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Regulation of the Cln3–Cdc28 kinase by cAMP in *Saccharomyces cerevisiae*

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The yeast *Saccharomyces cerevisiae* grows at widely varying rates in different growth media. In order to maintain a relatively constant cell size, yeast cells must regulate the rate of progress through the cell cycle to match changes in growth rate, moving quickly through G_1 in rich medium, and slowly in poor medium. We have examined connections between nutrients, and the expression and activity of Cln3–Cdc28 kinase that regulates the G_1–S boundary of the cell cycle in yeast, a point referred to as Start. We find that Cln3 protein levels are highest in glucose and lower in poorer carbon sources. This regulation involves both transcriptional and post-transcriptional control. Although the Ras–cAMP pathway does not appear to affect *CLN3* transcription, cAMP increases Cln3 protein levels and Cln3–Cdc28 kinase activity. This regulation requires untranslated regions of the *CLN3* message, and can be explained by changes in protein synthesis rates caused by cAMP. A model for *CLN3* regulation and function is presented in which *CLN3* regulates G_1 length in response to nutrients.

Keywords: cAMP/Cln3–Cdc28 kinase/regulation/ *Saccharomyces cerevisiae*

Introduction

Although yeast grow well on a variety of carbon sources, they grow fastest on glucose. For example, yeast growing logarithmically in glucose medium grow at least 3- to 4-fold faster than they do after the diauxic shift to ethanol. In order for the cells to maintain the proper size at these different growth rates, they must adjust their rate of progress through the cell division cycle to match the rate at which they accumulate size. Cells growing rapidly have short cell-cycle times, while slowly growing cells have longer cell cycles. These changes in cell-cycle length can be accounted for as changes in the length of time spent in G_1 (Jagadish and Carter, 1977; Johnston *et al*., 1977).

Several proteins that regulate the timing of Start have been identified (Tyers *et al*., 1993; Epstein and Cross, 1994; Di Como *et al*., 1995; Stuart and Wittenberg, 1995). Among these, the Cln3–Cdc28 kinase complex is thought to play an important role in regulating transcriptional activation of *CLN1* and *CLN2* at the end of G_1. In turn, the Cln1–Cdc28 and Cln2–Cdc28 kinase complexes are thought to trigger downstream events that allow cells to pass through Start. Recent results indicate that translation of Cln3 is decreased in poor nutrients. This regulation is thought to involve a leaky scanning mechanism that produces inefficient translation under conditions in which translational pre-initiation complexes are low in abundance (Polymenis and Schmidt, 1997).

It has long been thought that the components of the Ras–cAMP pathway play a role in connecting progress through the cell cycle with nutrient signals. Genes encoding two components of this pathway, *CDC25* and *CYR1* [allelic to *CDC35* (Boutelet *et al*., 1985)], originally were isolated as cell division cycle mutations producing a terminal phenotype that closely resembles cells arrested in G_1 due to lack of nutrients (Hartwell, 1974; Hartwell *et al*., 1974). Cdc25p is a Ras guanine nucleotide exchange factor (GEF) (Jones *et al*., 1991), while Cyr1p catalyzes the production of cAMP in response to activated Ras (Matsumoto *et al*., 1984; Toda *et al*., 1985). Glucose activates the Ras–cAMP pathway to cause increased cAMP production. Inactivation of the Ras–cAMP pathway leads to a G_1 cell-cycle arrest that is indistinguishable from the G_1 arrest seen in cells in nutrient-depleted medium (Matsumoto *et al*., 1985). The Ras–cAMP pathway therefore carries nutrient signals and plays a role in promoting passage through Start.

To understand better the processes by which glucose shortens G_1 length, we have measured the effects of different carbon sources on cell-cycle regulatory components. We find that Cln3–Cdc28 kinase activity is regulated by carbon source, being highest in glucose, and low in cells growing on non-fermentable carbon sources such as ethanol. *CLN3* mRNA levels are regulated by carbon source. In addition, Cln3 protein levels are regulated positively post-transcriptionally by the Ras–cAMP pathway. Cln3 appears to be an important target for the Ras–cAMP pathway, in that overexpression of *CLN3* is sufficient to bypass the essential requirement for cAMP. These results support a model in which rich nutrients increase expression of the Cln3–Cdc28 kinase complex to promote rapid passage through G_1.

Results

Carbon source regulation of CLN3

When cells shift from rapid growth on glucose to the slower, oxidative phase of growth on ethanol, they substantially increase the length of time that they spend in G_1. Consistent with this, we find that as cells reach the diauxic shift the population as a whole decreases the expression of mRNAs for *PCL1*, *PCL2*, *CLN1* and *CLN2*, encoding four cyclins that are expressed at Start (not shown).

Since the Cln3–Cdc28 kinase regulates the timing of

CLN1 and *CLN2* expression at Start, we tested the idea that cells might adjust Cln3–Cdc28 kinase activity in response to different growth conditions. We found that cells in log phase have substantially more Cln3 protein than cells in the slower post-log growth phase. An epitope-tagged *CLN3* was expressed from the normal *CLN3* promoter in a CEN plasmid (Levine *et al.*, 1996) to replace the normal Cln3 protein in a *cln3*Δ background strain. Extracts were prepared for immunoprecipitation from cells growing in log phase (L1), post-log phase (PL), or in post-log cells restored to log phase growth (L2) in fresh rich medium (YEPD). Immunoprecipitation from log phase extracts showed a relatively high Cln3 protein signal by Western blotting but, in post-log phase, the levels of Cln3 protein and the activity of histone H1 kinase were substantially reduced (Figure 1A). Cln3 protein levels were restored when fresh medium was added to these cells.

We also measured Cln3 protein levels and Cln3–Cdc28 kinase activity in extracts from cells cultured in different carbon sources (Figure 1B). Cells growing in glucose had considerably higher Cln3 protein and Cln3–Cdc28 kinase activity levels than cells growing in the non-fermentable carbon sources. These results show that the decrease in Cln3 protein and kinase activity observed when cells pass the diauxic shift can be accounted for by the change in carbon source from glucose to ethanol. Changes in *CLN3* mRNA levels appear to account for much of the carbon source regulation of Cln3 protein levels (Figure 1B); however, some of the decreases observed appeared to be greater than would be expected on the basis of changing message levels alone (Figure 1C).

The role of cAMP in G_1 progression

In addition to affecting the availability of *CLN3* mRNA, glucose is known to regulate the Ras–cAMP pathway, which in turn regulates progress through G_1 (Ishikawa *et al.*, 1986). We therefore wanted to explore the role that cAMP plays in regulating G_1. For these experiments, we used a strain carrying a deletion of *CYR1*, the gene encoding adenylate cyclase (Matsumoto *et al.*, 1982). This strain is dependent on cAMP in the medium. When cAMP was withdrawn from these cells, they entered a G_1-arrested state, and decreased the expression of the G_1 cyclin genes *CLN1*, *CLN2*, *PCL1* and *PCL2* (Figure 2A). In contrast, the levels of *CLN3* mRNA were only slightly decreased by withdrawal of cAMP. Readdition of cAMP reversed this process, producing a peak of *CLN1* and *CLN2* mRNA (Figure 2B), and a subsequent appearance of budded cells as the population passed through Start (not shown, but see Figure 3).

Induction of *CLN1* and *CLN2* mRNAs by cAMP was strongly inhibited by deletion of *CLN3* (Figure 3). In this experiment, cells were arrested in G_1 by cAMP withdrawal, and then allowed to progress through the cell cycle by readdition of cAMP. Readdition of 1 mM cAMP to the control *cyr1*Δ *CLN3* strain produced a rapid increase in *CLN1* and *CLN2* mRNAs, and the appearance of budded cells after ~45 min. In contrast, the response to cAMP in the strain carrying a *cln3* deletion was prolonged over a period of >2 h.

To determine the nature of the connection between cAMP and *CLN3*, we examined the effects of cAMP on Cln3 protein and activity levels. Samples of cells

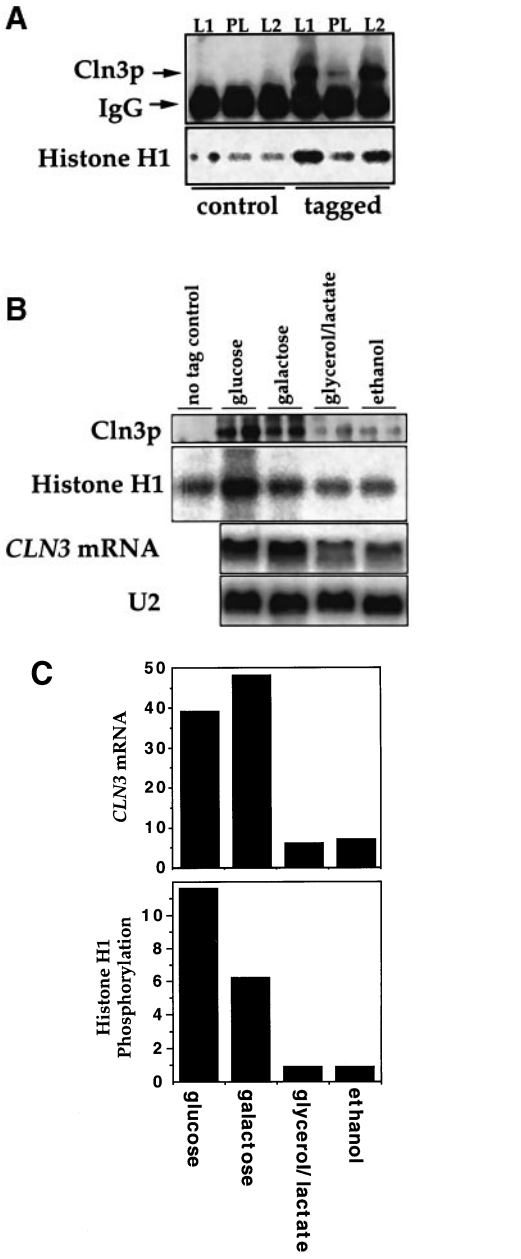

Fig. 1. Cln3 protein, Cln3–Cdc28 kinase activity and *CLN3* mRNA levels in cells growing on different carbon sources. (**A**) Cells (TDM251) carrying an epitope-tagged *CLN3* gene on a CEN plasmid were grown in YEPD and samples were collected from cells in log phase (L1) growth (1 OD_{660}), cells in post-log phase growth (PL) after the diauxic shift (6 OD_{660}) and cells that had returned to log phase (L2) growth after resuspension at a density of 1 OD_{660} for 1 h in fresh YEPD. Extracts were prepared for immunoprecipitation with the 12CA5 monoclonal antibody, and the immunoprecipitated Cln3 was then used for either Western blotting to measure Cln3 protein, or in histone H1 kinase assays to measure the activity of the immunoprecipitated Cln3–Cdc28 kinase complex. Control cells (DS10) carried an untagged wild-type copy of *CLN3*. (**B**) Cells (TDM251) were grown to mid-log phase in YEP with either glucose, galactose, glycerol/lactate or ethanol (each at 2%) as the carbon source, and samples were collected for extract preparation and immunoprecipitation of the tagged Cln3p, and RNA preparation for Northern blots. Control cells (DS10) carried an untagged wild-type copy of *CLN3*. Samples were loaded in duplicate for the immunoprecipitation–Western blot. (**C**) Numerical data from the phosphoimager scan shown for histone phosphorylation and the Northern blot. Baseline phosphorylation of histone H1 in control samples has been subtracted. The *CLN3* mRNA signal was normalized for loading using the U2 signal. The scales are arbitrary.

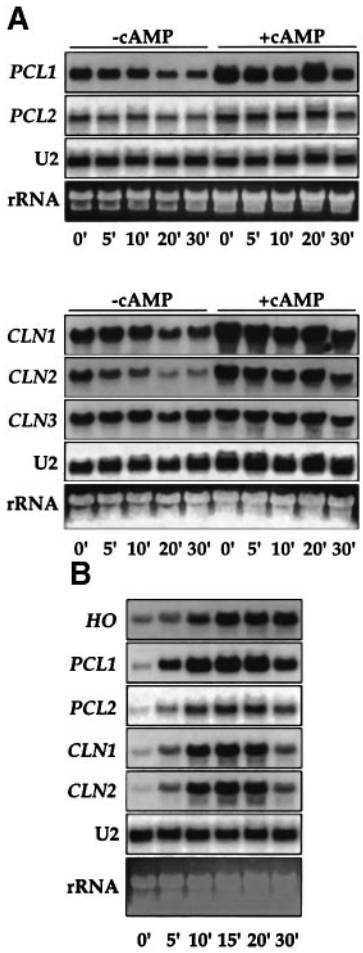

Fig. 2. cAMP promotes transcription of Start-specific genes. (**A**) A *cyr1*Δ strain (TC41) growing in mid-log phase in YEPD/1 mM cAMP was centrifuged and resuspended at a density of 1 OD_{660} in YEPD with no cAMP. Samples were collected for Northern blotting at the indicated times after cAMP removal (–cAMP). Controls (+cAMP) remained in the YEPD/1 mM cAMP medium. (**B**) Cells (TC41) were arrested by cAMP withdrawal as above for 4 h, cAMP was added back to 1 mM, and samples were collected for Northern blotting at the indicated times after cAMP addition.

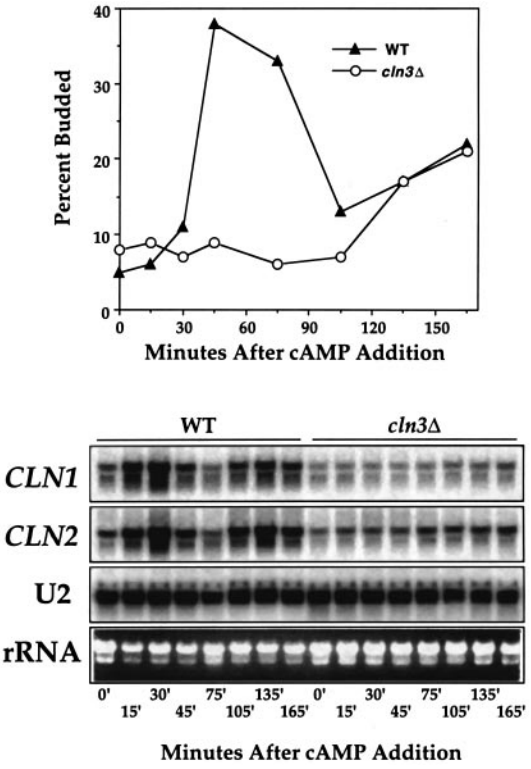

Fig. 3. Deletion of CLN3 inhibits the induction of Start-specific transcripts by cAMP. A *cyr1*Δ *CLN3* strain (TH5) and a *cyr1*Δ *cln3*Δ strain (TH5ΔCLN3) were transferred to and incubated in cAMP-free medium as in Figure 2B. At time 0, cAMP (1 mM) was added and samples were collected for measuring budding index (top) or Northern blotting (bottom) at the indicated times. The *cyr1*Δ strain carrying a wild-type allele of *CLN3* is indicated as WT.

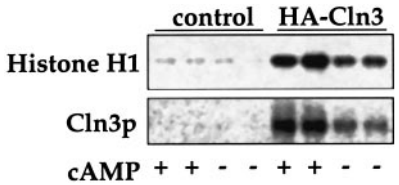

Fig. 4. cAMP regulates Cln3 protein and Cln3–Cdc28 activity levels. A *cyr1*Δ *cln3*Δ strain (TH122) carrying a plasmid expressing an epitope-tagged Cln3 protein from the normal *CLN3* promoter was grown in selective medium with 1 mM cAMP to mid-log phase. A sample was collected, the remaining cells were transferred to the same medium without cAMP and the cells were harvested after 1 h. Extracts were prepared from duplicate samples for immunoprecipitation of the tagged Cln3 protein with the monoclonal antibody 12CA5, and the immunoprecipitates were then used for kinase assays using histone H1 as a substrate (top), or Western blotting with the 12CA5 antibody (bottom). Control cells (TH5) carried an untagged *CLN3*.

carrying epitope-tagged *CLN3* in medium either with or without 1 mM cAMP were collected, and cell extracts were prepared for immunoprecipitation of the Cln3 protein (Figure 4). We found that Cln3–Cdc28 kinase activity was higher in extracts from cells cultured in the presence of cAMP than in those cultured in its absence. Western blotting of the immunoprecipitated hemagglutinin (HA)-tagged Cln3p showed that Cln3 protein levels were substantially higher in cells growing in cAMP, compared with cells without cAMP. Thus, while having little effect on *CLN3* mRNA levels (Figures 2 and 5), cAMP produced a strong effect on Cln3 protein and activity levels.

Mechanism of Cln3p regulation by cAMP

A region in the C-terminus of the Cln3 protein sequence has been shown to confer instability to the protein, and is important in Cln3 protein destruction by the Cdc34 ubiquitination pathway (Tyers *et al.*, 1992; Yaglom *et al.*, 1995). The C-terminus also contains a contiguous set of three potential sites for phosphorylation by the cAMP-dependent protein kinase A (PKA) in the sequence KKRSTS (559–564, potential phosphorylated residues are underlined) (Glass and Krebs, 1980; Denis *et al.*, 1991). Therefore, one possible model is that PKA could regulate the stability of Cln3p by direct phosphorylation at one or more of these serine and threonine residues. We used site-directed mutagenesis to convert these residues to alanines, thereby blocking the potential for PKA phosphorylation. We then expressed these proteins from the normal *CLN3* promoter on either a CEN or 2μ plasmid in a *cyr1*Δ strain carrying a deletion of the chromosomal copy of *CLN3*. Loss of the PKA sites in the mutant Cln3p did not alter

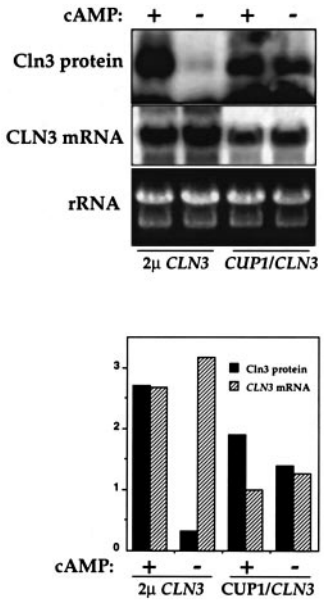

Fig. 5. Regulation of Cln3 by cAMP requires untranslated flanking regions. Strain TH122 carrying an epitope-tagged *CLN3* gene with normal flanking sequences on a 2μ plasmid (2μ CLN3) and TH100 carrying a 2μ plasmid with the *CUP1* promoter and *CYC1* terminator sequences in place of the normal flanking sequences (*CUP1–CLN3*) were grown overnight to mid-log phase in selective medium containing 1 mM cAMP. Cells were spun and resuspended to 0.4 OD_{660} in fresh medium either with or without cAMP, as indicated, and grown for 1 h. Samples were collected for immunoprecipitation–Western blotting of the tagged Cln3, and for RNA preparation and Northern blotting. Numerical data from the densitometer scan of the Western blot and the phosphoimager scan from the Northern blot are shown in the lower panel.

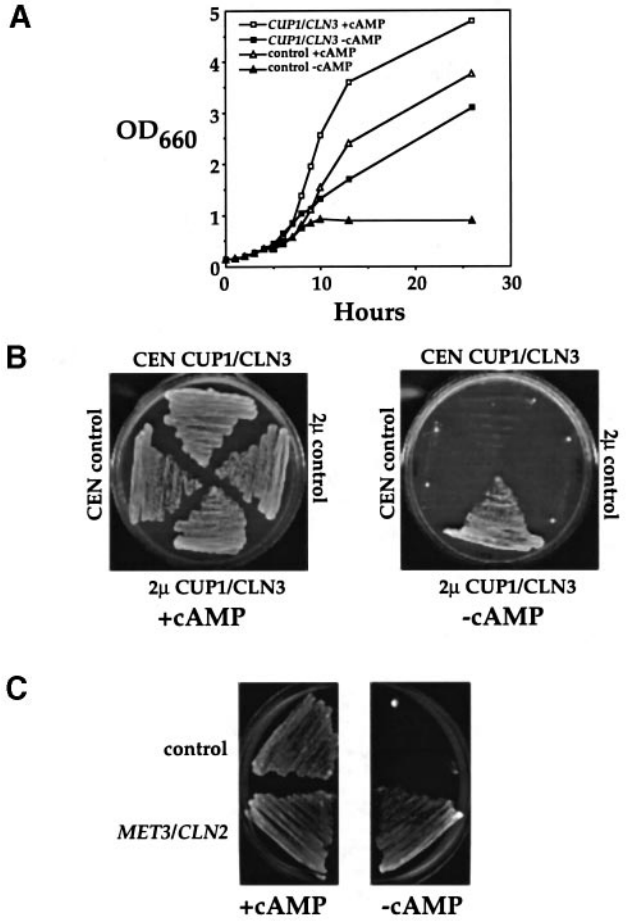

Fig. 6. Overexpression of *CLN3* bypasses the requirement for cAMP. (**A**) Cells carrying a deletion in *CYR1* (TH5) were transformed with either a 2μ plasmid (pCU1) expressing *CLN3* from the *CUP1* promoter, or the control plasmid with no *CLN3* insert. Cells were grown in selective medium with 1 mM cAMP, inoculated into YEPD/1 mM cAMP and grown for 5 h, when samples were removed and transferred to YEPD with no cAMP. Optical density was monitored at 660 nm for cells carrying the control plasmid with cAMP (△); control plasmid without cAMP (▲); *CUP1–CLN3* plasmid with cAMP (□); and *CUP1–CLN3* plasmid without cAMP (■). (**B**) The strains described above, along with strains carrying the same *CUP1–CLN3* construct in a CEN plasmid (pCU2) and the control CEN plasmid, were streaked onto either YEPD/1 mM cAMP or YEPD without cAMP. The plate with cAMP was incubated for 2 days at 30°C, the plate without cAMP was incubated for 3 days. (**C**) TH5 cells with either a plasmid carrying *MET3–CLN2* (pMET3–CLN2) (Espinoza *et al.*, 1994) or a control plasmid were streaked onto methionine dropout plates either with or without cAMP, as indicated, and incubated as in (B).

the regulation of Cln3 protein levels by cAMP, nor was the pattern of downstream induction of *CLN1* and *CLN2* altered (not shown). We conclude that phosphorylation of these residues by PKA is not important for regulating Cln3 protein levels.

We found that untranslated regions of the *CLN3* message play an important role in cAMP regulation of Cln3 protein levels. While levels of Cln3 protein translated from a normal mRNA were regulated by cAMP, this regulation was abolished when the *CLN3* coding region was moved into a message with heterologous 5′- and 3′-untranslated regions (Figure 5). In this experiment, the epitope-tagged *CLN3* coding region was transferred to the vector YpJ66 (a gift from James Hendricks) so that the *CUP1* promoter was upstream of the first codon, and the transcriptional terminator sequence from *CYC1* was downstream of the *CLN3* stop codon. This 2μ plasmid was then transformed into yeast carrying a *CLN3* deletion, and Cln3 protein levels in these cells were compared with Cln3 protein levels in cells transformed with a 2μ plasmid carrying *CLN3* with its normal flanking sequences. As shown in Figure 5, cAMP did not affect the transcript level from either plasmid. However, although the level of Cln3 protein made from the normal message was strongly influenced by cAMP, the level of Cln3 protein expressed from the *CUP1–CLN3* mRNA was unaffected by cAMP.

Ectopic G_1 cyclin expression bypasses the need for cAMP

The finding that cAMP regulates Cln3 protein levels, and that cells respond poorly to cAMP when *CLN3* is deleted, suggested that cAMP controls progress through Start largely by regulating the Cln3–Cdc28 protein kinase. If this is the case, artificially raising *CLN3* expression should bypass the requirement for cAMP. The *CUP1–CLN3* construct allowed us to test this prediction. We found that overexpression of *CLN3* was indeed able to bypass the essential requirement for cAMP, allowing *cyr1*Δ cells to grow after withdrawal of cAMP (Figure 6). In contrast, neither the same construct in a CEN-based, single-copy plasmid, nor control plasmids without inserts were able to bypass the need for cAMP. Overexpression of *CLN3* did not restore the normal growth rate completely: in the absence of cAMP, the TH11 strain carrying the 2μ *CUP1–*

CLN3 plasmid did not grow as rapidly as wild-type cells, nor as rapidly as *cyr1*Δ cells do in the presence of cAMP.

Spontaneous mutations such as loss-of-function mutations in *BCY1* can also bypass the need for cAMP (Matsumoto *et al.*, 1983), as is evident from the isolated colonies emerging from the control sectors on the plate lacking cAMP in Figure 6B. To be sure that the bypass of the cAMP requirement is actually due to the *CUP1–CLN3* plasmid, we have conducted five separate transformation experiments comparing the *CUP1–CLN3* plasmid with the control plasmid. In each of these experiments, transformants carrying *CUP1–CLN3* were cAMP independent, while the cells with the control construct were not. In addition, allowing the cells to lose the plasmid in non-selective medium produces a return to cAMP dependence. We conclude that it is the overexpression of *CLN3* that allows this bypass, rather than a suppressor mutation in the *cyr1*Δ strain.

Since deletion of *CLN3* does not cause cell-cycle arrest, withdrawal of cAMP cannot arrest cells in G_1 solely by decreasing Cln3 protein levels. However, cAMP may regulate Cln3 together with one or more additional genes to control the expression of an essential set of downstream genes including *CLN1* and *CLN2*. If this is the case, ectopic expression of *CLN2* should also bypass a *cyr1*Δ mutation. To test this, we transformed the *cyr1*Δ strain with the plasmid pMET3–CLN2 (Espinoza *et al.*, 1994), expressing *CLN2* from the methionine-repressible *MET3* promoter. Overexpression of *CLN2* from this construct also allowed the *cyr1*Δ cells to grow in the absence of cAMP (Figure 6C).

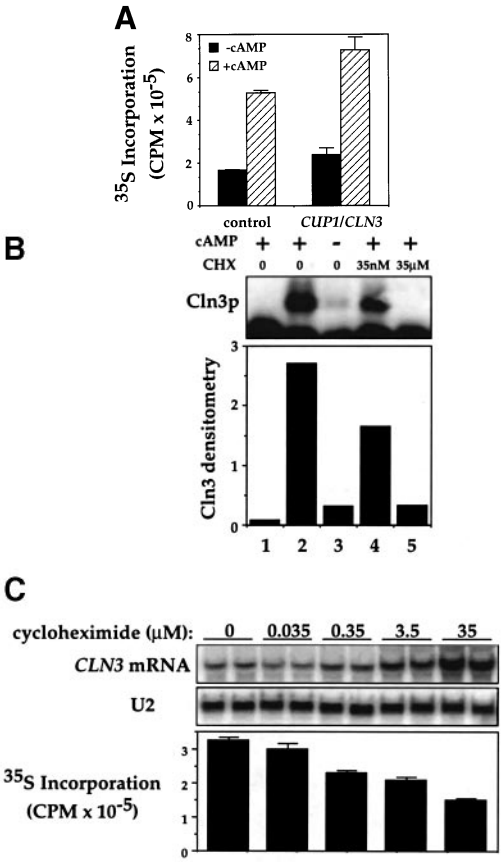

Fig. 7. cAMP, protein synthesis and Cln3 protein levels. (**A**) Cells carrying a deletion in *CYR1* (TH5) were transformed with a 2μ plasmid (pCU1) expressing *CLN3* from the *CUP1* promoter and were grown in selective medium with 1 mM cAMP. Cells were spun and resuspended at 0.3 OD_{660} in fresh medium either with or without cAMP, as indicated. After 4 h, samples were collected for [^{35}S]methionine incorporation measurements as described in Materials and methods. Error bars indicate standard deviations of triplicate samples. (**B**) Cells (TH122) carrying an epitope-tagged *CLN3* gene were grown overnight to mid-log phase in selective medium containing 1 mM cAMP. Cells were spun and resuspended to 0.4 OD_{660} in fresh medium either with (lanes 1, 2, 4 and 5) or without (lane 3) cAMP, and either no cycloheximide (lanes 1, 2 and 3), 35 nM (lane 4) or 35 μM cycloheximide (lane 5). Cells were grown for 1 h and 100 ml samples were collected for extract preparation and immunoprecipitation of the tagged Cln3p. Control cells (TH5, lane 1) carried an untagged wild-type copy of *CLN3*. The immunoprecipitated Cln3p was then used for Western blotting to measure Cln3 protein levels. Numerical data from a densitometer scan of the Western blot are shown in the bar graph. (**C**) Samples from cells treated with cycloheximide as described above were also collected for Northern blotting as indicated, and [^{35}S]methionine incorporation measurements.

Effects of cAMP and CLN3 on protein synthesis

cAMP has been shown to increase the transcription of genes involved in protein translation through a mechanism involving an increase in the levels of the Rap1 protein, a transcription factor that is important in the expression of genes important for translation (Klein and Struhl, 1994; Neuman-Silberberg *et al.*, 1995). This is consistent with the results of Iida and Yaharo (1984) and Boutelet *et al.* (1985) showing that *cdc35* mutations produce a decrease in protein synthesis at the restrictive temperature. It has been proposed that levels of the Cln3 protein should be especially sensitive to decreases in protein synthesis (Tyers *et al.*, 1992; Tokiwa *et al.*, 1994; Polymenis and Schmidt, 1997). Therefore, cAMP could regulate Cln3 protein levels through effects on protein synthetic rate. We confirmed that cAMP does indeed affect the rate of protein synthesis. [^{35}S]methionine incorporation in cells in medium with cAMP was ~3-fold higher than in cells without cAMP (Figure 7A). Protein synthesis in the cells carrying the *CUP1–CLN3* plasmid was slightly higher, perhaps due to the increased rate of growth seen in this strain. Nevertheless, the rate of label incorporation remained sensitive to cAMP, falling to levels similar to those seen in the controls when cAMP was withdrawn. This indicates that overexpression of *CLN3* does not bypass the decreased protein synthesis produced by cAMP withdrawal.

Addition of 35 μM cycloheximide produced a decrease in [^{35}S]methionine incorporation of ~50%, and a halt in cell division. This concentration of cycloheximide also caused a substantial fall in Cln3 protein levels relative to the levels of other proteins in the cell extract (Figure 7B). This was despite the fact that *CLN3* mRNA increased in cells treated with cycloheximide (Figure 7C). Cycloheximide has been shown to increase the levels of a wide variety of messages by increasing mRNA half-life (Herrick *et al.*, 1990). We find that cycloheximide also increases the stability of *CLN3* mRNA (not shown). Cln3 protein levels are clearly sensitive to decreased protein synthesis, suggesting that the low Cln3 protein levels observed in the absence of cAMP may be a response to the decrease in protein synthesis produced by cAMP withdrawal.

Discussion

Ras–cAMP and Cln3

A cell in glucose medium grows ~3- to 4-fold faster than a cell in ethanol (Jagadish and Carter, 1977; Lorincz and Carter, 1979), requiring a corresponding increase in the rate of passage through G_1 in order to maintain constant size (Jagadish and Carter, 1977). The Ras–cAMP pathway serves as one connection between glucose and passage from G_1 into S phase. The *CDC25* and *CDC35* genes have long been known to be required for Start (Hartwell, 1974; Hartwell *et al.*, 1974). As the products of these genes were identified, it became clear that they are involved in the glucose-regulated synthesis of cAMP (Boutelet *et al.*, 1985; Camonis *et al.*, 1986; Broek *et al.*, 1987; Daniel *et al.*, 1987; Robinson *et al.*, 1987), and that cAMP is needed in order for cells to pass Start (Matsumoto *et al.*, 1982). Our experiments connect the Ras–cAMP pathway to regulation of the Cln3–Cdc28 kinase and transcription of Start-specific genes. We find that cAMP is necessary for the expression of *CLN1* and *CLN2*, that this response is inhibited by deletion of *CLN3*, that cAMP regulates Cln3 protein levels, and that overexpression of *CLN3* is sufficient to bypass the essential requirement for cAMP.

We initially had thought that Cln3 might be a direct target for phosphorylation by PKA. However, our experiments substituting alanines at the potential PKA phosphorylation sites on Cln3 rule this model out. On the other hand, replacement of the untranslated portions of the *CLN3* mRNA with heterologous flanking regions in our *CUP1–CLN3* construct blocks the down-regulation of Cln3 protein when cAMP is withdrawn. Since *CLN3* mRNA levels are unaffected by cAMP, and the coding sequence for the Cln3 protein was not altered in this experiment, this suggests that cAMP regulates translation of Cln3 through a mechanism that involves elements within the flanking regions. It is interesting that while the *CUP1–CLN3* construct can bypass the need for cAMP, overexpression of *CLN3* from a 2μ plasmid (pKL034) carrying the normal untranslated *CLN3* sequences, while producing more *CLN3* mRNA than the 2μ *CUP1–CLN3* plasmid, fails to bypass the cAMP requirement (D.D.Hall, unpublished), presumably because Cln3 protein expressed from normal mRNA can still be downregulated when cAMP is withdrawn.

Our results are in apparent contradiction to previous work showing that a sudden upshift from a poor carbon source to glucose, which is known to create an increase in intracellular cAMP, produces a transient decrease in *CLN1* and *CLN2* (Baroni *et al.*, 1994; Tokiwa *et al.*, 1994). The basis for this apparent paradox may lie in the fact that we are investigating a different set of responses than the previous investigators: our work has been focused on understanding how cells shorten G_1 in glucose medium, and why loss of the Ras–cAMP pathway leads to G_1 arrest, while the previous workers were examining the pause in the cell cycle produced by a glucose upshift. Both of these processes are physiologically relevant. How then can cAMP both increase and decrease *CLN1* and *CLN2* expression? We suggest that the decrease seen in glucose upshift experiments is in some way related to the stress involved in a sudden change in carbon source.

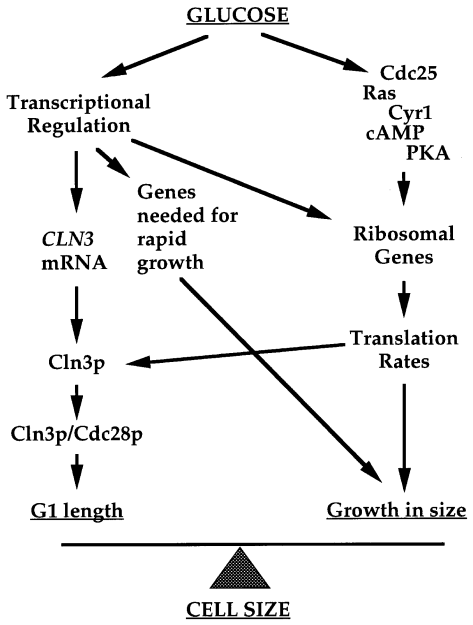

Fig. 8. Model: systems that produce rapid growth in response to glucose are also coupled via *CLN3* to rapid progression through the cell cycle.

Rowley *et al.* (1993) found that stress produces a transient fall in *CLN1* and *CLN2* mRNA. Transient stress would be expected in a cell that must change metabolism from a carbon source that is suddenly no longer present, to a new carbon source to which the cell has not yet adapted. It is also known that activation of the Ras–cAMP pathway causes cells to become extremely sensitive to stress. Cells carrying *bcy1*, *ira1*, *ira2* or *RAS2*^{val19} mutations die in response to temperature stress, nitrogen starvation and withdrawal from fermentable carbon sources (Toda *et al.*, 1985, 1987; Tanaka *et al.*, 1989, 1990).

Transcriptional regulation of CLN3

Glucose also produces a significant increase in the availability of *CLN3* messages for translation. Artificial manipulations of *CLN3* mRNA levels repeatedly have been found to produce changes in both Cln3–Cdc28 kinase activity and G_1 length (Cross, 1988; Nash *et al.*, 1988; Tyers *et al.*, 1993; Dirick *et al.*, 1995; Stuart and Wittenberg, 1995). We show that transcriptional regulation of *CLN3* occurs under normal physiological conditions, and correlates with changes in Cln3 protein levels, Cln3–Cdc28 kinase activity and G_1 length. However, since the changes in Cln3 protein levels are somewhat larger than the changes in *CLN3* mRNA, we propose that glucose controls Cln3 protein levels through both transcriptional regulation and the Ras–cAMP pathway. Two pleiotropic responses to glucose are an increase in mRNA levels and an increase in protein synthesis. Both of these responses appear to be connected to Cln3 protein levels (Figure 8).

Protein synthesis and CLN3

A concentration of cycloheximide that is sufficient to decrease protein synthesis by 50% produces a much larger decrease in Cln3 protein levels. This indicates, as predicted by Polymenis and Schmidt (1997), that compared with other cellular proteins Cln3 stands out as being especially

Table I. *Saccharomyces cerevisiae* strains

Strain	Description
HR125[a]	*MAT* **a** *leu2-3,112 ura3-52 trp1-1 his3-532 his4*
TC41[a]	*MAT* **a** *leu2-3,112 ura3-52 trp1-1 his3-532 his4 cyr1::URA3 cam*
TH5[b]	*MAT* **a** *leu2-3,112 ura3-52 trp1-1 his3-532 his4 cyr1::LEU2 cam*
TH9[b]	TC41 carrying pCU2 (CEN *CUP1–CLN3 LEU2*)
TH10[b]	TC41 carrying YCplac118 (CEN *LEU2*)
TH11[b]	TH5 carrying pCU1 (2μ *CUP1–CLN3 URA3*)
TH12[b]	TH5 carrying pRD10 (2μ *URA3*)
TH6[b]	TH5 carrying pMET3-CLN2
TH5ΔCLN3[b]	*MAT* **a** *leu2-3,112 ura3-52 trp1-1 his3-532 his4 cyr1::LEU2 camΔ cln3Δ::URA3*
TH124[b]	TH5ΔCLN3 carrying pKL001 (CEN epitope-tagged *CLN3, TRP1*)
TH125[b]	TH5ΔCLN3 carrying pDH51 (CEN epitope-tagged *CLN3pka–, TRP1*)
TH122[b]	TH5ΔCLN3 carrying pKL034 (2μ epitope-tagged *CLN3, TRP1*)
TH123[b]	TH5ΔCLN3 carrying pDH52 (2μ epitope-tagged *CLN3pka–, TRP1*)
TH100[b]	TH5ΔCLN3 carrying pCU1-HA (2μ epitope-tagged *CUP1–CLN3 URA3*)
DS10[c]	*MAT* **a** *his3-11,15 leu2-3,112 lys1 lys2 ura3-52 trp1Δ*
TG3[b]	*MAT* **a** *his3-11,15 leu2-3,112 lys1 lys2 ura3-52 trp1Δ cln3Δ::URA3*
TDM251[b]	TG3 carrying pKL001 (CEN epitope-tagged *CLN3, TRP1*)

[a]Heideman *et al.* (1990); [b]this study; [c]gift from E.A.Craig.

sensitive to changes in protein synthetic rate. The level of Cln3 protein could, therefore, serve as a trip wire to halt the cell division cycle under conditions of low protein synthesis. Withdrawal of cAMP also produces a decrease in total protein synthesis (Iida and Yahara, 1984; Boutelet *et al.*, 1985), and decreased levels of Cln3 protein. Ectopic expression of *CLN3*, while not bypassing the fall in protein synthesis produced by cAMP withdrawal, is sufficient to allow a bypass of the cell-cycle arrest. As expected for cells with decreased protein synthesis, the *CUP1–CLN3* cells grew more slowly in the absence than in the presence of cAMP. Nevertheless, the decrease in protein synthesis did not cause a halt in growth if G_1 cyclins were provided. This suggests a model in which loss of cAMP produces a fall in protein synthesis that triggers a decrease in Cln3 protein levels and cell-cycle arrest. Since *CLN3* is not essential for passing Start, presumably other redundant components must also be affected as well. This then prevents the expression of a downstream set of genes, including *CLN1* and *CLN2*, that are essential for G_1–S phase progression. In support of this model, we find that ectopic expression of *CLN2* is also able to bypass the G_1 arrest produced by cAMP withdrawal. This result is consistent with the previous findings of Hadwiger *et al.* (1989) that expression of a hyperstable Cln2 protein is able to bypass the G_1 arrest produced by nutrient limitation. The G_1-specific arrest of cells with defects in the Ras–cAMP pathway indicates that cells without cAMP can progress through all phases of the cell cycle except Start. Having removed the G_1 block by ectopic G_1 cyclin expression, the cells are able to proceed through the cell cycle, although the decrease in protein synthesis appears to slow their growth.

Regulation of Cln3 protein levels by protein synthesis rates may play a role in the lengthening of G_1 in small daughter cells. The rate of cellular growth steadily increases as yeast cells become larger (Woldringh *et al.*, 1993). This suggests an increase in the rate of protein synthesis with increasing cell size. Thus, Cln3 protein levels may be higher in larger cells, allowing them to traverse G_1 more rapidly than smaller cells.

Role of CLN3

The finding that overexpression of *CLN3* makes cells smaller originally was taken to indicate that *CLN3* serves to modulate the critical size for bud emergence. Measurements of Cln3 protein and activity correlate poorly with a role in setting critical size: if Cln3 serves to decrease the critical size for bud emergence, why are Cln3 levels highest in the large cells produced by growth in glucose, and lowest in the smaller cells formed in poor medium? The dramatic changes in Cln3 levels in different media are also difficult to reconcile with a model in which Cln3–Cdc28 kinase activity triggers Start at some critical threshold level. We propose instead that the function of the Cln3–Cdc28 kinase is to shorten the length of G_1. In this model, rapidly growing cells in glucose express high levels of Cln3 in order to shorten the cell cycle, while slowly growing cells lengthen G_1 by downregulating Cln3.

The fact that cells in glucose are larger than cells growing in poorer medium can be interpreted in different ways, depending on one's point of reference. In terms of size at budding, the larger size at bud emergence indicates that glucose delays Start until the cell reaches a larger size. On the other hand, in terms of time, G_1 is demonstrably shorter in glucose, but because yeast grow in size so much faster in glucose the cells bud at a larger size. A relatively unexplored model that would explain the larger cells in glucose is that rich medium increases the rate of growth in size slightly more than it accelerates progress through the cell cycle. In this model, cells are larger in glucose because they grow faster, rather than because Start is delayed.

CLN3 could regulate G_1 length by modulating the rate of a process that begins at the end of mitosis, and culminates in transcription of SCB genes to trigger Start. Such a model brings up interesting similarities between Cln3 and mammalian D-type cyclins (Sherr, 1996). Both D-type cyclins and Cln3 have similarities in expression, being less affected by cell-cycle position than other cyclins, and both types of cyclin appear to function in G_1. The D cyclins serve to link signals from the cellular environment to the process of exiting G_1. Like the D cyclins, Cln3

protein levels are regulated by a variety of mechanisms in response to environmental signals, including regulation of transcription by both nitrogen and carbon source (Parviz and Heideman, 1998), and post-transcriptional regulation by rich medium (Polymenis and Schmidt, 1997), the Ras–cAMP pathway, protein synthesis rates and nitrogen limitation (Gallego *et al.*, 1997). The D cyclins allow cells to move out of G_1 through a mechanism that involves the phosphorylation of the retinoblastoma protein Rb. This allows the release and activation of the E2F transcription factor that in turn produces, among other things, transcription of downstream G_1 cyclins. It is notable that Rb is reset to the dephosphorylated form at the end of M phase. The Cln3–Cdc28 kinase could serve a similar function to move cells through G_1, the rate of this process determining G_1 length. Whether such a system exists in yeast remains to be seen; however, it is worth noting that an E2F-like activity (termed SCELA) has been identified in yeast (Vemu and Reichel, 1995). As is the case with E2F, SCELA binds to elements that regulate the expression of the downstream cyclins, in this case *CLN1* and *CLN2*. SCELA binds to SCB elements but does not appear to be the Swi4–Swi6 complex. Transcription from SCB elements is known to be positively regulated by Cln–Cdc28 kinase complexes (Koch *et al.*, 1996), and SCELA activity peaks at the G_1–S boundary.

Materials and methods

Yeast strains and media

Yeast strains are listed in Table I. Cells were grown in either YEPD containing 1% yeast extract, 2% bactopeptone and 2% glucose, or in synthetic complete medium (SC) containing 6.7 g/l yeast nitrogen base (Difco) supplemented with adenine, uracil, amino acids and 2% glucose (Sherman, 1991). Budding indexes are the average of triplicate determinations of 100 cells each.

Incorporation of [^{35}S]methionine

Determination of the rate of [^{35}S]methionine incorporation into protein was as previously described (Boucherie, 1985). Triplicate 250 µl samples containing equal numbers of cells for each treatment were labeled in microfuge tubes at 30°C, with 5 µCi of Trans^{35}S-Label (ICN) for 2 min and stopped with 250 µl of ice-cold 10% trichloroacetic acid (TCA). Incorporation was determined to be linear through the 2 min assay.

Preparation of RNA and RNA blots

RNA was prepared using the method described by Ellwood and Craig (1984). Following blotting, ethidium-stained rRNA was visualized on the blots by UV illumination and photographed. The blots were probed with a 1 kb *Sac*I–*Eco*RI fragment from *CLN1*, a 1 kb *Sac*I–*Xho*I fragment from *CLN2*, a 1 kb *Eco*RI fragment of *CLN3*, a 0.6 kb *Pst*I fragment of *PCL1*, a 1.2 kb *Bam*HI fragment from *PCL2* and a 0.6 kb *Hin*dIII fragment from *HO*. A 0.6 kb *Sac*I fragment was used to probe for U2 RNA as a loading and transfer control. Probes were radiolabeled with [α-^{32}P]dCTP by the random primer method to a specific activity of 10^9 c.p.m./mg.

Immunoprecipitations, Western blots and kinase assays

Immunoprecipitation–Western blots and immunoprecipitation–kinase assays using epitope-tagged Cln3 protein were as described by Tyers *et al.* (1993). For each immunoprecipitation assay point, 5 mg of extract protein was used. The 12CA5 monoclonal antibody was used as the primary antibody, and an ECL detection kit was used to develop the Western blots (Amersham).

Plasmid constructs

The constructs expressing triple epitope-tagged *CLN3*, driven by a normal *CLN3* promoter on a CEN plasmid (pKL001) and on a 2µ plasmid (pKL034), were a gift from Fred Cross (Levine *et al.*, 1996). We subcloned an *Eco*RI–*Sac*II fragment from pKL001, encoding the HA-tagged C-terminus of *CLN3*, into the vector pBSKS+ to generate pDH50 for site-directed mutagenesis (Sambrook *et al.*, 1989). To mutagenize the potential PKA phosphorylation sites in the Cln3 protein, an oligonucleotide was designed (Operon Technologies, Inc.; 5′-CAATCCACAGAGGCAGCTGCTCTCTTTTTCAGTTG-3′) which mutates the codons for the consensus cAPK sites from KKRSTS to KKRAAA. *Eco*RI–*Sac*II fragments from plasmids containing mutagenized sequences were subcloned back into pKL001 and pKL034 and transformed into *cln3*Δ yeast as indicated. The mutated *CLN3* is designated as *CLN3pka*– in Table I.

The *CUP1–CLN3* 2µ plasmid, pCU1, was made by attaching *Eco*RI linkers to the 1.8 kb *Bam*HI fragment containing the *CLN3* coding region from pW16 (Cross, 1990). The 1.8 kb fragment was obtained by partial digestion with *Eco*RI and was then isolated and inserted in place of the *Eco*RI fragment containing the *galK* gene into the plasmid YpJ66 (a gift from James Hendricks) to place the *CLN3* coding region in the proper orientation between the *CUP1* promoter and the *CYC1* terminator sequences. YpJ66 was made by insertion of the *Cla*I–*Sma*I fragment containing the *CUP1–CYC1* sequences from YpYSK1215 (Butt *et al.*, 1984) into YEp352 (Hill *et al.*, 1986). The *CUP1–CLN3–CYC1* sequences were transferred to the CEN-based plasmid YCplac111 (Gietz and Sugino, 1988) as a *Sal*I–*Sac*I fragment to make pCU2.

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