3 ura3 trpl met14</td>
</tr>
<tr>
<td>K699 (= W303a)</td>
<td>MATa ade2-1 trpl-1 leu2-3,112 his3-11,15 ura3 can1-100 [psi+]</td>
</tr>
<tr>
<td>RN200-6d</td>
<td>MATa cdc34-2 his3 leu2 ura3</td>
</tr>
<tr>
<td>RN202-2b</td>
<td>MATa CDC34 his3 leu2 ura3</td>
</tr>
</tbody>
</table>

*Derivative strains were created from the parental strains by transformation. Only the portion of the genotype changed by the transformation is indicated.

<table>
<thead>
<tr>
<th>Derivativea</th>
<th>Parent strain</th>
<th>Relevant genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF305-15d CLN3-1</td>
<td>BF305-15d</td>
<td>cln3::URA3—CLN3-1</td>
</tr>
<tr>
<td>BF #21 MW12-1</td>
<td>BF305-15d #21</td>
<td>cln3::URA3—CLN3M</td>
</tr>
<tr>
<td>BF #21 CW3</td>
<td>BF305-15d #21</td>
<td>ctn3::URA3—CLN3C</td>
</tr>
<tr>
<td>BF #21 CM3</td>
<td>BF305-15d #21</td>
<td>CLN3-1C</td>
</tr>
<tr>
<td>BF338-3b #23p</td>
<td>BF338-3b</td>
<td>ctn3::URA3—GAL1—CLN3-1C</td>
</tr>
<tr>
<td>BF338-4b #23</td>
<td>BF338-4b</td>
<td>CLN3M</td>
</tr>
<tr>
<td>BF338-4b #43p</td>
<td>BF338-4b</td>
<td>ctn3::URA3—GAL1—CLN3M</td>
</tr>
<tr>
<td>BF411-2cF ΔΔ</td>
<td>BF411-2cF</td>
<td>cln1::HIS3 cln2::LEU2</td>
</tr>
<tr>
<td>GT104</td>
<td>K699</td>
<td>CLN1C</td>
</tr>
<tr>
<td>GT108</td>
<td>K699</td>
<td>CLN3C</td>
</tr>
<tr>
<td>MT137</td>
<td>BF305-15d #21</td>
<td>CLN1C</td>
</tr>
<tr>
<td>MT212</td>
<td>RN200-6d</td>
<td>cln3::URA3—CLN3C</td>
</tr>
<tr>
<td>MT236</td>
<td>RN200-6d</td>
<td>cln3::URA3—CLN3C CDC34</td>
</tr>
<tr>
<td>MT238</td>
<td>BF305-15 #21</td>
<td>CLN2C</td>
</tr>
<tr>
<td>MT242</td>
<td>GT102</td>
<td>cln3::URA3—CLN3-1C</td>
</tr>
<tr>
<td>MT263</td>
<td>K699</td>
<td>cln2::CLN2C—LEU2</td>
</tr>
<tr>
<td>MT313</td>
<td>K699</td>
<td>cln3::URA3—GAL1—CLN3</td>
</tr>
<tr>
<td>MT335</td>
<td>K699</td>
<td>[pGAL1—CLN1 LEU2 URA3 CEN6]</td>
</tr>
</tbody>
</table>
the unaltered Cln3 activity in α-factor arrested cells, the inability of CLN3 to support viability of a swi6Δ swi6Δ double mutant, the α-factor sensitivity of cells living on heterologous cyclins or the dependence of HCS26, ORFD and CLB5 transcription on CLN3. A difference in substrate specificity might, however, explain why Cln3 is a more efficient activator of the positive feedback loop than Cln1. We have found that the Cln3–Cdc28 complex has at least one associated substrate that is not in the Cln1 or Cln2 complexes (Tyers et al., 1991).

A prediction of the model is that a CLN3 strain bearing mutations in some combination of downstream G1 cyclin genes (e.g. a CLN3 cln1 cln2 hcs26 orfD clb5 strain) should be inviable. We are currently testing this. However, inviability of such a strain might mean that some of the downstream cyclins have an additional function that CLN3 cannot supply. Conversely, viability of such a strain might mean there are additional downstream G1 cyclins.

**Upstream and downstream cyclins: the division of labour**

We suspect that Cln3 is a ‘pilot light’ or ‘boosting’ activity needed at a low level to activate other G1 cyclins. It is for precisely this reason that Cln3 does not oscillate. The constitutive Cln3 activity does not cause constitutive activation of Start because it is so weak. Since Cln3 has a short half-life, its steady-state abundance may be regulated by the rate of growth, protein synthesis and cell size, and this may affect the rate of activation of other G1 cyclins and hence the timing of Start. This could be the link between cell growth and cell division (Moore, 1988; Nash et al., 1988). The strong phenotype of CLN3-1 (Nash et al., 1988) and the genetic interactions of FUS3, KSS1 and SGV1 with CLN3 (Cournchese et al., 1989; Elion et al., 1990; Irie et al., 1991) suggest that control mechanisms regulate Cln3 at the top of the Start cascade rather than at the point of near maximal Cln activity where Cln1 and Cln2 are required. Perhaps the reason that CLN3 transcription is induced and not repressed by α-factor (Nash et al., 1988) is that Cln3 activity is needed after the arrest to reactivate other G1 cyclins, perhaps including Cln1 and Cln2. The effect of CLN3-1 on Cln2 associated kinase activity is consistent with this idea.

**Summary**

We imagine that Cln3 monitors growth and size, but does not provide any oscillatory input. Rather, the oscillations are generated at the level of the downstream cyclins. In early G1, Cln3 activity is present and leads to transcription of downstream cyclins and SWI4, which lead to increased Cln activity and Start. Passage through Start causes an interruption in the positive feedback loop (Figures 8B and 9B), causing loss of the downstream cyclins. Obviously, discovering and understanding this resetting mechanism will be crucial to a complete understanding of the cell cycle oscillation. This model segregates two different cell cycle functions to two different classes of Clns; the Cln3 class is responsible for linking growth to division, while the Cln1 and Cln2 class is primarily responsible for oscillation.

We have proposed a Cln kinase cascade for the regulation of Start. A similar cascade for mitosis, in which cyclin A–Cdc2 complexes activate cyclin B–Cdc2 complexes, has been proposed by Minshull et al. (1990). Perhaps other structural classes of cyclins are also involved in cascades, helping explain why so many classes of mammalian cyclins exist.

**Materials and methods**

**Yeast strains, growth media and plasmids**

Yeast strains are listed in Table II. Standard methods were used for cultivating and manipulating yeast (Guthrie and Fink, 1991). YEP was 1% yeast extract, 2% peptone and was supplemented to 2% with carbon sources (e.g. 2% glucose for YEPD; 2% raffinose for YEP + raf; 2% raffinose and 2% galactose for YEP + raf + gal). CLN3 encodes Cln3 with a C-terminal triple hemagglutinin (HA1 epitope tag); and CLN3:1 encodes Cln1-1 (the truncated protein) with a C-terminal triple HA1 tag (Tyers et al., 1992). CLN1 and CLN2 were tagged by inserting Nod sites at the end of each open reading frame. A triple HA1 epitope cassette on a Nod fragment was cloned into the site in the same reading frame. The CLN1 mutagenic oligonucleotide used was 5'-AATAGCTTCAGACCCGCGGTACCAATCTTCCAGATC-3'; the CLN2 oligonucleotide was 5'-AAATACCCAGAATAAACGTGGCCGCTAGTTCCTGTTCTTT-3'; the cassette sequence was 5'-GCCGCGGCACTTATTTACACCGAGTTCTGCTGCTCAGTGCGGCCGCTAGTTCCTGTTCTTT-3'. The tagged genes were called CLN1C and CLN2C. These were integrated into strain BYF05-15d #21 (Xiong et al., 1991) by a single step gene replacement with selection for growth on glucose. Transformants were identified by PCR of the triple epitope and by immunoprecipitation of kinase activity. CLN1C and CLN2C were followed in crosses by PCR. In some cases, unmarked tagged CLN alleles were integrated into a strain by replacing the genomic disrupted copy of the CLN, selecting for co-transformation with an unrelated marker plasmid and screening for loss of the disrupting marker.

Where indicated cdc34-2 strains were transformed with Yep-LEU34-1 (a LEU2 derivative of YepEp34-1, initial plasmid kindly provided by Mark Goeb), which carries wild type CDC34. The ADH1-driven heterologous B-type cyclin plasmids were produced in the work of Xiong et al. (1991). All the complementing heterologous cyclins were N-terminal truncations T. Connelly, Y. Xiong, M. Calleja, B. Fütterer and D. Beach, unpublished observations). The GAL1–CLN1 plasmid is described in Cross and Tinkelenberg (1991).

Quantitative sensitivity to α-factor was measured as described by Nash et al. (1988), except that 3000 cells were spotted at each grid position. Grids were incubated at 25°C and photographed after 2–3 days. Percentage budding was assessed by light microscopy of at least 200 cells per timepoint. The population distribution of cellular DNA content was determined by FACS analysis as described by Nash et al. (1988).

**Cell synchrony experiments**

Cell arrests were carried out by treatment for 3–4 h with 5 μM α-factor, 100 mM hydroxyurea or 10 μg/ml nocodazole. Arrest was confirmed by microscopic examination and FACS analysis.

Synchronization by release from an α-factor block was done by arresting cells (typically 600 ml grown in YEPD to 106 cells/ml) for 3.3 h at room temperature with 5 μM α-factor, washing twice with 100 ml YEPD and resuspending in the original volume of YEPD. At 20 min intervals aliquots were removed, rapidly pelleted and washed once with ice-cold water before analysis. Typically 30 ml of culture were used for protein lysate, 7 ml for total RNA preparation and 1 ml for FACS analysis and budding index.

Homogeneous small G1 cells were isolated by centrifugal elutriation. For analysis of wild type levels of Cln3, 3 l of a CLN3 strain (GT108) were grown to 5 × 107 cells/ml in YEP + sucrose, concentrated to 100 ml by centrifugation and sonicated. Cells were quickly loaded into a Beckman elutriator at 2400 r.p.m. and a pump speed of ~25 ml/min. Cells were elutriated at room temperature with the clarified medium of the original culture. The pump speed was increased ~20% to yield an initial fraction of ~300 ml at ~2 × 107 cells/ml of small G1 cells, which had a typical mode volume of 25 fl. As soon as this fraction was collected (~25 min from the initial centrifugation), aliquots were taken at time intervals and processed as described for α-factor release experiments.

Elutriation experiments using a GAL1–CLN3 strain (MT313) were similar except that cells were grown and elutriated in YEP + raf. Experiments were initiated by adding galactose to 2% to two-thirds of the elutriated fraction; after the indicated time, half of this culture was either washed free of galactose (with pre-conditioning YEP + raf + gal), or were taken into a glucose to shut off the GAL1 promoter. Time-points were taken in parallel from the raffinose, galactose pulse and galactose cultures and harvested as

1967
above. Experiments with a GAL1–CLN1 strain (MT355) were carried out in the same way except that cells were grown in YNB-Ura and inoculated 1:10 into YEP + raf 10 h before collection; this ensured that at least 90% of the cells retained the GAL1–CLN1 centromeric plasmid.

**Immunoprecipitation, immunoblot analysis and kinase assays**

These methods were carried out essentially as described by Tyers et al. (1992). Cell lysates were prepared by vortexing cell suspensions in lysis buffer (1% NP40, 50 mM NaCl, 50 mM NaF, 5 mM EDTA and 50 mM Tris–HCl pH 7.5) in the presence of glass beads. Crude clarified lysates were made by pelleting cell debris at 15 000 g for 15 min. Protein concentration in the lysate was determined by a dye binding assay (Bio-Rad); lysates typically contained 25–50 mg/ml of total protein.

Ascites fluid generated from the 12CA5 mouse monoclonal antibody hybridoma was used to detect the HA1 epitope (Field et al., 1988). An ECL system (Amersham) was used for immunoblot detection according to the directions of the manufacturer. Primary and secondary antibodies were diluted 1:10 000. Direct immunoblot analysis was carried out with 45 µg of total protein; cell lysates were diluted into protein sample buffer on ice and boiled immediately before loading on 10% polyacrylamide–SDS minigels. A rabbit serum (kindly provided by Dr F.S. Solomon) against the C-terminus of β-tubulin was used to confirm equal loading of each lane.

Immunoprecipitations were typically carried out on 3 mg of cell lysate with 0.15 µl of 12CA5 ascites fluid for 1–2 h on ice. Immune complexes were collected on protein A-Sepharose beads by rocking at 4°C for 1 h. For detection of immunoprecipitated proteins, beads were pelleted by gentle centrifugation (1000 g for 5 s), washed four times with lysis buffer and boiled in protein sample buffer immediately before SDS-PAGE. For determination of immunoprecipitated kinase activity, the washed protein A beads were transferred to a fresh tube and washed twice more with kinase reaction buffer. Reactions were for 10 min at 37°C under conditions described previously by Tyers et al. (1992); incorporation of [32P] into histone H1 was linear with respect to time. Kinase activity was quantitated by excising Coomassie Blue-stained histone H1 bands and counting Cerenkov emissions in a scintillation counter.

**Northern analysis**

Total RNA was isolated, transferred to nylon membrane and probed as described by Tyers et al. (1992). The probes used were: a 2.5 kb EcoRI fragment that encompassed CLN1 (Hadwiger et al., 1989); a Ndel–NcoI fragment corresponding to CLN2 (Hadwiger et al., 1989); a Ndel–NcoI fragment that corresponded exactly to the CLNJ reading frame (Nash et al., 1988); a 1.5 kb HCS26 PCR fragment (Ogas et al., 1991); a 1.4 kb ORFD PCR fragment (Frolich et al., 1991); a 1.9 kb CLB5 PCR fragment (Epstein and Cross, 1992); a 3 kb BglII–BglII fragment that encompassed most of the SWT4 reading frame (Andrews and Herskowitz, 1989); a 600 bp EcoRI–HindIII internal fragment of ACT1. Blots were probed sequentially, two probes at a time. The last probing was done with ACT1 to ensure that RNA had been equally loaded and retained on the membrane.

**Acknowledgements**

We thank Fred Cross, Mark Goebel and Kim Nasmyth for providing strains and plasmids, Frank Solomon for providing the anti-β-tubulin antibody, and Chuck Epstein and Fred Cross for providing information about CLB5 prior to publication. Eric Richards, Kim Arndt and Kim Nasmyth and members of the Futcher lab are thanked for insightful comments and stimulating discussions. Finally, we thank Phil Renna for help with the figures. This research was supported by NIH grant GM39978 to B.F.; M.T. was supported by an MRC of Canada Fellowship.

**References**


Received on December 28, 1992