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Connections between the Ras-Cyclic AMP Pathway and G_1 Cyclin Expression in the Budding Yeast *Saccharomyces cerevisiae*

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We have identified two processes in the G_1 phase of the *Saccharomyces cerevisiae* cell cycle that are required before nutritionally arrested cells are able to return to proliferative growth. The first process requires protein synthesis and is associated with increased expression of the G_1 cyclin gene *CLN3*. This process requires nutrients but is independent of Ras and cyclic AMP (cAMP). The second process requires cAMP. This second process is rapid, is independent of protein synthesis, and produces a rapid induction of START-specific transcripts, including *CLN1* and *CLN2*. The ability of a nutritionally arrested cell to respond to cAMP is dependent on completion of the first process, and this is delayed in cells carrying a *CLN3* deletion. Mating pheromone blocks the cAMP response but does not alter the process upstream of Ras-cAMP. These results suggest a model linking the Ras-cAMP pathway with regulation of G_1 cyclin expression.

A fundamental goal in biology is to understand how cells control proliferative growth. In recent years, progress toward this goal has been made in two important areas. The first area involves the identification and study of oncogenes—genes that in many cases encode proteins that carry signals regulating cellular proliferation. Mutations in these genes lead to aberrant signalling, unregulated proliferation, and cancer. The second area involves the discovery of two families of proteins, the cyclins and the cell cycle-dependent protein kinases (CDKs), that are believed to allow cells to pass checkpoints in the cell cycle (26, 28). Included among these cell cycle checkpoints is the G_1-to-S phase transition that is known as START in the yeast *Saccharomyces cerevisiae* or as the restriction point in mammals (16, 25).

In *S. cerevisiae*, three cyclin genes that affect passage through START have been identified: *CLN1*, *CLN2*, and *CLN3*. The protein products of these genes activate the only member of the CDK family known to exist in *S. cerevisiae*, encoded by *CDC28*. Activation of $p34^{CDC28}$ enables cells to pass the START checkpoint. Cells remain viable after loss of any two of the G_1 cyclin genes; however, loss of all three G_1 cyclin genes leaves the cells arrested permanently at START, an effect similar to that produced by loss of the *CDC28* kinase (29). In contrast, activating mutations in any of the G_1 cyclin genes, or the overexpression of any of these genes, results in small cells with an abbreviated or absent G_1 phase (8). These and other results suggest a pathway in which three redundant cyclins associate with and activate the $p34^{CDC28}$ kinase in order to carry cells through START.

Although the three mitotic cyclins appear to serve redundant functions, there are distinct differences between them. *CLN1*, *CLN2*, and *CLN3* all show sequence homologies with the mitotic cyclins (7, 22, 32); however, *CLN1* and *CLN2* show much greater similarity to each other than to *CLN3*. *CLN3* also stands apart in its expression pattern. While *CLN1* and *CLN2* expression peaks dramatically at the G_1/S boundary, the level of *CLN3* message remains relatively constant throughout the cell cycle (22, 32). In addition, *CLN1* and *CLN2* expression is inhibited by mating factor, while *CLN3* expression remains unaffected. These differences suggest that despite the fact that any one of these genes allows the cell to survive the loss of the other two, the G_1 cyclins probably play somewhat different roles in regulating the activity of $p34^{CDC28}$.

The transcription of *CLN1* and *CLN2* at START is itself enhanced by activated $p34^{CDC28}$ (9), suggesting a positive feedback loop in which activation of $p34^{CDC28}$ by Cln1 and Cln2 further increases *CLN1* and *CLN2* transcription (9, 23, 24). Such a positive feedback loop would allow some critical level of $p34^{CDC28}$ activity to trigger a rapid rise in G_1 cyclin levels, consequent snowballing of $p34^{CDC28}$ activity, and progression of the cell through START. *CLN3* expression is not regulated by this process, but as might be expected, *CLN3* (presumably by activation of $p34^{CDC28}$) enhances accumulation of *CLN1* and *CLN2* message (11, 23).

If the cyclins and CDKs act as switches to allow cells to pass cell cycle checkpoints, then signal transduction pathways that regulate cellular proliferation should in some way regulate the cyclins and CDKs. While much has been learned about signal transduction pathways that ultimately regulate the cell cycle, little is known about how signals from these pathways affect CDK activity. In *S. cerevisiae*, three signals are known to regulate passage through START. These signals regulate cell cycle arrest in late G_1 in response to cell size, nutrient availability, and mating factor. We have only limited information about how these regulatory signals affect the cyclin-$p34^{CDC28}$ pathway. For example, because mutations in *CLN3* affect cell size, *CLN3* is thought to play a role in the process that maintains cell size. Beyond this, we know very little about how cell size is translated into changes in the cyclin-$p34^{CDC28}$ pathway. Mating pheromone appears to block the cell cycle by preventing the accumulation of *CLN1* and *CLN2* message (12, 22, 32). This process involves the products of the *FAR1* and *FUS3* genes (5, 12) and posttranslational regulation of the activity of Cln2 (6, 31). Finally, nutrient availability also regulates the cell cycle of *S. cerevisiae* at START. Although it is known that the Ras-cAMP pathway is involved in this process and that $p34^{CDC28}$ kinase activity is low in nutrient-arrested cells (21),

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TABLE 1. Yeast strains used in this study

Strain	Description	Genotype	Reference
HR125	Wild type	*MATa leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4*	3
NW23-9C	*cyr1-1* mutant isogenic with HR125	*MATa leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 cyr1-1 CAM*	3
TC28-1-1	*RAS1* and *RAS2* deletions in NW23-9C	*MATa leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 ras1-545(URA3) ras2-530(LEU2) cyr1-1 cam*	3
TC41-1	*CYR1* deletion in NW32-9C	*MATa leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 cyr::URA3 CAM*	17
TL2-1	*CLN3* deletion in NW23-9C	*MATa leu2-3 leu2-112 ura3-52 trp1-1 his3-532 his4 cyr1-1 CAM cln3::URA3*	

we know very little about how these pathways might be linked.

In this study, we have explored connections between the Ras-cyclic AMP (cAMP) pathway and the G_1 cyclin and mating pheromone pathways. We have identified two processes in the G_1 phase of the cell cycle that limit passage through START. The first process is independent of *RAS* and cAMP and must be completed before cells can proliferate in response to cAMP. This process is slow, is nutrient sensitive, requires protein synthesis, and is associated with increased expression of *CLN3*. The second process is initiated by cAMP and is associated with a rapid increase in *CLN1* and *CLN2* message levels. This step is not blocked by cycloheximide. From these results, we have proposed a model that links the G_1 cyclin, Ras-cAMP, and pheromone pathways.

MATERIALS AND METHODS

Yeast strains and media. Yeast strains are listed in Table 1. All of the strains used were created by gene replacement in the parent strain HR125 and are isogenic to HR125 except as noted. The *CLN3* deletion in strain TL2-1 was made by using one-step gene disruption (30) to insert the *URA3* gene between the *Xho*I site at position 586 and the *Hin*dIII site at position 1710 of *CLN3*. Disruption of *CLN3* was confirmed by Southern blotting. Cells were grown on rich medium (YEPD) containing 1% yeast extract, 2% Bacto Peptone, and 2% glucose. YEP contained 1% yeast extract and 2% Bacto Peptone.

Preparation of nutrient-arrested cells. Cells were cultured with shaking at 30°C in YEPD supplemented with 1 mM cAMP. The cells were grown for 2 to 4 days without replenishing the nutrients or cAMP. After this time, the optical density at 660 nm (OD_{660}) of the cell cultures was approximately 5, and the cells were mostly unbudded (0 to 2% budded).

Cell budding in response to cAMP. Nutrient-arrested TC41-1 cells were pelleted by centrifugation at 1,800 × *g* for 10 min. The cells were then resuspended in YEPD or in their original nutrient-depleted medium and incubated at 30°C. At the indicated times, cAMP was added to a final concentration of 5 mM. Single aliquots of the cell suspensions were taken at 15-min intervals and fixed in 3.7% formaldehyde–0.1 M K_2PO_4. Cell budding was determined in triplicate by light microscopy (at least 300 cells counted per point). The low budding index values that we observed (<20%) is probably due to our counting only cells with newly emerged, small buds as budded cells. Log-phase TC41-1 cells showed a similarly low budding index.

Preparation of RNA and Northern (RNA) blotting. RNA was prepared by the method described by Ellwood and Craig (13). The RNA samples (15 μg per lane) were separated by formaldehyde agarose gel electrophoresis and transferred to a GeneScreen Plus membrane as instructed by the manufacturer (New England Nuclear). Uniform loading and transfer were confirmed by staining of rRNA prior to loading of the gel. Following blotting, ethidium-stained rRNA was visualized on the blots by UV illumination and photographed, and the negatives of the photographs were scanned with a Molecular Dynamics computing densitometer. rRNA loading and transfer varied by less than 5% between lanes as measured by this method. The blots were probed with the 2.5-kb *Hin*dIII fragment from *HO*, a 1-kb *Sac*I-*Xho*I fragment from *CLN2*, or the 1-kb *Eco*RI fragment from *CLN3* as indicated. Probes were radiolabelled with ^{32}P by the random primer method to a specific activity of 10^9 cpm/μg. The bands were quantitated by scanning with a Molecular Dynamics computing densitometer.

cAPK assays. Cells were disrupted by vortexing with glass beads in YMB buffer, containing 50 mM 2[*N*-morpholino]-ethanesulfonic acid (pH 6.0), 0.1 mM EDTA, 0.1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 50 μg of leupeptin per ml, as previously described (4). The extract was then incubated for 10 min at 30°C with the synthetic peptide cAMP-dependent protein kinase (cAPK) substrate Kemptide (18) in the presence or absence of 10 μM cAMP in a buffer containing 10 mM $MgCl_2$, 250 μg of bovine serum albumin per ml, 0.5% aprotinin, and 100 μM [γ-^{32}P]ATP (1,000 dpm/pmol). The labelled peptide was then bound to phosphocellulose filters, the filters were washed, and the labelled peptide was counted.

Western blotting (immunoblotting). Protein samples (prepared as described above) were separated on 10% polyacrylamide gels and transferred to nitrocellulose, and the membranes were blocked with BLOTTO (5% nonfat powdered milk in 50 mM Tris [pH 7.4]–150 mM NaCl–5 mM EDTA–0.01% sodium azide–0.05% Tween 20 [polyoxyethylene sorbitan monolaurate]) and developed with anti-PSTAIRE antibodies (Santa Cruz Biologicals), 2 μg/ml in Blotto. Immunoreactive proteins were visualized by the addition of horseradish peroxidase-labelled goat anti-rabbit antibody, using 4-chloronaphthol as the substrate.

RESULTS

A pathway upstream of Ras-cAMP. For many of the experiments described below, we have used a yeast strain (TC41-1) that allows easy manipulation of the Ras-cAMP pathway. These cells carry a deletion of the structural gene for adenylate cyclase (*CYR1*) and mutations of the *CAM* genes which allow cAMP uptake from the medium (20). In the absence of adenylate cyclase, these cells cannot synthesize cAMP; however, the cells can be maintained by supplying exogenous cAMP in the growth medium. The Ras-cAMP pathway in TC41-1 cells can therefore be controlled by altering the concentration of cAMP in the medium. Grown at

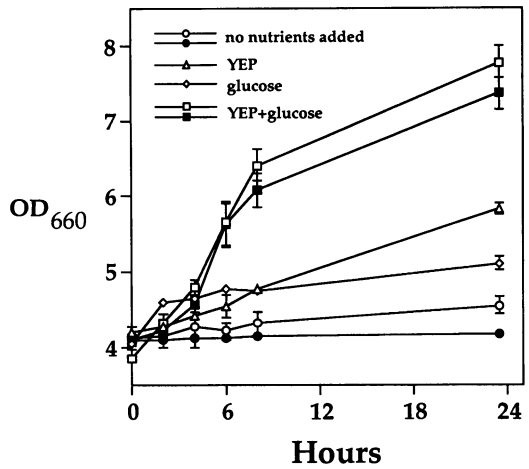

FIG. 1. Post-log-phase cells are unresponsive to cAMP. TC41-1 cells were grown with shaking at 30°C for 4 days in YEPD–1 mM cAMP. The nutrients and cAMP were not replenished during this time. Concentrated YEP and/or glucose was then added to the cells at $t = 0$ as indicated to give final concentrations of 1× and 2%, respectively. Cultures represented by closed symbols also received sufficient cAMP to increase the concentration in the medium by 1 mM. Growth of the cells was monitored by measuring the OD_{660} of cell suspensions. Each point is the mean ± standard deviation ($n = 3$). Similar results were obtained in three independent experiments.

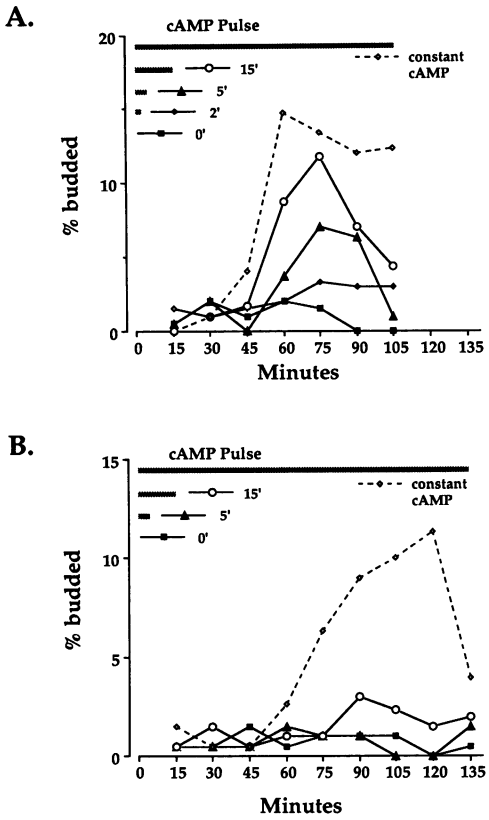

FIG. 2. Ability of YEPD-pretreated cells to bud after short pulses of cAMP. Nutrient-arrested TC41-1 cells were resuspended in fresh YEPD at an OD_{660} of 1 either 3 h prior to initiation of growth with cAMP (A) or immediately before treatment with cAMP (B). cAMP (1 mM) was added for the time indicated. After these times, the cells were pelleted, washed once with YEPD in the absence of cAMP, and returned to incubation with YEPD alone. Aliquots of the cell suspension were taken at 15-min intervals and fixed in 3.7% formaldehyde–0.1 M K_2HPO_4. The percentage of budded cells was measured as described in Materials and Methods. Similar results were obtained in three independent experiments. Cells were maintained with shaking at 30°C during all incubations.

constant levels of cAMP, these cells behave as though they have a constitutively active Ras-cAMP pathway. When TC41-1 cells were initially cultured in YEPD supplemented with 1 mM cAMP, early-log-phase growth was followed by a stationary-like, post-log phase. Cells in the post-log phase were unbudded, indicating that they were arrested in the G_1 phase of the cell cycle. Since log-phase cells arrest growth in G_1 when cAMP is removed (19), it seemed likely that the cAMP had been depleted from the culture. However, the post-log-phase cells did not grow when we added more cAMP to the medium (Fig. 1). On the other hand, the addition of either concentrated YEP or glucose to the medium induced growth. Addition of both YEP and glucose together produced an even larger growth response. The addition of cAMP did not augment the growth effect of fresh nutrients. Therefore, the post-log-phase cells had stopped growing as a result of nutrient depletion rather than cAMP depletion.

To examine this observation further, we conducted an experiment to determine whether the nutrient-depleted cells had stopped growth upstream of the point in the cell cycle where cells are arrested by lack of cAMP or whether they had already passed the cAMP point and had become arrested at a point in G_1 closer to START. In this experiment, the cAMP-insensitive post-log-phase cells were transferred from nutrient-depleted medium containing cAMP into fresh medium without cAMP. When placed in fresh YEPD, these cells clearly responded, changing from refractile cells with prominent vacuoles to phase-dark cells. However, in the absence of cAMP they did not go on to produce buds (this result is not shown but can be seen in the no-cAMP control in Fig. 2B). Thus, cells receiving fresh nutrients became arrested for lack of cAMP before they were able to reach START. If cAMP was added along with the fresh medium, the cells underwent identical changes in appearance, but in contrast to the cells with YEPD alone, these cells began to bud after approximately 2 h (Fig. 2B). This result indicates that the step limiting the nutrient-arrested cells cannot lie downstream of the point in the cell cycle that is affected by cAMP. This conclusion is reinforced by the experiments described below.

We saw similar responses with *ras1*$^-$ *ras2*$^-$ *cyr1*$^-$ cells (see, for example, Fig. 5). Therefore, the YEPD effect is Ras as well as cAMP independent. To simplify further discussion, we will refer to this as the Ras-cAMP-independent process.

YEPD pretreatment primes cells for budding in response to cAMP. Shifting the post-log-phase cells into fresh YEPD without cAMP allowed them to move past a stage in which they were limited by nutrients to one in which they became limited by lack of cAMP. We expected that after YEPD pretreatment the cells would have progressed beyond the nutrient arrest point and would be lined up at the cAMP arrest point, poised to respond to cAMP. This expectation was confirmed in the experiment shown in Fig. 2. Nutrient-arrested TC41-1 cells were diluted into fresh YEPD and incubated for 3 h without cAMP. They were then exposed to

short pulses of cAMP and returned to YEPD without cAMP, and samples were counted for buds every 15 min (Fig. 2A). Treatment with cAMP for as little as 5 min produced cells that were committed to budding, while a 15-min cAMP exposure produced a budding response similar to that seen in cells treated with cAMP for the duration of the assay. Thus, after being pulsed with the cAMP for 5 min or more, many of the YEPD-pretreated cells had passed START. This result indicated that the cAMP response was rapid (5 to 15 min).

The rapid response to cAMP did not occur unless the cells had been pretreated with fresh medium (Fig. 2B). Post-log-phase cells that were not YEPD pretreated were unable to pass START after a short pulse of cAMP. It appeared that they needed to complete all or part of the upstream, Ras-cAMP-independent process before they were able to respond to cAMP. On the other hand, cells that had been pretreated with YEPD appeared to have passed the nutrient checkpoint, continued in the cell cycle until they reached the cAMP arrest point, and were poised to rapidly respond to cAMP.

Effects of cAMP and YEPD on G_1 cyclin gene expression. Since commitment to budding after YEPD pretreatment required only a short exposure to cAMP, we determined whether the rapid cAMP effects coincided with the accumulation of early markers for passage through START. These markers included the genes *HO* and *CLN2*. These genes contain distinct SWI4/SWI6-dependent cell cycle box (SCB) promoter elements that allow cell cycle-dependent increases in transcription at START (1, 2). Figure 3 shows that cAMP induced a dramatic increase in *CLN2* mRNA after only 10 min of cAMP exposure in YEPD-pretreated cells. Like the budding response, this response to cAMP was completely dependent on YEPD pretreatment (Fig. 4). cAMP also induced a similar increase in *HO* mRNA (Fig. 3) and *CLN1* message (not shown). The addition of 10 μg of cycloheximide per ml during cAMP treatment did not block the accumulation of *CLN2* mRNA (not shown). This result indicates that cAMP was not acting by increasing protein synthesis.

This finding is in apparent contradiction with the results of Fernandez-Sarabia et al. (14), who found that a temperature-sensitive *cdc25* mutation had no effect on *CLN1* or *CLN2* expression. As yet, we have no explanation for the difference between our results.

Overall, the time required for accumulation of *CLN2* mRNA was similar to the time course for commitment to the cell cycle in response to cAMP (Fig. 2A). However, it is difficult to determine exactly how closely these time courses match. Although some cells were committed to budding after as little as a 5-min exposure to cAMP, a 15-min exposure was more effective. Additionally, it is not clear exactly how long the effects of cAMP persist after the cells are transferred back into medium without cAMP.

Time course of the Ras-cAMP-independent process. Because YEPD pretreatment was essential for the accumulation of *CLN1* and *CLN2* message in response to cAMP, we were able to measure the time course of the process by which responsiveness to cAMP was restored. Nutrient-arrested TC41-1 cells were pretreated with YEPD for times varying from 0 to 2 h and then incubated for an additional 20 min in the presence or absence of cAMP to allow accumulation of *CLN2* mRNA. As seen in Fig. 4A, nutrient-arrested cells were unable to respond to cAMP until after about 60 min of pretreatment with fresh YEPD. A maximum response to cAMP was obtained after about 90 min of pretreatment.

The ability of fresh nutrients to restore cAMP-induced *CLN2* mRNA accumulation was blocked by the addition of cycloheximide during the YEPD pretreatment, indicating that the Ras-cAMP-independent process requires protein synthesis (Fig. 4C). These results highlight the contrasts between the Ras-cAMP pathway and the Ras-cAMP-independent process. While each is involved in a response to nutrients, the Ras-cAMP-independent process is relatively slow and cycloheximide sensitive whereas the cAMP pathway is rapid and cycloheximide insensitive.

We were also able to measure the time course of the Ras-cAMP-independent process by monitoring a biological response. Because completion of the Ras-cAMP-independent process was necessary before cells could respond to cAMP, cells that were transferred to YEPD-cAMP directly without pretreatment had to complete the first process before they were able to bud in response to cAMP. This was manifested as a lag in growth. For example, cells pretreated with YEPD for several hours were able to bud within 45 to 60 min after addition of cAMP, while cells that were not pretreated, but instead transferred to fresh medium at the time of cAMP addition, did not bud until about 70 to 120 min after cAMP addition (Fig. 2). This effect on growth lag was

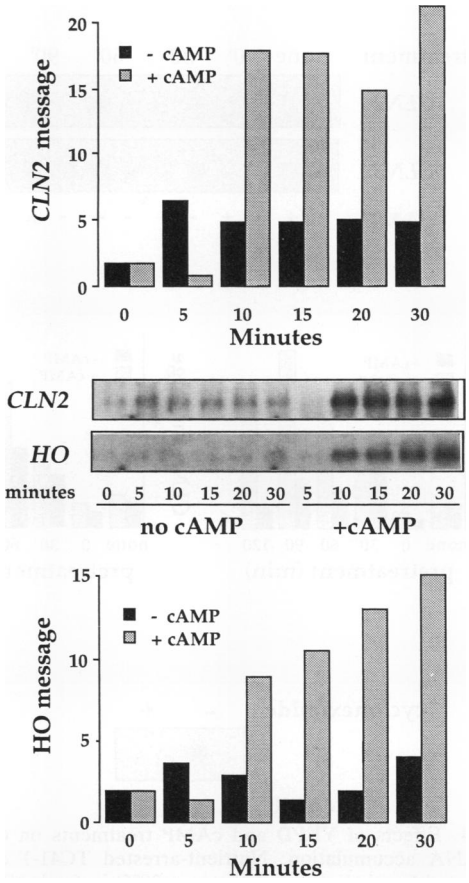

FIG. 3. Rapid increase in cell cycle box gene expression in response to cAMP. Nutrient-arrested TC41-1 cells were preincubated with shaking at 30°C at an OD_{660} of 1 with fresh YEPD for 3 h. Cells were then treated with or without 2 mM cAMP at $t = 0$. After the indicated time periods, the cells were harvested for RNA preparation and Northern blotting. The blot was probed for *CLN2* and *HO* mRNA. Loading and transfer of rRNA varied by no more than 5% per lane. The bands were quantitated by scanning with a Molecular Dynamics computing densitometer. Similar results were obtained in three independent experiments.

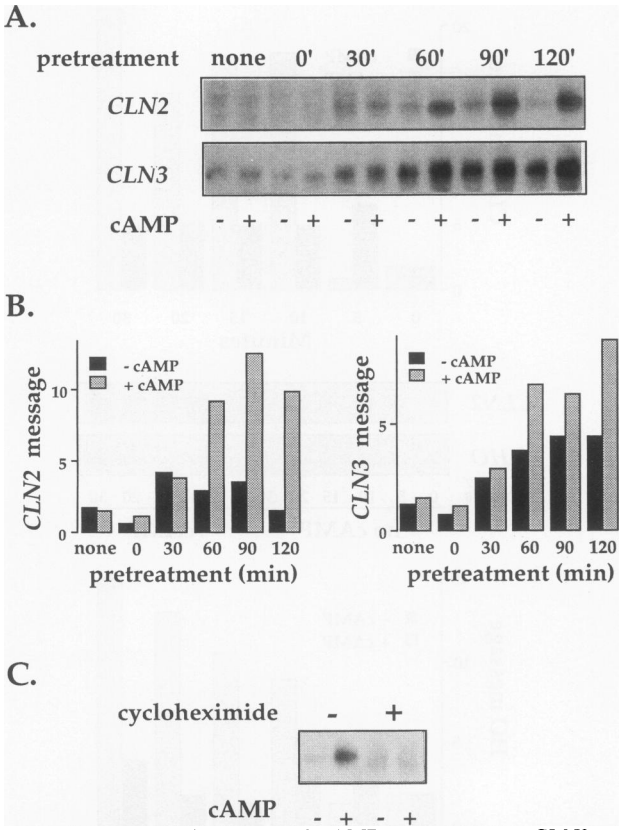

FIG. 4. Effects of YEPD and cAMP treatments on *CLN2* and *CLN3* RNA accumulation. Nutrient-arrested TC41-1 cells were preincubated for the indicated times at 30°C in fresh YEPD at an OD_{660} of 1. Aliquots of cells were then treated with or without 2 mM cAMP for an additional 20 min. The cells were harvested for RNA preparation and Northern blotting. (A) Blot probed for *CLN2* and *CLN3* mRNA. In the first two lanes, no fresh YEPD was present during the preincubation or cAMP treatment. (B) The bands were quantitated by scanning with a Molecular Dynamics computing densitometer. (C) Cells were preincubated with YEPD for 120 min and challenged with cAMP as described for panel A. In one set of samples, cycloheximide (10 μg/ml) was added during the preincubation as indicated and washed away prior to cAMP addition. The cells were harvested for RNA preparation and Northern blotting with a probe for *CLN2* mRNA. Similar results were obtained in three independent experiments.

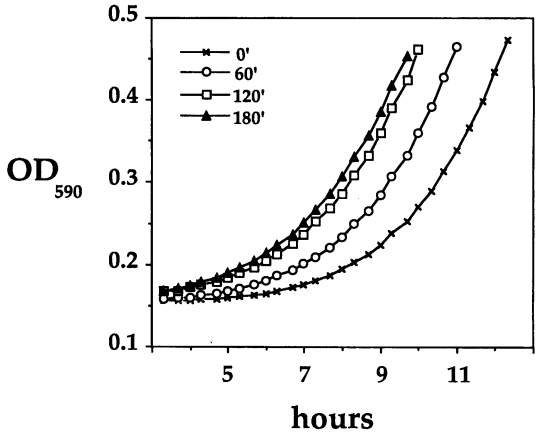

FIG. 5. YEPD pretreatment reduces lag in culture growth. Nutrient-arrested cells carrying disruptions in *RAS1* and *RAS2* (TC28-1) were added to fresh YEPD (200 μl of cells into 15 ml of YEPD) with no cAMP and incubated for the indicated time prior to addition of cAMP (2 mM) at time zero. Cells (200 μl) were then added to wells of a 96-well microtiter plate and loaded into an automated plate reader. The plate reader maintained a temperature of 25°C and shook the plates every 15 min prior to reading of OD_{590} of the wells. Numbers are averages of replicates of six. Similar results were obtained in three independent experiments.

also detectable by monitoring culture density. When cells were pretreated with YEPD before initiation of growth with cAMP, the growth curve was shifted about 2 h ahead of the curve for a culture that was transferred into fresh nutrients at the time of cAMP addition (Fig. 5). In these experiments, post-log-phase cells were pretreated with fresh YEPD for various times before addition of cAMP. Cultures were grown in 96-well microtiter plates, and culture densities were measured in an automated plate reader. Pretreatment of the cells with fresh medium allowed them to complete the Ras-cAMP-independent process before cAMP was added. Once this process was completed, longer pretreatment times had no further effect in decreasing the lag in growth. In agreement with other experiments, pretreatment for 120 min produced a maximum effect. The reciprocal experiment had no effect. Pretreatment of cells with cAMP did not shorten the lag in growth when growth was initiated by addition of fresh medium (not shown). The experiment shown in Fig. 5 used a *ras1*$^-$ *ras2*$^-$ *cyr1*$^-$ strain otherwise isogenic to the *cyr1*$^-$ TC41-1 strain. Similar results were obtained with the TC41-1 cells.

Effects of YEPD and cAMP on *CLN3* mRNA levels. One hypothesis that is consistent with these results is that addition of fresh medium allows the synthesis of components that are required for cAMP induction of *CLN1* and *CLN2* mRNA. Accumulation of these components during YEPD pretreatment would then allow cells to respond rapidly to cAMP. Because a functional *CLN3* gene has been previously shown to increase *CLN1* and *CLN2* transcript levels, we considered Cln3 as a candidate for this role. We reprobed the blot shown in Fig. 4A for *CLN3* message to determine whether *CLN3* expression changes during YEPD pretreatment (Fig. 4). In contrast to the results seen with the *CLN2* probe, YEPD pretreatment alone was sufficient to stimulate production of *CLN3* mRNA approximately fivefold. The maximum response was seen after about 90 to 120 min of pretreatment, corresponding well with the time required for the acquisition of the ability to respond to cAMP. Thus, the time course of the Ras-cAMP-independent process correlates well with the time course of *CLN3* mRNA accumulation.

In addition to changes in *CLN3* expression, there are several other possible mechanisms that might restrict a cell's ability to respond to cAMP. One possibility is that the post-log-phase cells have reduced levels of cAPK and that pretreatment with fresh medium restores the levels of the kinase sufficiently to allow a response to cAMP. Kinase assays revealed no significant difference in cAPK activity between post-log-phase and medium-pretreated cells. Another possibility is that the $p34^{CDC28}$ itself was limiting in the post-log-phase cells. Western blotting with PSTAIRE antibodies showed no significant increase in $p34^{CDC28}$ during the course of nutrient pretreatment.

Changes in *CLN2* and *CLN3* transcript levels in wild-type cells released from nutritional arrest. Although we were able to dissect the nutrient response of starved TC41-1 cells into

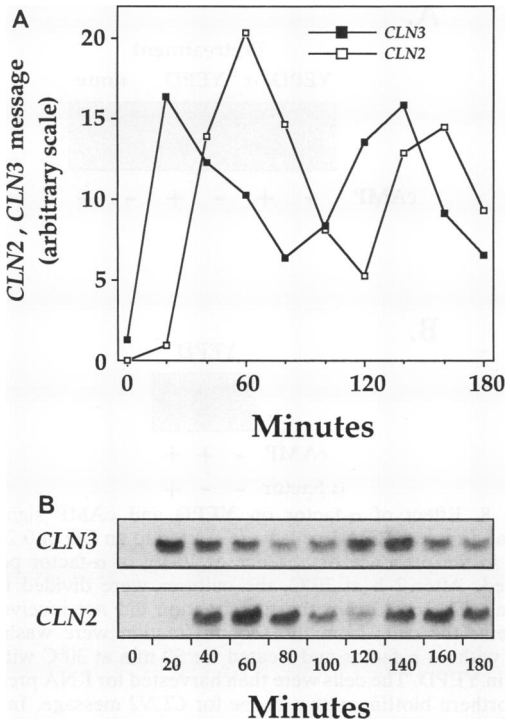

FIG. 6. Changes in *CLN2* and *CLN3* transcript levels in wild-type cells released from nutritional arrest. Wild-type HR125 cells were grown to stationary phase in YEPD (3 days). Cells were then diluted to an OD_{660} of 1 in fresh YEPD at $t = 0$ and incubated with shaking at 30°C. After the indicated times, the cells were harvested for RNA preparation and Northern blotting with probes for *CLN2* and *CLN3* mRNA (B). The bands were quantitated by scanning with a Molecular Dynamics computing densitometer (A). Similar results were obtained in three independent experiments.

Ras-cAMP-independent and Ras-cAMP-mediated processes, these two processes normally are not separable in wild-type cells containing an intact Ras-adenylate cyclase system. The addition of fresh medium to stationary-phase, wild-type cells should activate both the Ras-cAMP-independent process and the Ras-cAMP pathway simultaneously. However, because of our results with the *cyr1*$^-$ cells, we expected that an increase in *CLN2* expression in response to increasing intracellular cAMP would be delayed until the Ras-cAMP-independent process was completed. This prediction was at least in part confirmed in the experiment shown in Fig. 6. Although intracellular cAMP must have risen immediately in response to fresh medium, *CLN2* expression did not begin to rise until more than 20 min after addition of fresh medium. Accumulation of *CLN2* mRNA did not begin until after *CLN3* expression had peaked. These results are consistent with an upstream Ras-cAMP-independent process involving *CLN3* expression and a downstream Ras-cAMP pathway triggering the accumulation of *CLN2* mRNA. The wild-type cells seemed to complete the Ras-cAMP-independent process considerably more rapidly than the *cyr1*$^-$ cells did. They were able to bud after only 45 to 60 min of exposure to fresh medium, while the *cyr1*$^-$ cells did not bud until 90 to 120 min after transfer to fresh YEPD-cAMP. Furthermore, the increase in *CLN3* expression in response to fresh medium, although similar in magnitude, was much faster in the wild-type cells than in the *cyr1*$^-$ cells. This may reflect the fact that cells held at high cAMP store carbohydrates poorly in response to starvation. In the wild-type cells, both the *CLN2* and *CLN3* transcript levels cycled during the first 3 h after YEPD treatment as the cells traversed the mitotic cycle. Although this appears to contradict reports that *CLN3* expression is cell cycle independent (22, 32), we do not know whether this cyclic pattern of *CLN3* expression continues as cells enter log phase growth. Our result may be specific to cells leaving stationary phase.

Deletion of *CLN3* delays the Ras-cAMP-independent process. To test the idea that *CLN3* plays a role in restoring cAMP sensitivity to nutrient-arrested cells, we tested the ability of cells carrying a deletion in *CLN3* to respond to fresh medium. The *CLN3* deletion was made in a strain carrying a *cyr1-1* mutation to produce strain TL2-1. These cells, along with the parent *CLN3*$^+$ *cyr1-1* cells (NW23-9C) as controls, were inoculated into YEPD–1 mM cAMP and grown for 2 days to an OD of 3.5. We then conducted an experiment similar to that described for Fig. 4, in which we measured the time course of the process by which fresh nutrients restore induction of *CLN2* message by cAMP. The nutrient-arrested cells were transferred to fresh YEPD with no cAMP to a final OD of 1.0 and incubated for the indicated times at 30°C. After this time, samples were collected and either challenged with cAMP or left in YEPD alone for an additional 20 min. Cells were then collected for Northern blotting with a *CLN2* probe. As seen in Fig. 7A, the ability to increase *CLN2* message in response to cAMP increased steadily in the control *CLN3*$^+$ cells. In contrast, the cells carrying the *CLN3* deletion took much longer to regain the capacity to respond to cAMP, requiring approximately 3 h in fresh medium before *CLN2* induction was observed. It should also be noted that in addition to the effect on the time course of *CLN2* induction, loss of *CLN3* resulted in much lower amounts of *CLN2* message overall; the blot from the TL2-1 cells was exposed five times longer than the blot for the control NW23-9C cells.

Cells carrying the *CLN3* disruption were also retarded in emerging from nutrient arrest in response to fresh medium and cAMP (Fig. 7B). When post-log-phase TL2-1 cells were transferred to fresh YEPD–1 mM cAMP, they did not begin increasing in density until approximately 1 to 2 h after the control NW23-9C cells had started growing. Although the *cln3*$^-$ cells were slow to increase in density, in another respect they responded to nutrients as rapidly as the *CLN3*$^+$ cells did. In both strains, there was a noticeable change in appearance in the cells after only 1 h of exposure to fresh nutrients. The cells had become phase dark, and the vacuoles prominent in the nutrient-arrested cells had disappeared. Thus, the *cln3*$^-$ cells were able to respond to fresh nutrients at some level. The loss of *CLN3*, however, delayed completion of the Ras-cAMP-independent process.

Effects of α-factor on the Ras-cAMP-dependent and Ras-cAMP-independent processes. Yeast mating pheromones have been shown to inhibit the accumulation of *CLN1* and *CLN2* mRNA (32). Since both the Ras-cAMP-independent and cAMP pathways were required for the accumulation of *CLN2* mRNA, we tested the ability of α-factor to block each of these processes. As shown in Fig. 8A, the presence of α-factor during the YEPD pretreatment did not inhibit the Ras-cAMP-independent process. In agreement with the experiment shown in Fig. 4, a 2-h YEPD pretreatment enabled cells to accumulate *CLN2* mRNA in response to a short pulse of cAMP. This was not changed by the presence of α-factor during the YEPD pretreatment, provided that the α-factor was removed prior to the addition of cAMP. The inclusion of α-factor during the pretreatment also did not

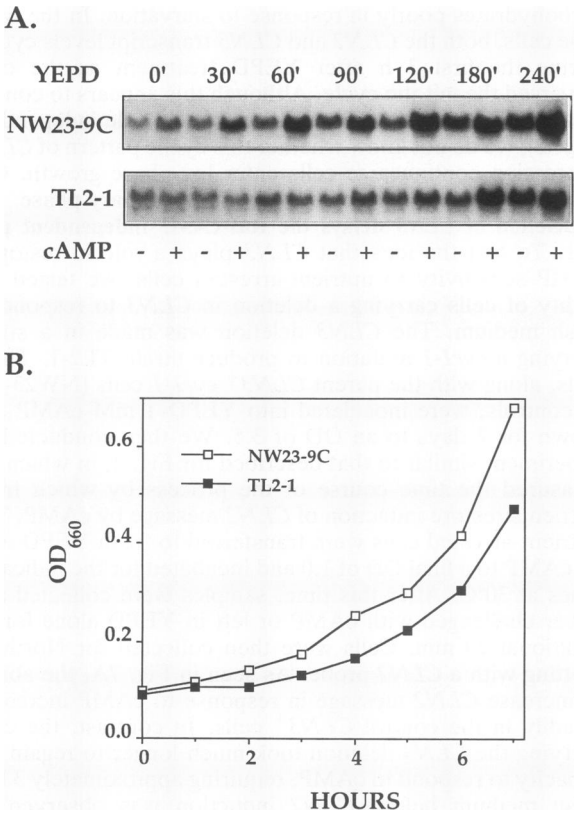

FIG. 7. Loss of *CLN3* delays the Ras-cAMP-independent process. (A) Nutrient-arrested (OD of 3.5) TL2-1 (*cln3*$^-$ *cyr1*$^-$) cells and control NW23-9C (*cyr1*$^-$) cells were preincubated for the indicated times at 30°C in YEPD at an OD_{660} of 1. Aliquots of cells were then treated with or without 2 mM cAMP for an additional 20 min. The cells were then harvested for RNA preparation and Northern blotting. The blots were probed together for *CLN2* mRNA. The blot for the TL2-1 cells was exposed to film for five times longer than the control blot was. (B) Post-log-phase cells were transferred to fresh YEPD–2 mM cAMP at an OD of 0.1 and incubated with shaking at 30°C. The OD_{660}s of the cells were measured at the indicated times. Open symbols, NW23-9C control cells; closed symbols, TL2-1 cells.

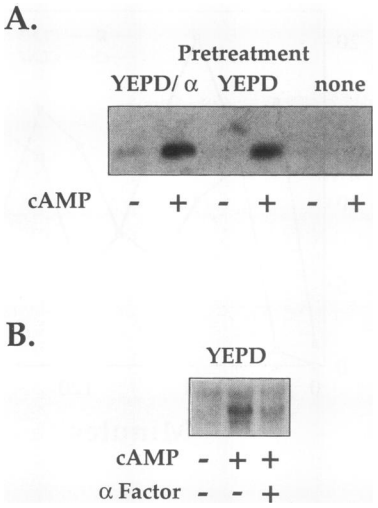

FIG. 8. Effect of α-factor on YEPD and cAMP signals. (A) Nutrient-arrested TC41-1 cells were diluted to an OD of 0.2 in fresh YEPD in the presence or absence of 10 μg of α-factor per ml as indicated. After 2 h at 30°C, the cultures were divided into two fractions. The cells from the first fraction did not receive cAMP treatment; the cells from the second fraction were washed with YEPD without α-factor and treated for 30 min at 30°C with 1 mM cAMP in YEPD. The cells were then harvested for RNA preparation and Northern blotting with a probe for *CLN2* message. In the last two lanes, cells received no YEPD pretreatment prior to cAMP exposure. (B) Nutrient-arrested TC41-1 cells were preincubated with YEPD for 3 h. cAMP (2 mM) was then added to the cells in the presence or absence of 10 μg of α-factor per ml as indicated. The cells were incubated 20 min at 30°C and harvested for Northern blotting with a probe for *CLN2* mRNA. Similar results were obtained in three independent experiments.

block the increase in *CLN3* message (not shown). In contrast, when α-factor was added during the cAMP treatment, the accumulation of *CLN2* mRNA was blocked (Fig. 8B). These results indicate that α-factor acts to block the cAMP response but has no effect on the Ras-cAMP-independent process.

We used a reciprocal shift experiment to show that α-factor arrests cells at the same point in the cell cycle as the cAMP arrest point. Cells pretreated with fresh YEPD were exposed to cAMP (2 mM) and α-factor (10 μg/ml) for 30 min; the cells were then washed and incubated in YEPD with no α-factor or cAMP. If the α-factor arrest point was downstream of the cAMP arrest point, then a 30-min treatment with cAMP in the presence of α-factor would be sufficient to allow the cells to pass the cAMP arrest point and proceed to the α-factor arrest point. When the α-factor was washed away, the cells would then be able to bud in the absence of further cAMP addition since they would have passed the upstream cAMP arrest point. But this was not the case. After release from α-factor, the cells were unable to bud unless cAMP was reintroduced into the medium (not shown). This finding indicates that the α-factor arrest point cannot be downstream in the cell cycle from the cAMP arrest point. On the other hand, a previous study indicates that the α-factor arrest point cannot be upstream of the cAMP arrest point either. Matsumoto and coworkers (19) used α-factor and a temperature-sensitive allele of *CYR1* to demonstrate that cells released from the restrictive temperature were blocked by simultaneous addition of α-factor. Taken together, these results indicate that cells need the simultaneous presence of a positive cAMP signal and the absence of a negative mating factor signal to advance from G_1 to S phase.

DISCUSSION

We have characterized two processes that must be completed before nutrient-arrested cells are able to pass START. The first process is Ras-cAMP independent and lies upstream of the Ras-cAMP pathway in the G_1 phase of the cell cycle. It is slow, cycloheximide sensitive, and associated with the accumulation of *CLN3* mRNA. The Ras-cAMP-independent process is not affected by α-factor. In contrast to the Ras-cAMP-independent process, the response to cAMP is rapid and insensitive to cycloheximide. The cAMP response is associated with a dramatic increase in *CLN1* and *CLN2* message. The effects of cAMP are effectively blocked by α-factor.

A simple model that fits our results is shown in Fig. 9. In this model, the Ras-cAMP-independent process acts to increase the expression of *CLN3* (and presumably other

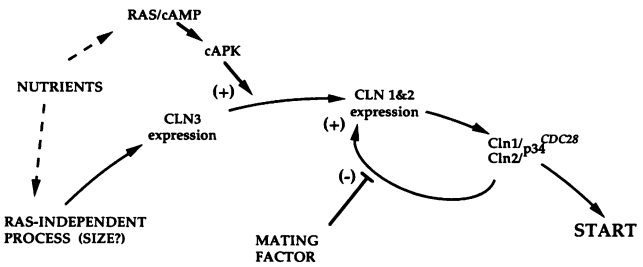

FIG. 9. Model of cAMP-dependent and -independent contributions to $p34^{CDC28}$ activity.

genes). This process is necessary but not sufficient for passage through START. Activation of the Ras-cAMP pathway is necessary for the accumulation of *CLN1* and *CLN2* message that is believed to propel cells past START. This positive feedback loop is blocked by mating factor. Two important features of this model remain unexplored. The first is the nature of the pathway that mediates the Ras-cAMP-independent process. Although an increase in *CLN3* expression appears to play a role in this process, we know little about the signals that regulate *CLN3* expression. The second area is the mechanism by which cAMP produces the rapid increase in *CLN1* and *CLN2* expression. We cannot say how many steps lie between cAMP and the increase in message levels. We have not yet determined whether cAMP acts to increase transcription or to increase the half-life of the transcript, so any number of mechanisms that do not require the synthesis of new proteins are possible.

With this uncertainty in mind, we can still construct a very simple model for our results if we propose that Cln3 is activated by the Ras-cAMP pathway. In this case, the increased *CLN3* expression produced by the Ras-cAMP-independent process would be necessary for the response to cAMP. The activated Cln3-$p34^{CDC28}$ complex would in turn produce increased *CLN1* and *CLN2* transcription, leading to START. This model is consistent with the increased *CLN3* expression associated with the Ras-cAMP-independent process. It is also consistent with the role that Cln3 is thought to play in increasing transcription of *CLN1* and *CLN2*, a function that is thought to be under posttranslational control (23). Finally, it is possible that Cln3 is modified directly by cAPK. The predicted amino acid sequence of Cln3 contains a consensus site for phosphorylation by cAPK at position 562 (10, 15).

This model must be tempered by the fact that each of the G_1 cyclins is by itself dispensable. Despite the fact that loss of *CLN3* delays the Ras-cAMP-independent process, cells survive the loss of *CLN3*. They must therefore be able to complete the Ras-cAMP-independent process without *CLN3*. Similarly, they can do without *CLN1* and *CLN2* and therefore do not need to make the products of these genes in order to respond to cAMP. We can conclude then that if our model is accurate, there must be redundant components that allow these processes to go on with only a single G_1 cyclin. Some evidence for this kind of redundancy can be found in the data presented above. Although the Ras-cAMP-independent process is associated with a more substantial increase in *CLN3* expression, *CLN2* message levels also consistently increased in response to fresh medium. It is therefore perhaps more reasonable to think in terms of distinct but overlapping functions for *CLN3*, *CLN1*, and *CLN2*.

Although the model presented above fits our data, there are many other possible mechanisms by which cAMP could increase *CLN1* and *CLN2* accumulation. In light of the apparent redundancies in both the Ras and cyclin pathways of *S. cerevisiae*, it would not be surprising if multiple mechanisms were found to mediate the effects of cAMP on *CLN* message levels and passage through START.

We have used an artificial system in which we can manipulate the Ras-cAMP pathway in order to dissect out responses to cAMP. With this system, we have identified a process related to nutrients that must be completed before cells can respond to cAMP. Although in this setting the Ras-cAMP-independent process must be completed before cells can respond to cAMP, it is important to recognize that in wild-type cells, cAMP production and the Ras-cAMP-independent process must be occurring simultaneously rather than in the stepwise fashion seen in our experiments.

Although TC41-1 cells have proven very valuable in defining cAMP-dependent and -independent nutrient responses, these cells present limitations as well. The biggest limitation is the obvious difference between the way that wild-type cells and TC41-1 cells respond to nutrient depletion. Cells with elevated cAMP levels fail to store carbohydrates and lose viability when nutrients are depleted. This loss of viability is probably the cause of the low budding indices shown in Fig. 2. Under the conditions used in this experiment, 50% of the cells remained viable when plated onto YEPD–1 mM cAMP. Thus, not all of the mutant cells were able to reenter the cell cycle following the period of starvation. Despite this difference, the processes that we observed in the TC41-1 cells appear to be qualitatively similar to those seen in wild-type cells. Specifically, in response to nutrient depletion, the levels of *CLN3* transcript fell to barely detectable levels in both TC41-1 and wild-type cells. Upon restoration of nutrients, the levels of *CLN3* message dramatically increased in both strains. The slower increase in *CLN3* transcript observed in the *cyr1*$^-$ strain may reflect the defect in nutrient storage produced by growing the cells at constant cAMP levels. Cells defective in laying down nutrient stores may require more time to replenish these stores before leaving stationary phase.

A nutrient-induced increase in *CLN3* expression also appeared to precede *CLN2* expression in both the TC41-1 and wild-type strains. In TC41-1 cells, cAMP induction of *CLN2* message clearly required a nutrient-dependent step associated with increased *CLN3* expression. Once this step was completed, however, the response to cAMP was quite rapid, with a substantial increase in *CLN2* after only 10 min. In contrast, although cAMP must have increased in the wild-type cells immediately upon transfer to fresh medium, an increase in *CLN2* expression was not observed until 40 min later, following an increase in *CLN3* message. This delay is consistent with the idea that the wild-type cells also need to complete a nutrient-driven process involving *CLN3* expression before they can increase *CLN2* message in response to cAMP and return to proliferative growth. Our results indicate that cAMP and mating pheromones act simultaneously to regulate expression of G_1 cyclins. This view is in contrast to the conclusions reached by workers using temperature-sensitive mutations and α-factor (27). An example of this type of experiment is the one performed by Matsumoto and colleagues in which cells carrying a temperature-sensitive allele of *CYR1* were arrested with α-factor and then simultaneously released from α-factor arrest and shifted to the restrictive temperature to stop cAMP formation (19). Because these cells were able to pass START and bud at the restrictive temperature, it was concluded that

α-factor arrests cells at a point in G_1 beyond the cAMP arrest point. The authors, however, were careful to point out that they could not rule out the possibility that cAMP levels remained sufficiently high for a period of time after the shift to allow the cells to pass START. Our results showing that a short exposure to cAMP can commit a cell to budding indicate that this was indeed the case.

Our results might also seem surprising in view of the distinct arrest phenotypes produced by these two pathways. Cells arrested by nutrient limitation or mutations that decrease cAMP stop in G_1 as quiescent rounded cells that are unable to mate, while cells arrested in G_1 by mating pheromone are mating competent and continue to grow, producing a distinctive morphology. These arrest phenotypes can be reconciled if we propose that in addition to affecting a common pathway in the cell cycle, these signal transduction systems produce many other effects that are not common to both pathways. In particular, the cAMP signal also regulates metabolism and growth in size (which may affect mating competence), while mating pheromones elicit the expression of mating-specific genes. These effects that are not directly related to the cell cycle and which are not held in common contribute to the different arrest phenotypes.

Our results are a step toward making connections between the signal transduction systems that regulate proliferative growth and the conserved cyclin-CDK pathways that regulate the machinery of the cell cycle. These experiments have allowed us to assemble a model that can be tested and refined in future experiments.

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REFERENCES

1. **Andrews, B. J., and I. Herskowitz.** 1989. Identification of a DNA binding factor involved in cell-cycle control of the yeast HO gene. Cell **57**:21–29.
2. **Breeden, L., and K. Nasmyth.** 1987. Similarity between cell-cycle genes of budding yeast and fission yeast and the Notch gene of Drosophila. Nature (London) **329**:651–654.
3. **Casperson, J. F., N. Walker, and H. R. Bourne.** 1985. Isolation of the gene encoding adenylate cyclase in the yeast *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **82**:5060–5063.
4. **Casperson, J. F., N. Walker, A. R. Brasier, and H. R. Bourne.** 1983. A guanine nucleotide sensitive adenylate cyclase in the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **258**:7911–7914.
5. **Chang, F., and I. Herskowitz.** 1990. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: *FAR1* is an inhibitor of a G1 cyclin, *CLN2*. Cell **63**:999–1011.
6. **Chang, F., and I. Herskowitz.** 1992. Phosphorylation of *FAR1* in response to alpha-factor: a possible requirement for cell-cycle arrest. Mol. Biol. Cell **3**:445–450.
7. **Cross, F.** 1988. *DAF1*, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:4675–4684.
8. **Cross, F.** 1990. Cell cycle arrest caused by *CLN* gene deficiency in *Saccharomyces cerevisiae* resembles START-1 arrest and is independent of the mating-pheromone signalling pathway. Mol. Cell. Biol. **10**:6482–6490.
9. **Cross, F. R., and A. H. Tinkelenberg.** 1991. A potential positive feedback loop controlling *CLN1* and *CLN2* gene expression at the start of the yeast cell cycle. Cell **65**:875–883.
10. **Denis, C. L., B. E. Kemp, and M. J. Zoller.** 1991. Substrate specificities for yeast and mammalian cAMP-dependent protein kinases are similar but not identical. J. Biol. Chem. **266**:17932–17935.
11. **Dirick, L., and K. Nasmyth.** 1991. Positive feedback in the activation of G1 cyclins in yeast. Nature (London) **351**:754–757.
12. **Elion, E. A., J. A. Brill, and G. R. Fink.** 1991. *FUS3* represses *CLN1* and *CLN2* and in concert with *KSS1* promotes signal transduction. Proc. Natl. Acad. Sci. USA **88**:9393–9396.
13. **Ellwood, M. S., and E. A. Craig.** 1984. Differential regulation of the 70K heat shock gene and related genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:1454–1459.
14. **Fernandez-Sarabia, M. J., A. Sutton, T. Zhong, and K. T. Arndt.** 1992. SIT4 protein phosphatase is required for the normal accumulation of SWI4, CLN1, CLN2, and HCS26 RNAs during late G1. Genes Dev. **6**:2417–2428.
15. **Glass, D. B., and E. G. Krebs.** 1980. Protein phosphorylation by cyclic AMP-dependent and cyclic GMP-dependent protein kinases. Annu. Rev. Pharmacol. Toxicol. **20**:363–388.
16. **Hartwell, L. H.** 1974. *Saccharomyces cerevisiae* cell cycle. Bacteriol. Rev. **38**:164–198.
17. **Heideman, W., G. F. Casperson, and H. R. Bourne.** 1990. Adenylyl cyclase in yeast: antibodies and mutations identify a regulatory domain. J. Cell. Biochem. **42**:229–242.
18. **Kemp, B. E., E. Benjamani, and E. G. Krebs.** 1976. Synthetic hexapeptide substrates and inhibitors of 3′:5′-cyclic AMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA **73**:1038–1042.
19. **Matsumoto, K., I. Uno, and T. Ishikawa.** 1983. Control of cell division in *Saccharomyces cerevisiae* mutants defective in adenylate cyclase and cAMP-dependent protein kinase. Exp. Cell Res. **146**:151–161.
20. **Matsumoto, K., I. Uno, Y. Oshima, and T. Ishikawa.** 1982. Isolation and characterization of yeast mutants defective in adenylate cyclase and cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA **79**:2355–2359.
21. **Mendenhall, M. D., C. A. Jones, and S. I. Reed.** 1987. Dual regulation of the yeast CDC28-p40 protein kinase complex: cell cycle, pheromone, and nutrient limitation effects. Cell **50**:927–935.
22. **Nash, R., G. Tokiwa, S. Anand, K. Erickson, and A. B. Futcher.** 1988. The *WHI+* gene of *Saccharomyces cerevisiae* tethers cell division to cell size and is a cyclin homolog. EMBO J. **7**:4335–4346.
23. **Nasmyth, K., and L. Dirick.** 1991. The role of *SWI4* and *SWI6* in the activity of G1 cyclins in yeast. Cell **66**:995–1013.
24. **Ogas, J., B. J. Andrews, and I. Herskowitz.** 1991. Transcriptional regulation of *CLN1*, *CLN2*, and a putative new G1 cyclin (*HCS26*) by *SWI4*, a positive regulator of G1-specific transcription. Cell **66**:1015–1026.
25. **Pardee, A. B.** 1989. G1 events and regulation of cell proliferation. Science **246**:603–608.
26. **Pines, J.** 1991. Cyclins: wheels within wheels. Cell Growth Differ. **2**:305–310.
27. **Pringle, J. R., and L. H. Hartwell.** 1981. The *Saccharomyces cerevisiae* cell cycle, p. 97–142. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces cerevisiae*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Reed, S. I.** 1991. G1-specific cyclins: in search of an S-phase-promoting factor. Trends Genet. **7**:95–99.
29. **Richardson, H. E., C. Wittenberg, F. Cross, and S. I. Reed.** 1989. An essential G1 function for cyclin-like proteins in yeast. Cell **59**:1127–1133.
30. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101**:202–211.
31. **Valdivieso, M., K. Sugimoto, K. Jahng, P. Fernandes, and C. Wittenberg.** 1993. *FAR1* is required for posttranscriptional regulation of *CLN2* gene expression in response to mating pheromone. Mol. Cell. Biol. **13**:1013–1022.
32. **Wittenberg, C., K. Sugimoto, and S. I. Reed.** 1990. G1-specific cyclins of *S. cerevisiae*: cell cycle periodicity, regulation by mating pheromone, and association with the $p34^{cdc28}$ protein kinase. Cell **62**:225–237.