G₁ cyclins *CLN1* and *CLN2* repress the mating factor response pathway at Start in the yeast cell cycle

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Transcriptional induction by the mating pheromone α -factor was monitored at different stages of the yeast cell cycle. G₂/M-phase and pre-Start cells showed strong *FUS1* mRNA induction, whereas in post-Start cells the signaling was reduced significantly. This reduction in signaling activity in post-Start cells was correlated with the presence of *CLN1* or *CLN2* transcripts and was not observed in synchronized cells lacking functional *CLN1* and *CLN2* genes. Activation of the Cln–Cdc28p kinase by overexpression of *CLN2* from the *GAL1* promoter strongly reduced *FUS1* mRNA induction. *CLN1* overexpression had a similar effect when the *FAR1* gene, encoding a negative regulator of *CLN1/2* function, was deleted. This reduction of pheromone signaling was specific for *CLN1* and *CLN2*, as it was not observed when *CLN3* was overexpressed. Inactivation of the Cln–Cdc28p kinase complex by thermal inactivation of temperature-sensitive Cdc28p prevented repression of *FUS1* signaling. *CLN2* overexpression suppressed the constitutive signaling and division–arrest phenotypes of cells with a disrupted *gpa1* gene, indicating that the site of action for repression is downstream of the α -subunit (Gpa1p) of the heterotrimeric G protein. The repression at Start of pheromone signaling by Cln1–Cdc28p or Cln2–Cdc28p kinase complexes may contribute to the acquisition of pheromone resistance as cells execute Start.

[Key Words: *CLN1*; *CLN2*; *CLN3*; *cdc28*; *Saccharomyces cerevisiae*; *FUS1*; *FAR1*; α -factor]

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Depending on the environmental conditions, *Saccharomyces cerevisiae* cells in early G₁ phase of the cell cycle can enter different developmental pathways. Nutrient starvation causes cells to arrest and to enter a quiescent state (Pringle and Hartwell 1981). When diploid cells are deprived for a nitrogen source, they can enter a developmental pathway leading to meiotic division and sporulation (Pringle and Hartwell 1981) or they can switch to a pseudohyphal growth form (Gimeno et al. 1992). Exposure of haploid cells to the peptide mating pheromone secreted by cells of opposite mating type will activate a developmental pathway that prepares the cells for conjugation and formation of diploid cells (Cross et al. 1988). This preparation involves the transcriptional induction of many genes necessary for mating (Sprague and Thorner 1992), and the arrest of cells in late G₁ phase at a point called Start (Hartwell 1974; Pringle and Hartwell 1981). The arrest of cells in G₁ may be required to guarantee the correct ploidy of the zygote after conjugation.

The mechanism by which the mating-pheromone signal is transduced, and by which it induces morphological changes, transcription and cell cycle arrest has been the focus of recent reviews (Kurjan 1992; Sprague and

Thorner 1992). In short, upon binding of pheromone to a transmembrane receptor (encoded by the *STE2* gene in *MATa* cells and by *STE3* in *MAT α* cells), a heterotrimeric G protein, consisting of an α , a β , and a γ subunit (encoded by the *GPA1*, *STE4*, and *STE18* genes, respectively) is thought to be activated by releasing a complex of β and γ subunits from the α subunit. The signal is further propagated from the Ste4p–Ste18p subunits through a cascade of several protein kinases. The last of these protein kinases (Fus3p/Kss1p) eventually phosphorylates and activates the transcription factor Ste12p. The activated Ste12p binds to specific elements in the promoters of many genes whose products are required for mating. This binding, in some cases in conjunction with other transcription factors, then results in transcriptional induction of these genes. Among the genes whose transcription is markedly induced after pheromone treatment, are *FUS1*, necessary for efficient cell fusion (McCaffrey et al. 1987; Trueheart et al. 1987), and *FAR1*, which is essential for cell cycle arrest in response to pheromone (Chang and Herskowitz 1990, 1992; McKinney et al. 1993; Peter et al. 1993; Tyers and Futcher 1993). The understanding of the mechanism by which mating pheromone causes cells to arrest at Start is incomplete. The activity of the Cdc28p protein kinase is required for cells to pass the Start point in the cell cycle.

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CDC28 encodes the catalytic component of this protein kinase (Wittenberg et al. 1990; Tyers et al. 1993) and three different cyclin genes, *CLN1*, *CLN2*, and *CLN3* encode stimulatory subunits of the kinase (Cross 1988; Nash et al. 1988; Hadwiger et al. 1989; Richardson et al. 1989; Wittenberg et al. 1990; Tyers et al. 1992, 1993). Any one of these stimulatory subunits is sufficient for activation of the Cdc28p kinase and the execution of Start, but lack of all three stimulatory subunits leads to cell cycle arrest at Start (Richardson et al. 1989; Cross 1990). Addition of mating pheromone leads to a decrease in the activity of the Cln–Cdc28p protein kinase (Wittenberg et al. 1990; Tyers et al. 1993), to elevated Far1p levels and to phosphorylation of Far1p by the Fus3p protein kinase (Chang and Herskowitz 1992; Peter et al. 1993). As Far1p has been reported to interact with both Cdc28p and Cln2p (Chang and Herskowitz 1990; Peter et al. 1993; Tyers and Futcher 1993; J. McKinney and F. Cross, unpubl.), one possible mechanism by which cells arrest at Start may involve a Far1p-dependent inactivation of the Cln–Cdc28p protein kinase.

Only cells in the G₁ phase of the cell cycle arrest in response to mating pheromone and the ability to mate is also restricted to the G₁ phase of the cell cycle (Reid and Hartwell 1977). The molecular mechanism ensuring the restriction of arrest and mating to the G₁ interval has remained elusive until recently. It has been demonstrated that maximal concentrations of Far1p are restricted to pre-Start G₁ cells (McKinney et al. 1993). Because Far1p is essential for cell cycle arrest in response to pheromone (Chang and Herskowitz 1990), the cell cycle regulated expression of Far1p may help to explain how post-Start cells acquire mating-pheromone resistance (McKinney et al. 1993). As Far1p also appears to be necessary for efficient mating (Chang and Herskowitz 1990), the cell cycle-regulated expression of Far1p may also help to explain why mating of haploid yeast cells is restricted to the G₁ phase of the cell cycle. We were interested to find out whether other mechanisms might contribute to the limitation of pheromone responsiveness and mating to the pre-Start G₁ interval of the cell cycle. Based on the observation that transcriptional induction of *FUS1* and *STE2* is cell cycle regulated (Zanolari and Riezman 1991), we studied the cell cycle timing of transcriptional regulation by pheromone in more detail. We found that a significant decrease of pheromone-induced transcription occurred in post-Start cells and that this decrease is dependent on the activation of the Cdc28p kinase by the G₁ cyclins Cln1p and/or Cln2p. The repression of pheromone signaling by the Cln1/2–Cdc28p protein kinase may contribute to the acquisition of pheromone resistance as cells execute Start.

Results

Transcriptional induction by α -factor is cell cycle regulated

It was shown previously that the response to pheromone varies at different cell cycle stages (Zanolari and Riez-

man 1991). The timing of changes in responsiveness to mating factor may be relevant for the acquisition of pheromone resistance when cells pass Start. As the original report did not describe cell cycle timing in detail, we wished to focus more on this aspect. The addition of α -factor to MATa cells rapidly induces (within 5 min) high levels of *FUS1* transcript (McCaffrey et al. 1987; data not shown). Therefore, the induction of *FUS1* mRNA by pheromone was used as a sensitive indicator to monitor the cell cycle regulation of transcriptional induction. MATa *bar1 cdc15-2* cells were synchronized, and samples for Northern analysis and morphological examination were taken every 12 min; similar samples were incubated for an additional 12 min with 0.1 μ M α -factor before harvesting them for Northern analysis and microscopic examination. The basal levels of *FUS1* mRNA were low at all stages of the cell cycle (Fig. 1A). Incubation with α -factor resulted in a transcriptional induction at all cell cycle positions, but the final level of induced transcript fluctuated markedly during the cell cycle, indicating a cell cycle variation in responsiveness to mating pheromone, consistent with previous work (Zanolari and Riezman 1991). The induced *FUS1* mRNA level was high in M-phase-blocked *cdc15* cells and early G₁-phase cells, low in late G₁-phase and S-phase cells, and returned to high levels later in the cycle (Fig. 1A). The expression of the *CLN1* and *CLN2* transcripts was low in M-phase-blocked and early G₁-phase cells, rose to maximal levels toward the end of G₁ phase, and then fell again, as expected (Wittenberg et al. 1990; Tyers et al. 1993). *CLN1* and *CLN2* transcript levels closely reflect levels of protein and Cln-associated H1 kinase activity (Wittenberg et al. 1990; Tyers et al. 1993). *CLN3* mRNA levels were not as strongly cell cycle regulated as observed for *CLN1* and *CLN2* (Fig. 1A), and the timing of these minor fluctuations seemed to be similar to data described previously (Tyers et al. 1993). *H2A* is transcribed throughout S phase and therefore the *H2A* transcript serves as an indicator of the beginning and end of S phase. The first transcription of *H2A* correlated with the initiation of bud formation (data on budding not shown). Protein 1 transcript does not vary during the cell cycle and was used as a loading control.

To determine more precisely the timing of the return to strong transcriptional induction after the significant decrease in signaling in late-G₁- and S-phase cells, we studied *FUS1* mRNA induction in synchronized cultures to which 15 μ g/ml of the microtubule poison nocodazole was added. Addition of nocodazole did not interfere with recovery from the block or with normal cell cycle progression through G₁ and S phase but then effectively prevented spindle formation and blocked cells in early M phase. As a result of this block, no second cycles of transcription for *CLN2* and *H2A* were observed (data not shown). The return to high *FUS1* induction levels was established before the nocodazole block in early M phase, as transcriptional induction of *FUS1* by α -factor was high both in these synchronized nocodazole-blocked cells and in asynchronously log-phase cultures blocked with nocodazole for 2.5 hr (data not shown).

Figure 1. Transcriptional induction by mating factor is cell cycle regulated. *MATa bar1::URA3 cdc15-2* (K2944) cells were grown at 25°C and then arrested in late M phase by shifting the culture to 36°C for 3 hr. Synchronous division was induced by shifting cells back to the permissive temperature of 25°C. Samples for Northern analysis, and microscopic examination were taken every 12 min. For mating pheromone-stimulated samples, aliquots of culture were incubated for another 12 min in the presence of pheromone. (A) The basal and induced levels for *FUS1* mRNA and the transcripts for *CLN1*, *CLN2*, *CLN3*, and protein 1 (Pr1)/H2A in the pheromone-induced samples. Basal and pheromone-induced samples in this experiment were processed in parallel. Northern gels were similarly loaded, and blots were probed simultaneously and exposed to film for the same amount of time. (B) The basal, uninduced, *FUS1* mRNA levels in samples from a different experiment where Northern gels were loaded with more RNA and the *FUS1*-probed blots were exposed to film for a longer time. *CLN2* and Pr1/H2A transcript in these samples are also shown.

Although the basal level of *FUS1* mRNA was low during all stages of the cell cycle, periodicity in basal levels could be observed. Increasing loading and exposure times made this cell cycle regulation in basal *FUS1* mRNA levels clearly visible (Fig. 1B). Basal levels of *FUS1* mRNA were highest in early G₁ phase, fell quickly in late G₁ phase as cells started expressing maximal levels of *CLN2* transcript, and remained low through S phase and G₂ phase before they rose again (Fig. 1B).

Deletion of CLN1 and CLN2 abolishes cell cycle regulation of FUS1 inducibility

FUS1 inducibility dropped sharply at about the beginning of S phase (Fig. 1A). The timing of this reduction

suggested that the reduction in signaling might be dependent on the execution of Start or on the activation of the Cln–Cdc28p kinase. To test this, we analyzed the cell cycle regulation of pheromone responsiveness in strains with deletions of different combinations of *CLN* genes. Strains were constructed that were *cdc28-13 CLN1⁺ CLN2⁺ CLN3⁺*, *cdc28-13 CLN1⁺ CLN2⁺ cln3⁻*, or *cdc28-13 cln1⁻ cln2⁻ 3xCLN3*. The *3xCLN3* strain contained approximately three repeats of the *CLN3* gene integrated at the *TRP1* locus (see Materials and methods). This strain was used because in the absence of *CLN1* and *CLN2*, it gave better synchrony than a strain with only one chromosomal copy of *CLN3*. Cells were arrested at Start by thermal inactivation of the temperature-sensitive Cdc28p, and synchronized cell cycle progression was then started by reactivation of the kinase through release to permissive temperature. At different time points after the release, cells were challenged with α -factor. As shown in Figure 2, A and C, *FUS1* mRNA inducibility was cell cycle regulated in *CLN1 CLN2 CLN3* and *CLN1 CLN2 cln3* cells. The timing of the period of lowest *FUS1* induction coincided roughly with the emergence of buds and was therefore similar to the previous pattern of signaling in *cdc15* synchronized cells (Fig. 1A; data not shown). In contrast, when *CLN1* and *CLN2* were deleted, no cell cycle variation in *FUS1* inducibility was observed (Fig. 2B). In similar synchronization experiments we also tested the cell cycle regulation of *FUS1* inducibility in *CLN1 cln2 cln3 cdc28-13* and in *cln1 CLN2 cln3 cdc28-13* cells. Although the degree of synchrony in these strains was less than in the experiments described above, we still could observe fluctuation in *FUS1* inducibility during the cell cycle (data not shown). These observations indicate that of the three Cln proteins, Cln1p, and/or Cln2p are necessary and sufficient for the transient repression of mating pheromone signaling at the time of Start.

Overexpression of CLN1 or CLN2, but not of CLN3, can lead to repression of α -factor-induced FUS1 transcription

To pursue the observations described above, *FUS1* induction was examined in *cln1⁻ cln2⁻ CLN3⁺* strains containing one of the three *CLN* genes under the control of the strong *GAL1* promoter. In a strain without any overexpressed *CLN* gene, the induction of *FUS1-lacZ* by α -factor was high, as was the case in cells overexpressing *CLN1* or *CLN3* (Fig. 3). In contrast, induction of *FUS1-lacZ* by mating factor was greatly reduced in cells overexpressing *CLN2*. Far1p is thought to act negatively on Cln function (Chang and Herskowitz 1990; Valdivieso et al. 1993; Peter et al. 1993; Tyers and Futcher 1993); therefore, disruption of the *FAR1* gene might further stimulate Cln–Cdc28p activity. Consistent with this idea, *FUS1* signaling in *far1⁻* strains overexpressing *CLN1* was found to be lower than in *FAR1⁺* strains. Strains expressing only *CLN3*, whether from the wild-type locus or from the *GAL1* promoter, and with or without *FAR1*, were all highly competent for *FUS1* induction

Figure 2. Knockout of *CLN1* and *CLN2* abolishes cell cycle regulation of transcriptional induction by mating pheromone. *MATa bar1 cdc28-13* cells with the indicated *CLN* genotypes were grown at 30°C and arrested at Start by shifting the culture to 37°C for 3 hr. Synchronous cell cycle progression was induced by shifting cells back to 30°C to reactivate the thermosensitive Cdc28p. Samples for Northern analysis and microscopic examination were taken every 12 min, and an aliquot of culture was stimulated for another 12 min with 0.1 μ M mating pheromone. The percentage of unbudded cells shown in the bar graphs represents the average of samples taken at the beginning and the end of this 12-min incubation with α -factor. Northern blots were probed for the presence of *Fus1* mRNA, *CLN2* and protein 1 (Pr1)/H2A transcripts. (A) Data for a *MATa bar1 cdc28-13 CLN1 CLN2 CLN3* strain (2130-3D); (B) data for a *MATa bar1 cdc28-13 cln1 cln2 CLN3 TRP1::3xCLN3* strain (2131-10D); (C) data for a *MATa bar1 cdc28-13 CLN1 CLN2 cln3* strain (2134-1A). The *MATa bar1 cdc28-13 cln1 cln2 CLN3 TRP1::3xCLN3* strain (B) contains the *cln2-delxs* allele, which results in a nonfunctional RNA that can be detected by Northern analysis (Cross and Tinkelenberg 1991). The data presented in the three different panels are from separate experiments. The differences in the intensity of the Pr1/H2A signals between the different panels reflect mostly differences in exposure to film. Data for lanes labeled AS represent asynchronous exponential phase cultures at 30°C.

(Fig. 3). As overexpression of *CLN3* from the *GAL1* promoter has been reported to give only moderate elevations of Cln3-associated Cdc28p kinase activity (Tyers et al. 1992), we tested *FUS1* induction also in cells carrying a plasmid with an activated allele of *CLN3*, *CLN3-2*, expressed from the *GAL1* promoter (Cross 1990). Although this construct is expected to result in very strong stimulation of Cln3-associated Cdc28p kinase activity (Tyers et al. 1992), no reduction in mating pheromone signaling was observed in cells containing the *GAL1::CLN3-2* plasmid (data not shown). Furthermore, galactose-grown strains containing *GAL1::CLB5* or *GAL1::CLB2* CEN plasmids showed no suppression of *FUS1* mRNA induction (data not shown). Consistent with the results in the previous section, suppression of mating pheromone signaling appears to be specific for *CLN1* and *CLN2*.

Cell cycle regulation of *FUS1* mRNA induction in *GAL1::CLN* synchronized cultures

To study the repression of mating pheromone signaling observed in *GAL1::CLN1* and *GAL1::CLN2* cells in more detail, *MATa bar1 cln1⁻ cln2⁻ cln3⁻* cells containing one of the three *CLN* genes expressed from the

GAL1 promoter were synchronized and then challenged with α -factor at different points of the cell cycle. Synchronization was achieved by conditional expression of the *GAL1*-controlled *CLN* genes. After arrest of galactose-grown cells in raffinose due to *CLN* deprivation, synchronous cell cycle progression was induced by addition of galactose and, consequently, the stimulation of *CLN* expression (Cross and Tinkelenberg 1991). *GAL1::CLN1* synchronized cells challenged with α -factor in the G₁ phase of the cycle induced *FUS1* mRNA well, but the signaling activity was lower at later stages of the cell cycle (Fig. 4A). If cells were synchronized by using *GAL1::CLN2*, significant *FUS1* mRNA induction was only observed in the first time points after addition of galactose, and induction was very low later in the time course (Fig. 4B). The low *FUS1* mRNA induction at these later time points is consistent with the low *FUS1-lacZ* induction by mating factor in asynchronous cells (Fig. 3). Figure 4B also shows that *CLN2*, once turned on, can rapidly repress pheromone signaling. This fast repression in *GAL1::CLN2* cells is consistent with the observation that there is little delay between the maximal expression levels of *CLN2* and the period of repressed pheromone signaling in *CLN1 CLN2 CLN3* cells (Fig. 1A). It shows further that the effect of *CLN2* can occur in cells in early

Figure 3. Repression of pheromone signaling by overexpression of G_1 cyclins. *FAR1*⁺ or *far1*⁻ strains containing different *GAL1::CLN* constructs integrated at the *leu2* locus were grown on SC-galactose-ura medium to mid-exponential phase. Strains used were 2101-40A (no *GAL1::CLN*), 2101-13C (*GAL1::CLN1*), 2110-16C (*GAL1::CLN2*), 2104-75B (*GAL1::CLN3*), 2110-33C-1a (*far1*⁻), 2101-32B-1a (*far1*⁻ *GAL1::CLN1*), 2110-20A-1a (*far1*⁻ *GAL1::CLN2*), 2104-67B-1a (*far1*⁻ *GAL1::CLN3*). All strains were *MATa bar1 cln1⁻ cln2⁻ CLN3⁺* and contained the pSB231 plasmid, which contains a pheromone-inducible *FUS1-lacZ* construct. Part of the cultures was stimulated with pheromone by incubation in the presence of 0.1 μ M α -factor for 3 hr. β -Galactosidase activity in uninduced and pheromone-induced samples was then assayed and quantified as described by Ausubel et al. (1987). The values presented are the mean of two duplicate samples.

stages of the cell cycle and is not linked to changes in the cell cycle distribution like the delay in G_2 , which is known to occur in cells that overexpress *CLN1* (Richardson et al. 1989). In contrast to the *GAL1::CLN1* and *GAL1::CLN2* cells, *FUS1* inducibility was high and constant throughout the cell cycle in *GAL1::CLN3* synchronized cells (Fig. 4C). This is in agreement with the lack of signaling-suppression at any cell cycle position in the *cdc28-13 cln1 cln2 3xCLN3* strain (Fig. 2B). In all three *GAL1::CLN* cultures, the beginning of S phase, as indicated by the *H2A* transcript, roughly coincided with the emergence of buds (data not shown).

Repression of mating pheromone signaling requires active Cln-Cdc28p kinase

Cln proteins are thought to function by activating Cdc28p protein kinase (Richardson et al. 1989; Wittenberg et al. 1990; Tyers et al. 1992, 1993; Cross and Blake 1993). Therefore, we wanted to ask whether the repression of *FUS1* inducibility by *CLN2* overexpression required active Cdc28p. When *cdc28-4* cells overexpressing the different *CLN* genes from the *GAL1* promoter were challenged with α -factor at restrictive temperature, *FUS1* induction was high, independent of which *CLN* gene was linked to the *GAL1* promoter (Fig. 5).

GAL1::CLN2 resulted in sharp reduction in *FUS1* mRNA induction in the *cdc28-4* strain grown at permissive temperature (data not shown). Therefore, both a Cln1p or Cln2p regulatory subunit and an active Cdc28p kinase subunit are required for repression of mating pheromone signaling.

Effects of CLN overexpression on pheromone sensitivity and mating efficiency

We wondered whether the repression of pheromone signaling by the Cln1-Cdc28p or Cln2-Cdc28p kinase was of biological significance to the cells. Therefore, pheromone sensitivity and mating efficiency of cells overexpressing the different *CLN* genes were determined. In all cases where overexpression of *CLN* genes caused strong reduction of *FUS1* induction (*GAL1::CLN2* cells and *far1*⁻ *GAL1::CLN1* cells), strains were α -factor resistant (Fig. 6A). In contrast, cells with high *FUS1* inducibility were found to be α -factor sensitive (Fig. 6A). All strains

Figure 4. Cell cycle regulation of *FUS1* induction in *GAL1::CLN* synchronized cells. *MATa bar1 cln1⁻ cln2⁻ cln3⁻* cells overexpressing one of the three *CLN* genes from the *GAL1* promoter were grown in YEP-galactose to early exponential phase. They were then arrested as unbudded cells by filtration, resuspension into YEP-raffinose medium, and incubation in raffinose medium for 2.5–3 hr (Cross and Tinklenberg 1991). Synchronous cell cycle progression was started by addition of galactose, and aliquots were incubated every 12 min in the presence of 0.1 μ M α -factor for another 12 min. At the end of this period, samples were prepared for Northern analysis. Northern blots were probed for *FUS1* and Protein 1 (Pr1)/*H2A* mRNAs. Strains used were 1242-7C (*GAL1::CLN1*) (A), 1242-7C::pJM212 (*GAL1::CLN2*) (B), 1608-21C (*GAL1::CLN3*) (C). Differences in intensity of the Pr1/*H2A* signals between the different panels mostly reflect differences in exposure to film.

MATa bar1 cln1 cln2 CLN3

Figure 5. Repression of pheromone signaling by overexpression of *CLN1* or *CLN2* requires active Cdc28p kinase. Cells were grown to early exponential phase at 25°C, shifted to 36°C, and incubated at the restrictive temperature for 2 hr and 15 min. At this time all *cdc28-4* populations had arrested as unbudded cells. Portions of cells then were incubated with 0.1 μ M mating pheromone for another 15 min at 36°C before preparing induced and uninduced samples for Northern and microscopic analysis. RNA blots were probed for *FUS1* and the loading control *TCM1*. Strains used were 2101-40A (no *GAL1::CLN*), 2101-13C (*GAL1::CLN1*), 2110-16C (*GAL1::CLN2*), 2104-75B (*GAL1::CLN3*), 2107-15C (*GAL1::CLN1 cdc28-4*), 2125-44D (*GAL1::CLN2 cdc28-4*), and 2126-7D (*GAL1::CLN3 cdc28-4*). All strains were *MATa bar1 cln1 cln2 CLN3*.

were pheromone sensitive when grown on glucose plates (data not shown).

As some *CLN*-overexpressing strains were severely affected in their response to mating pheromone, we tested whether they were able to mate. In quantitative mating assays, *GAL1::CLN2* strains were reduced in efficiency by 30- to 40-fold; *GAL1::CLN1* and *GAL1::CLN3* strains were reduced in mating efficiency only 3- to 4-fold compared with *cln1 cln2 CLN3* controls (Fig. 6B), an effect that may be linked to the shortening of G₁ phase in these *GAL1::CLN* cells. When cells were grown and mated on glucose, the *GAL1::CLN* strains had the same mating efficiency as the strains without *GAL1::CLN* (data not shown). The mating defects correlate with the observed defects in *FUS1* transcriptional induction. *FAR1* inactivation strongly reduces mating efficiency, independent of *CLN* gene composition (Fig. 6B), by an unknown mechanism (Chang and Herskowitz 1990).

Recovery pathways dependent on either the pheromone receptor carboxyl terminus or Sst2 are not required for repression of signaling

In addition to repressing transcriptional induction by pheromone, overexpression of *CLN2* also lowered the basal level of transcription of various *STE12*-dependent genes, including *FUS1* and the α -factor receptor gene *STE2* (data not shown). To test the possibility that lowered basal expression of *STE2* would lead to lowered receptor protein levels and, thereby, to reduced respon-

siveness of cells to mating pheromone, *STE2* was overexpressed from a 2 μ -based plasmid or from a *CEN*-based *GAL1* promoter. *FUS1* mRNA induction in *GAL1::CLN2* cells overexpressing *STE2* in either of these two ways was found to be repressed just as in *GAL1::CLN2* cells carrying a control plasmid (data not shown), indicating that the repression of mating pheromone signaling is unlikely to occur primarily by affecting the receptor transcript levels.

One potential mechanism for repression of pheromone signaling could be the activation of desensitization or recovery pathways. Truncation of the carboxy-terminal part of the α -factor receptor results in supersensitivity to mating factors (Konopka et al. 1988; Reneke et al. 1988); therefore, the carboxyl terminus of the receptor has been proposed to be required for a recovery pathway. Involvement of this recovery pathway in the repression of pheromone signaling was tested by looking at *FUS1* induction in strains expressing either a full-length *STE2* gene or a mutant gene producing carboxy-terminally truncated receptors (*STE2-T326*; Konopka et al. 1988). As shown in Figure 7A, the repression of signaling by overexpression of *CLN2* did not require the presence of the receptor carboxyl terminus.

Mutations in the *SST2* gene also result in supersensitivity and strong defects in recovery from pheromone treatment; these defects are independent of the receptor carboxyl terminus-dependent pathway (Chan and Otte 1982; Dietzel and Kurjan 1987b; Konopka et al. 1988; Reneke et al. 1988). We tested whether repression of *FUS1* induction by the *Cln2*-Cdc28p kinase complex was dependent on the *SST2* gene product. As shown in Figure 7B, overexpression of *CLN2* in *sst2*⁻ cells resulted in repression of mating pheromone signaling, indicating that the *GAL1::CLN2* repression of signaling does not act through the *Sst2p*-dependent recovery pathway.

Overexpression of CLN2 suppresses gpa1 phenotypes

Disruption of the *GPA1* gene, which encodes the α -subunit of the heterotrimeric G protein involved in pheromone-signal transduction, results in a constitutive signaling phenotype, presumably due to release of the β - γ subunits (Nakayama et al. 1988; Dietzel and Kurjan 1987a; Miyajima et al. 1987; Kurjan et al. 1991; White-way et al. 1989, 1990). Inactivation of several components downstream of the α -subunit (including the β - and γ -subunits) can suppress inviability due to *Gpa1* inactivation (Nakayama et al. 1988; Blinder and Jenness 1989). If repression of pheromone signaling by the *Cln1/2*-Cdc28p protein kinase is achieved by inactivation of one or more of these downstream components, the inviability of *gpa1*⁻ cells might also be suppressed by overexpression of *CLN2*. To test this, spores were analyzed from a diploid of genotype *leu2::LEU2::GAL1::CLN2/leu2 gpa1::URA3/GPA1 ura3/ura3*. Results from this analysis are presented in Table 1 and in Figure 8A. Among the strains recovered from this tetrad dissection were Ura⁺ Leu⁺ strains (*gpa1::URA3 leu2::LEU2::*

Figure 6. Effects of repression of pheromone signaling on pheromone resistance and mating efficiency. The same *FAR1*⁺ and *far1*[−] strains as described in the legend to Fig. 3, but without the pSB231 plasmid, were grown in liquid YEP–galactose to early exponential phase. (A) Pheromone sensitivity (halo) assay. A small portion of the culture (0.2 ml) was spread on YEP–galactose plates and filter papers containing 20 μ l of 0.1 mM α -factor (upper/middle disc), 0.05 mM (lower/right disc) or 0.025 mM (lower/left disc) were placed on the surface. Plates were incubated at 30°C for 2 days. (B) Mating efficiencies of the various strains to a *MAT α* tester strain are shown. Equal numbers of the *MAT α* and *MAT α* mating partners were concentrated on nitrocellulose filters and allowed to mate on YEP–galactose plates for 4 hr at 30°C. Cells were then resuspended in water, briefly sonicated, and serial dilutions in water were spread on YC–galactose minimal and YEP–galactose plates for determination of the proportion diploids cells formed per number of viable cells. The mean percentage of diploids and the standard error for duplicate samples from three separate experiments are presented.

GAL1::CLN2) that were alive on galactose but inviable on glucose. This indicates that the inviability of *gpa1::URA3* cells was effectively suppressed by *CLN2* overexpression. We then tested whether the constitutive *FUS1* transcriptional induction phenotype of *gpa1::URA3* cells (Jahng et al. 1988) was also suppressed in *GAL1::CLN2* cells. *MAT α gpa1::URA3 GAL1::CLN2*, *MAT α GPA1 GAL1::CLN2*, and *MAT α GPA1* cells were grown to early exponential phase in liquid YEP–galactose medium. No significant differences in growth rate, cell morphology, and the percentage of budded cells were observed between the *MAT α gpa1::URA3 GAL1::CLN2* cells and *MAT α GPA1 GAL1::CLN2* cells (Fig. 8B; data not shown). When cultures were shifted to glucose medium, both *GPA1* strains continued to divide. In contrast, shift of the *gpa1::URA3 GAL1::CLN2* to YEP–glucose resulted in an arrest as unbudded cells (Fig. 8B). Six hours after the shift to glucose, samples were treated with α -factor for 15 min and *FUS1* mRNA was analyzed. Figure 8C shows that the *MAT α gpa1::URA3 GAL1::CLN2* cells shifted to glucose had high levels of *FUS1* mRNA, independent of α -factor treatment. The same cells incubated in YEP–galactose had low levels of *FUS1* mRNA in the presence or absence of mating pheromone. *GAL1::CLN2* repressed *FUS1* induction in response to α -factor in *GPA1*⁺ cells, as expected from the results described above. These data indicate that overexpression of *CLN2* can effectively suppress the constitutive signaling phenotype of *gpa1::URA3* cells. The suppression of constitutive pheromone signaling and inviability of *gpa1*[−] cells by the Cln2–Cdc28p protein kinase may therefore involve the inactivation of one or more components downstream from the G protein α -subunit.

In an initial effort to further characterize the site of repression in the pheromone signaling pathway, we found that *GAL1::CLN2* cannot suppress the lethality caused by overexpression of either *STE4* or *STE11-1* from the *GAL1* promoter (L. Oehlen and F. Cross, unpubl.).

Although this seems to suggest that *GAL1::CLN2* may have a repression effect upstream of, or at the level of the G protein β -subunit, the results from this experiment are difficult to interpret. Because both the lethal inhibitor genes (*STE4* and *STE11-1*) and the potential antagonist gene (*CLN2*) are expressed from the same *GAL1* promoter, the result of this experiment may depend on which of the two pathways, the inhibitory or the antagonistic, becomes established first. This order is important, as we found that when raffinose-grown cells are first arrested with α -factor before turning on *GAL1::CLN2* by addition of galactose, cells remain arrested (L. Oehlen and F. Cross, unpubl.). We are therefore currently trying to characterize the site of repression by other genetic and biochemical means.

Discussion

Cell cycle regulation of pheromone signaling

In response to mating pheromone, haploid cells in early G₁-phase of the cell cycle will arrest quickly at Start, whereas post-Start G₁-phase cells have acquired resistance to mating pheromone: They progress through the mitotic cycle and divide before they arrest at the next Start (Pringle and Hartwell 1981). It has been shown previously that pheromone-induced transcription of *FUS1* and *STE2* is cell cycle regulated (Zanolari and Riezman 1991). Here, we investigated this in further detail. Transcriptional induction of *FUS1* mRNA by mating pheromone is high in pre-Start cells and is markedly reduced as cells execute Start and commit themselves to the mitotic division cycle. The temporal repression of pheromone signaling is correlated with the activation of the Cdc28p protein kinase by either Cln1p or Cln2p. An independent mechanism leading to mating pheromone insensitivity at Start was described previously (McKinney et al. 1993). Thus, multiple mechanisms, including the

Figure 7. Suppression of pheromone signaling does not require α -factor receptor carboxyl terminus or *SST2*. (A) Cells with various sources of α -factor receptor genes were grown in YC-galactose-ura medium to mid-exponential phase and then challenged with 0.1 μ M α -factor for 10 min. Northern blots were probed for *FUS1* and *TCM1* transcripts. Strains used were 1255-5C (*STE2*), 2150-39A (*STE2 GAL1::CLN2*), 2150-45C (*ste2::LEU2*), and 2150-1A (*ste2::LEU2 GAL1::CLN2*). The latter two strains carried YCp50-based *URA3*-CEN plasmids with either a normal copy of the *STE2* (YCp50-*STE2*) gene or a truncated *STE2* (YCp50-*STE2-T326*) gene. The former two strains with wild-type chromosomal receptor genes carried the plasmid YCp50. All strains were *CLN1 CLN2 CLN3* except for strain 2150-39A, which was *CLN1 cln2 CLN3*. (B) Cells of the indicated genotypes were grown in YEP-galactose medium to mid-exponential phase and then challenged with 0.1 μ M α -factor for 12 min. Samples were prepared for Northern analysis, and blots were probed for *FUS1* and *TCM1* mRNAs. Strains used were 2116-7B (*BAR1 sst2 GAL1::CLN2*), 2116-1C (*BAR1 sst2*), 2116-3A (*BAR1 SST2 GAL1::CLN2*), 2116-5B (*BAR1 SST2*), 2110-14C (*bar1 SST2*), and 2110-16C (*bar1 SST2 GAL1::CLN2*). These strains were *MATa bar1 cln1 cln2 CLN3*.

repression of signaling by the Cln1/2-Cdc28p kinase, may contribute to the restriction of pheromone responsiveness and mating to the pre-Start G₁ interval of the cell cycle.

Repression of mating pheromone signaling is specific for Cln1p and Cln2p

The Start event is known to lead to several processes, including DNA replication and bud emergence (Pringle and Hartwell 1981). The repression of pheromone signaling by activation of Cln1-Cdc28p and Cln2-Cdc28p kinase complexes constitutes a novel pathway associated with the normal execution of Start. Although the three

known *CLN* genes are functionally redundant in the sense that any single one of them is sufficient for the Start event to occur (Richardson et al. 1989), they differ in other aspects. Based on DNA sequences, Cln1p and Cln2p are very similar to each other, but their homology with Cln3p is much weaker (Hadwiger et al. 1989). Cln1 and Cln2 expression is strongly cell cycle regulated, peaking at about the time of Start, whereas Cln3 expression is close to constitutive in the cell cycle (Nash et al. 1988; Hadwiger et al. 1989; Wittenberg et al. 1990; Tyers et al. 1993; Fig. 1A). Furthermore, Cln1p and Cln2p levels and the associated Cdc28p H1 kinase activities were reported to be much higher than for Cln3p (Tyers et al. 1993). These differences between *CLN1/2* on the one hand and *CLN3* on the other may also extend to different biological functions for Cln1/2-Cdc28p and Cln3-Cdc28p kinases. Cln1/2-Cdc28p kinase may be the predominant kinase activity involved in morphogenic processes (Lew and Reed 1993; Benton et al. 1993; Cvrcková and Nasmyth 1993) and Cln3-Cdc28p kinase has been proposed to be more efficient in transcriptional activation of other cyclins and thus in driving a positive feedback loop at Start (Tyers et al. 1993). Here, we describe another way in which the biological activities of Cln1/2-Cdc28p and Cln3-Cdc28p kinase clearly differ: Unlike the Cln1/2-Cdc28p kinase, the Cln3-Cdc28p kinase is ineffective in repression of mating pheromone

Table 1. Suppression of inviability of *gpa1* strains by *GAL1::CLN2*

		Genotype of spores at				
		<i>GAL1::CLN2</i>	+	–	+	–
		<i>GPA1</i>	–	–	+	+
Glucose	viable	0	0	38	39	
	inviable	39	38	0	0	
Galactose	viable	21	1 ^a	39	26	
	inviable	5	38	0	0	

A diploid of genotype *leu2::LEU2::GAL1::CLN2/leu2 gpa1::URA3/GPA1 ura3/ura3* (D109-1a) was constructed and sporulated, and tetrads were dissected on glucose or galactose medium. In dissections on glucose, no tetrads had more than two viable spores. In dissections on galactose, tetrads with three or four viable spores were recovered. For tetrads with two or more viable spores, the genotype of viable and inviable spores was determined assuming 2:2 segregation of both the *LEU2* and *URA3* markers (allowing inference of the genotype of most of the inviable spores (Sherman et al. 1989); 2:2 segregation was observed in all four-viable-spore tetrads; see also Fig. 8A). Very tiny colonies observed in many tetrads (presumably because of *gpa1::URA3*, Dietzel and Kurjan 1987a) were scored as inviable. Direct determination of markers in such spore colonies was not attempted.

^aSmall slow-growing colony. *gpa1::URA3* spores were observed previously to give rise to such colonies at a low frequency, presumably because *gpa1::URA3* does not result in complete lethality (Dietzel and Kurjan 1987a; J. Hirsch, pers. comm.). In contrast, *gpa1::URA3 GAL1::CLN2* spores gave rise to large, fast-growing colonies (see also Fig. 8A).

Figure 8. Overexpression of *CLN2* can suppress constitutive signaling and inviability in *gpa1*-disrupted cells. (A) From the tetrad analysis described in Table 1, eight tetrads with four viable spores were selected and small quantities of cells from each spore colony were diluted in water and spotted on YEP-galactose, YEPD, YC-galactose-ura, and YC-galactose-leu plates. Cell growth on the different plates is shown after 2 days of incubation at 30°C. (B,C) Cells were grown to early exponential phase in YEP-galactose, filtered, resuspended, and further incubated in YEP-dextrose medium at 30°C. Strains were *GPA1* (W2153-1C), *GPA1 GAL1::CLN2* (W2154-18N), and *gpa1::URA3 GAL1::CLN2* (W2151-6C). These strains were *MATa BAR1 CLN1 CLN2 CLN3*. (B) The percentage of unbudded cells was followed at various time points after the shift to glucose. (C) After 6 hr of incubation in glucose, portions of the cultures were challenged for 15 min with 1 μ M α -factor, and then samples were taken for analysis of the levels of *FUS1* and *TCM1* mRNA.

signaling, even under conditions where it is equally effective at driving cell cycle progression (Figs. 2 and 4).

The Cdc28p kinase is thought to be stimulated at Start by its interaction with the Cln proteins and at later points in the cell cycle by its interaction with cyclins of the *CLB* class (Ghiara et al. 1991; Surana et al. 1991; Epstein and Cross 1992; Richardson et al. 1992; Schwob and Nasmyth 1993). Clb proteins are present in high concentrations and are potent activators of the Cdc28p kinase. However, our data give no indication that activation of the Cdc28p kinase by Clb proteins can repress mating pheromone signaling. No significant repression of pheromone-induced *FUS1* transcription was observed in *GAL1::CLB5* or in *GAL1::CLB2* cells. Furthermore, in synchronized cells lacking *CLN1* and *CLN2* no repression of signaling was observed at any phase of the cycle (Figs. 2 and 4), whereas the regulation of the *CLB* class of cyclins is presumably normal in these cultures. Signaling repression, therefore, does not occur if Cdc28p kinase is activated with Clbs and is probably specifically linked to the activation of Cdc28p by Cln1p and Cln2p.

It is interesting to note that *GAL1::CLN1 far1⁻* cells are pheromone resistant and have reduced pheromone-induced *FUS1* transcription, whereas *GAL1::CLN1 FAR1⁺* cells are pheromone sensitive and display normal *FUS1* induction (Figs. 3 and 6A). This shows that Far1p cannot only interact with Cln2p, as suggested by genetic and biochemical studies (Chang and Herskowitz 1990; Peter et al. 1993; Tyers and Futcher 1993; J. McKinney and F. Cross, unpubl.) but can also interfere, probably at the post-transcriptional level, with the functioning of Cln1, as has been suggested earlier by others (Valdivieso et al. 1993).

Mechanism of signaling repression

Sst2p and the carboxy-terminal part of the pheromone receptor have been implicated in recovery from mating pheromone-induced cell cycle arrest (Dietzel and Kurjan 1987b; Konopka et al. 1988; Reneke et al. 1988) but are not required for the repression of pheromone signaling by the Cln2-Cdc28p kinase (Fig. 7A, 7B). Furthermore, our data on overexpression of the *STE2* receptor indicate that the repression mechanism does not depend on the transcriptional down-regulation of receptor mRNAs. The suppression of *gpa1* by *GAL1::CLN2* shows that the target for repression is likely to be downstream of the α -subunit of the heterotrimeric G protein. Further epistatic and biochemical studies could provide a more precise definition of the site and mechanism of repression for the Cln1/2-Cdc28p kinase complex.

It is clear that at least some components of the pheromone signal transduction route and of the cell cycle control machinery in *Saccharomyces cerevisiae* are conserved in other unicellular and multicellular eukaryotes. Heterotrimeric G proteins and protein kinase cascades consisting of triplets of kinases are all to be found in a wide variety of eukaryotic organisms (Kaziro et al. 1993; Kosako et al. 1993; Neiman et al. 1993). Furthermore, the cell cycle machinery based on cyclin-dependent pro-

tein kinases appears to be remarkably well conserved among eukaryotes (Solomon 1993; Hunter 1993). Elucidation of the mechanism by which the Cln1/2–Cdc28p kinase can repress pheromone signaling in yeast may therefore be of relevance also for the cell cycle regulation of signal transduction pathways in other eukaryotes.

Materials and methods

Strains and plasmids

The genotypes of the strains used in this study are given in Table 2. Unless indicated otherwise, strains were isogenic to BF264-15D (*trp1-1a leu2-3,112 ura3 ade1 his2*) in most of which a nonreverting null allele of *ARG4* was installed (Epstein and Cross 1992). Strain K2944 [provided by K. Nasmyth (Research Institute for Molecular Pathology, Vienna, Austria)], the diploid strain D109-1a [derived from the D109 strain provided to us by J. Hirsch (Mt. Sinai Medical Center, NY)] and strains W2153-1C, W2154-18B and W2151-6C (all three from spores of D109-1a) had a W303 background. Mutant alleles were constructed for

cln1 or *cln2* deletion (Cross and Tinkelenberg 1991), *cln1::TRP1* (Richardson et al. 1989), *cln3* deletion (Cross 1990), *cdc28-4* (Reed 1980), *cdc28-13* (Reed 1980), *sst2::URA3* (Dietzel and Kurjan 1987b) and *gpa1::URA3* (Dietzel and Kurjan 1987a). The *leu2::LEU2::GAL1::CLN1* was constructed by cutting the *YlpLEU2::GAL1::CLN1* plasmid (see below) with *HpaI* to target integration at *leu2*. The *leu2::LEU2::GAL1::CLN2* was constructed by *KpnI*-targeted integration at *leu2* of plasmid *YlpJM212* (see below). *YlpLEU2::GAL1::CLN3* (see below) was cut with *ClaI* to target integration at *leu2* and to make *leu2::LEU2::GAL1::CLN3*. The copy number after integration was tested by Southern analysis and found to be one for all three *GAL1::CLN* integrants, except in the D109-1a strain, where the copy number of *leu2::LEU2::GAL1::CLN2* was estimated to be higher than one — probably two or three. The *far1::ura3-ns* allele was constructed from a *far1::URA3* allele (Chang and Herskowitz 1990) by integration of a nonfunctional *ura3-del-ns* (Richardson et al. 1989) at *URA3* and selection on fluoro-orotic acid (FOA) for *ura⁻* cells (Ausubel et al. 1987). The 3x*CLN3* expression construct was made by *BglII*-targeted integration of a *CLN3* containing YRp7 plasmid at the *TRP1* locus. The copy number after integration was estimated to be approximately

Table 2. Strain list

Strain	Genotype	Source
1242-7C	<i>MATa bar1 cln1::TRP1 cln2Δ cln3Δ YCpURA3::GAL1::CLN1</i>	
1242-7C::pJM212	<i>MATa bar1 cln1::TRP1 cln2Δ cln3Δ leu2::LEU2::GAL1::CLN2</i>	
1255-5C	<i>MATa bar1 CLN1 CLN2 CLN3</i>	
1608-21C	<i>MATa bar1 cln1::TRP1 cln2Δ cln3Δ leu2::LEU2::GAL1::CLN3</i>	
2101-13C	<i>MATa bar1 cln1::TRP1 cln2Δ CLN3 leu2::LEU2::GAL1::CLN1</i>	
2101-32B-1a	<i>MATa bar1 cln1::TRP1 cln2Δ CLN3 far1::ura3-ns</i>	
2101-40A	<i>MATa bar1 cln1Δ cln2Δ CLN3</i>	
2101-4B-1a	<i>MATa bar1 cln1Δ cln2Δ CLN3 far1::ura3-ns</i>	
2104-67B-1a	<i>MATa bar1 cln1::TRP1 cln2Δ CLN3 far1::ura3-ns leu2::LEU2::GAL1::CLN3</i>	
2104-75B	<i>MATa bar1 cln1::TRP1 cln2Δ CLN3 leu2::LEU2::GAL1::CLN3</i>	
2110-14C	<i>MATa bar1 cln1 cln2Δ CLN3</i>	
2110-16C	<i>MATa bar1 cln1 cln2Δ CLN3 leu2::LEU2::GAL1::CLN2</i>	
2110-20A-1a	<i>MATa bar1 cln1 cln2Δ CLN3 far1::ura3-ns leu2::LEU2::GAL1::CLN2</i>	
2110-33C-1a	<i>MATa bar1 cln1 cln2Δ CLN3 far1::ura3-ns</i>	
2116-1C	<i>MATa BAR1 sst2::URA3 cln1 cln2Δ CLN3</i>	
2116-3A	<i>MATa BAR1 cln1 cln2Δ CLN3 leu2::LEU2::GAL1::CLN2</i>	
2116-5B	<i>MATa BAR1 cln1 cln2Δ CLN3</i>	
2116-7B	<i>MATa BAR1 sst2::URA3 cln1 cln2Δ CLN3 leu2::LEU2::GAL1::CLN2</i>	
2125-44D	<i>MATa bar1 cdc28-4 cln1 cln2Δ CLN3 leu2::LEU2::GAL1::CLN2</i>	
2126-7D	<i>MATa bar1 cdc28-4 cln1 cln2Δ CLN3 leu2::LEU2::GAL1::CLN3</i>	
2130-3D	<i>MATa bar1 cdc28-13 CLN1 CLN2 CLN3</i>	
2131-10D	<i>MATa bar1 cdc28-13 cln1 cln2-delxs CLN3 TRP1::3xCLN3</i>	
2134-1A	<i>MATa bar1 cdc28-13 CLN1 CLN2 cln3Δ</i>	
2150-39A	<i>MATa bar1 CLN1 cln2Δ CLN3 leu2::LEU2::GAL1::CLN2</i>	
2150-45C	<i>MATa bar1 CLN1 CLN2 CLN3 ste2::LEU2</i>	
2150-1A	<i>MATa bar1 CLN1 CLN2 CLN3 ste2::LEU2 leu2::LEU2::GAL1::CLN2</i>	
K2944	<i>MATa bar1::URA3 cdc15-2</i>	a
D109-1a	<i>MATa/MATα gpa1::URA3/GPA1 leu2/leu2::LEU2::GAL1::CLN2</i>	b
W2151-6C	<i>MATa BAR1 CLN1 CLN2 CLN3 leu2::LEU2::GAL1::CLN2 gpa1::URA3</i>	b
W2153-1C	<i>MATa BAR1 CLN1 CLN2 CLN3</i>	b
W2154-18B	<i>MATa BAR1 CLN1 CLN2 CLN3 leu2::LEU2::GAL1::CLN2</i>	b

Unless indicated otherwise, strains were isogenic derivatives of BF264-15D (*trp1-1a leu2-3,112 ura3 ade1 his2*) from our laboratory collection (see also Materials and methods).

^aStrain K2944 was provided by K. Nasmyth and is isogenic to strain W303-1a, whose genotype is *MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3 ssd1*.

^bThe diploid strain D109-1a was derived from the W303 isogenic D109 strain (*MATa/MATα GPA1/gpa1::URA3 leu2/leu2*) provided to us by J. Hirsch. Strains W2153-1C, W2154-18B, and W2151-6C were derived from D109-1a by sporulation.

three by Southern analysis. Strains were constructed by standard techniques (Ausubel et al. 1987).

The YipLEU2::GAL1::CLN3 plasmid was constructed by cutting pW16 (GAL1::CLN3 in YCp50; Cross 1990) with *SalI* and *BglII* to remove a *URA3-CEN-ARS* fragment. This fragment was then replaced with a *LEU2*-containing *SalI*-*BamHI* fragment from YCpG2 (provided by S. Reed, Research Institute of Scripps, La Jolla, CA). YipLEU2::GAL1::CLN1 was constructed by partially cutting the YCpG2-GAL1::CLN1 plasmid (GAL1::CLN1-LEU2-URA3-CEN-ARS, [Richardson et al. 1989] with *PstI*. A fragment deleted for *URA3*, *CEN*, and *ARS* was then religated. YipJM212 (YipLEU2-GAL1::CLN2) was constructed by J. McKinney (Rockefeller University). First, *BamHI* and *HindIII* restriction sites were created by PCR for a 5' *CLN2* fragment with ~1.1 kb of the *CLN2*-coding sequence (a 5'-TTATTAGGATCCGTACCATGGCTAGTGCTGAACC-AAGAC-3' oligonucleotide was used to create the *BamHI* site just 5' of the ATG start codon and a 5'-GATATGGTAAGCTT-TCTCGAAA-3' oligonucleotide to create the *HindIII* site). This *BamHI*-*HindIII* fragment was ligated into pRS316 to yield pJM208. A *BclII*-*Clal* fragment containing the 3' *CLN2*-coding sequence and some 3' flanking DNA (from a YCp50-based plasmid with a large genomic *CLN2* fragment) was then ligated into *BclII*-*Clal*-digested pJM208 to yield pJM211. Finally, YipJM212 was constructed by exchanging a 1.9-kb *BamHI*-*SalI* fragment from pJM211 (containing the full *CLN2* open reading frame and some 3' flanking sequence) with the *CLN3*-containing *BamHI*-*SalI* fragment from YipLEU2::GAL1::CLN3 (see above). YEp24-*STE2* and YCpURA3-GAL1::*STE2* were provided by J. Hirsch. Both plasmids for *STE2* overexpression fully complemented a *ste2* deletion as judged from a restoration of α -factor sensitivity, mating, and *FUS1* mRNA inducibility by both plasmids in *ste2::LEU2* strains (data not shown). pSB231 (a *CEN*-based *URA3* plasmid containing a *FUS1*-linked *lacZ* gene) [Trueheart et al. 1987] was provided by G. Fink. YCp50-*STE2* and YCp50-*STE2*-*T326* [Konopka et al. 1988] were provided by J. Konopka. YCpGAL1::*CLB2* [Surana et al. 1993] was provided by B. Futcher (Cold Spring Harbor Laboratory) and YCpGAL1::*CLB5* by C. Epstein (Princeton University, NJ). YCp50-GAL1::*CLN3*-2 was described previously [Cross 1990].

Growth conditions and synchronization procedures

Cells were grown in YEP medium (1% Difco yeast extract, 2% Difco Bacto-peptone) containing 2% glucose, 3% galactose, or 3% raffinose as a carbon source. For growth on solid media 2% Difco agar was added. In experiments where retainment of plasmids was required, synthetic dropout medium was used with 1.7 g/l of Difco Yeast Nitrogen base (without amino acids and ammonium sulfate), 5 g/l of ammonium sulfate, 10 g/l of succinic acid, 6 g/l of NaOH, and amino acids in concentrations according to Ausubel et al. (1987).

Synchronous division was induced by one of several different protocols. *cdc15-2* cells were grown in YEPD at a permissive temperature of 25°C to an OD₆₆₀ of 0.5. Cells were arrested in late M phase by incubation at restrictive temperature of 36°C for 3 hr, and synchronous division was then induced by adding ice-cold YEPD until the starting OD₆₆₀ was ~0.7 and further cooling the cultures on ice to 25°C. Samples for Northern mRNA analysis and for microscopic examination were withdrawn every 12 min. For α -factor-stimulated samples, 10 ml of culture was added to a tube containing 10 μ l of 0.1 mM α -factor and this tube was then incubated for 12 min at permissive temperature before harvesting for Northern analysis (see below).

Synchronization of *cln1*⁻ *cln2*⁻ *cln3*⁻ cells with either a functional *CLN1*, *CLN2*, or *CLN3* gene expressed from the

GAL1 promoter was as described previously [Cross and Tinklenberg 1991]. In short, cells were grown to early exponential phase in YEP-galactose at 30°C, filtered, resuspended in YEP-raffinose and incubated for 2.5 hr. Because the *CLN* gene driven by the *GAL1* promoter is not expressed in raffinose medium, cells arrested during this time at Start because of *Cln* deprivation [Richardson et al. 1989; Cross 1990]. Synchronous growth was then induced by the addition of concentrated galactose [30% (wt/vol)] to a final concentration of 3%.

cdc28-13 cells with different *CLN* genotypes were synchronized essentially as described above for *cdc15* cells, except that the permissive temperature was 30°C and the restrictive temperature was 37°C.

Quantitative mating and halo assays

For quantitative mating assays, 2.5×10^6 or 5×10^6 of exponentially growing MATa cells were mixed with an equal number of MATa tester cells. Cells were concentrated on filters (0.65 pore size, 25-mm diam.), and the filters were placed, cell side up, on YEPD or YEP-galactose plates. After 4 hr of incubation at 30°C, the filtered cells were resuspended in water and briefly sonicated. Serial dilutions of 200 μ l in water were spread on YEPD or YEP-galactose for determination of the titer of viable cells and on YC-glucose or YC-galactose minimal plates for determination of the titer of diploid cells. Diploid cell colonies were selected on the basis of complementation of amino acid auxotrophies.

For halo assays, early exponential phase cultures were spread on YEPD or YEP-galactose plates and paper discs (0.635-cm diam., no. 740-E, Schleicher & Schuell, Keene, NH) containing 20 μ l of 0.1, 0.05, or 0.025 mM α -factor were placed on the surface. Plates were incubated at 30°C for 2 days.

Northern blot analysis

Procedures for mRNA analysis were as described previously [McKinney et al. 1993]. In short, 10 ml of log-phase culture was poured onto an equal volume of crushed ice and cells were collected by centrifugation. Cells were disrupted by vortexing with glass beads, and proteins were extracted with phenol/chloroform/isoamyl alcohol 25:24:1. These crude RNA extracts were run on formaldehyde gels, and the RNA was transferred to GeneScreen-plus nylon membranes by capillary blotting for at least 12 hr. Membranes were airdried for 30 min, X-linked in a Stratalinker (Stratagene, La Jolla, CA) and baked in vacuo in an oven at 80°C for 2 hr.

DNA restriction fragments for probing were excised from low melting point agarose gels and labeled by random-prime labeling using a Prime-It kit (Stratagene). For *CLN3* a 1-kb *EcoRI* fragment from pBSI6 (containing almost the entire *CLN3*-2 open reading frame) was used and for *H2A*/protein 1 transcripts, a 2.3 kb *SacI* fragment from plasmid pDN1 was used [provided by M. Osley [Osley et al. 1986]]. Other fragments used in hybridizations were described previously [Cross and Tinklenberg 1991; McKinney et al. 1993].

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