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FAR1 Is Required for Posttranscriptional Regulation of *CLN2* Gene Expression in Response to Mating Pheromone

M. HENAR VALDIVIESO, KATSUNORI SUGIMOTO,† KWANG-YEOP JAHNG,‡
PATRICIA M. B. FERNANDES, AND CURT WITTENBERG*

Departments of Molecular Biology and Cell Biology, The Scripps Research Institute, La Jolla, California 92037

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Yeast cells arrest during the G_1 interval of the cell cycle in response to peptide mating pheromones. The *FAR1* gene is required for cell cycle arrest but not for a number of other aspects of the pheromone response. Genetic evidence suggests that *FAR1* is required specifically for inactivation of the G_1 cyclin *CLN2*. From these observations, the *FAR1* gene has been proposed to encode an element of the interface between the mating pheromone signal transduction pathway and the cell cycle regulatory apparatus. We show here that *FAR1* is necessary for the decrease in *CLN1* and *CLN2* transcript accumulation observed in response to mating pheromone but is unnecessary for regulation of the same transcripts during vegetative growth. However, the defect in regulation of *CLN1* expression is dependent upon *CLN2*. We show that pheromone regulates the abundance of Cln2 at a posttranscriptional level and that *FAR1* is required for that regulation. From these observations, we suggest that *FAR1* function is limited to posttranscriptional regulation of *CLN2* expression by mating pheromone. The failure of mating pheromone to repress *CLN2* transcript levels in *far1* mutants can be explained by the stimulatory effect of the persistent Cln2 protein on *CLN2* transcription via the *CLN/CDC28*-dependent feedback pathway.

The capacity of cells to regulate cell cycle progression in response to both internal and environmental stimuli is essential for their continued viability and, in the case of metazoans, for the viability of the entire organism. To achieve such regulation, cells must be capable of translating the signals generated by those stimuli into cell cycle regulatory responses. As in most other organisms, cell proliferation in the budding yeast *Saccharomyces cerevisiae* is responsive to a number of external and physiological signals. Nutrient limitation and exposure to mating pheromone, the primary external signals, inhibit cell cycle progression specifically during the G_1 interval (see references 26 and 33 for reviews). Thus, both act through transduction pathways that must ultimately affect the elements that govern cell cycle progression. Although a detailed description of these elements is beginning to emerge, it is unclear how these signal transduction systems act to modulate their activity. Achieving an understanding of that process depends on the identification and characterization of the elements that act at the interface between these signal transduction systems and the cell cycle regulatory machinery.

In *S. cerevisiae*, the mating pheromones **a** factor and α factor, elaborated by haploid cells of the **a** and α mating types, respectively, induce a number of responses in cells of the opposite mating type (reviewed by Marsh et al. [26]). These include changes in morphology and the pattern of gene expression as well as inhibition of cell cycle progression during the G_1 interval. All of these effects are known to occur through stimulation of a signal transduction pathway which is initiated by binding of the peptide pheromone to a heterotrimeric G protein-coupled cell surface receptor (encoded by *STE3* or *STE2*). Many of the other components of this transduction pathway are known. The induction of mating pheromone-specific genes occurs through the action of the *STE12* gene product as a consequence of its binding to a pheromone-specific transcription-activating sequence known as the pheromone response element (11, 14). Several of these pheromone-specific genes are known to be involved in the mating process. In contrast, the mechanism by which the same pathway results in G_1-specific cell cycle arrest is not understood. Although *STE12* function has been implicated in this arrest, the nature of its involvement is not known (10).

Cell cycle progression in budding yeasts is known to require the activity of the *CDC28* gene product (20, 34, 35), a serine/threonine protein kinase of the Cdk (cyclin-dependent kinase) family, which includes the Cdc2 protein kinase (reviewed by Pines and Hunter [32]). The function of the *CDC28* gene product is essential for passage through the G_1/S and G_2/M transitions. Its role at each of these transitions is performed in conjunction with those of distinct families of cyclin proteins, the G_2/M function requiring B-type cyclins encoded by the *CLB* genes (16, 40) and the role during G_1 phase requiring the G_1 cyclins encoded by the *CLN* genes. The *CLN* gene family consists of three genes, *CLN1*, *CLN2*, and *CLN3*, which perform an overlapping function that is essential for progression through G_1 phase (5, 18, 27, 36). Inactivation of all three *CLN* genes but not any of the pairwise combinations results in arrest at START in a state reminiscent of cells arrested by inactivation of *CDC28* or by mating pheromone (36). The transcripts of the *CLN1* and *CLN2* genes, as well as the Cln2 protein, have been demonstrated to accumulate periodically during the cell cycle, peaking during late G_1, at the time of their essential function (43). While it is presumed that the Cln1 protein behaves similarly, that has not been demonstrated. During the G_1 interval, the Cln proteins associate with $p34^{cdc28}$ to

* Corresponding author.
† Present address: Department of Molecular Biology, School of Science, Nagoya University, Nagoya 464-01, Japan.
‡ Permanent address: Department of Biology, College of Natural Sciences, Chonbuk National University, Chonbuk, Chonju 560-756, Korea.

TABLE 1. List of strains

Strain[a]	Relevant genotype	Source or reference
15Dau	*MATa ade1 his2 leu2 trp1 ura3Δns*	4
15Daub	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ*	S. Reed
D13	*MATα ade1 his2 leu2 trp1 cdc28-13*	S. Reed
FC280	*MATa ade1 his2 leu2 trp1 ura3Δns bar1::LEU2 far1::URA3*	2
CWY149	*MATα ade1 his2 leu2 trp1 ura3Δns cln1::TRP cln2::LEU2 cln3::ura3*/YCpG2CLN1	36
DL4	*MATa ade1 his2 leu2 trp1 ura3Δns cln3Δ*	6
CWY181	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ cdc28-13*	Segregant from 15Daub × D13
CWY222	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ cdc28-13 far1::URA3*	This study
CWY228	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ cln1::TRP*	Segregant from CWY149 × 15Dau
CWY229	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ cln2::LEU2*	Segregant from CWY149 × 15Dau
CWY230	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ cln1::TRP cln2::LEU2*	Segregant from CWY149 × 15Dau
KJY47	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ cln1::TRP cln3Δ*	Segregant from CWY230 × DL4
KJY102	*MATa ade1 his2 leu2 trp1 ura3Δns cln3Δ*	Segregant from CWY230 × DL4
FC310	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ cln2::LEU2 far1::URA3*	2
FC322	*MATa ade1 his2 leu2 trp1 ura3Δns bar1::LEU2 cln1::TRP cln3::ura3Δ far1::URA3*	2
HVY33	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ cln2::LEU2 HIS2::CLN2^{3p}*	This study
KJY95	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ cln1::TRP cln2::LEU2 HIS2::CLN2^{3p}*	This study
HVY35	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ cln2::LEU2 far1::URA3 HIS2::CLN2^{3p}*	This study
KJY98	*MATa ade1 his2 leu2 trp1 ura3Δns bar1Δ cln1::TRP cln2::LEU2 HIS2::CLN2^{3p} far1::URA3*	This study
1258-14B	*MATa ade1 his2 leu2 ura3Δns bar1Δ cln1Δ cln2Δxs cln3Δ TRP1::GAL-CLN3*	J. McKinney and F. Cross
DLY518	*MATa ade1 his2 leu2 ura3Δns bar1Δ cln1Δ cln2Δxs cln3Δ TRP1::GAL-CLN3 far1::URA3*	D. Lew

[a] All strains are isogenic derivatives of BF264-15D.

form an active protein kinase complex. That association is thought to be essential for activation of the G_1-specific functions of the kinase. Most important in terms of the studies reported here, the accumulation of the *CLN1* and *CLN2* transcripts and their protein products is negatively regulated by exposure of cells to mating pheromone (43). From these observations, we have proposed that the *CLN* genes or their products are the targets of the mating pheromone signal transduction pathway and that their inactivation ultimately results in G_1 arrest.

Support for this hypothesis is derived from the study of the mating pheromone resistance mutation *far1* (2). Inactivation of the *FAR1* gene causes cells to fail to arrest in response to mating pheromone without interrupting other aspects of the mating pheromone response. Thus, while *far1* mutant cells undergo the morphological alterations associated with pheromone exposure and show pheromone-specific gene expression, they do not arrest during G_1 and consequently continue to proliferate. Strikingly, this defect is efficiently suppressed by inactivation of the *CLN2* gene but not by inactivation of either *CLN1* or *CLN3*. This observation led to the proposal that mating pheromone acts through *FAR1* to inactivate *CLN2* as well as through other *FAR1*-independent mechanisms to inactivate *CLN1* and *CLN3*. This predicts that, whereas *CLN1* and *CLN3* would be inactivated by mating pheromone in the absence of functional *FAR1*, *CLN2* would not, and as a result, cells would continue to proliferate. Alternatively, in cells lacking *CLN2*, *FAR1* would be nonessential for mating pheromone-induced arrest, since other mechanisms exist for the elimination of *CLN1* and *CLN3*.

Exposure of cells to mating pheromone results in repression of *CLN1* and *CLN2* gene expression (43), leading to the suggestion that *FAR1* acts specifically to mediate pheromone-induced transcriptional repression of *CLN2*. However, recent evidence (7, 9) demonstrates that transcription of *CLN1* and *CLN2* is coordinately regulated through a positive feedback loop that requires functional *CDC28* and a functional *CLN* gene. From these observations, Cross and Tinklenberg (7) have argued that the "trident model" proposed by Chang and Herskowitz (2) is oversimplified and predicted that loss of *FAR1* should disrupt pheromone regulation of both *CLN1* and *CLN2* gene expression.

In the work described here, we tested these hypotheses and attempted to elucidate the role of *FAR1* in cell cycle regulation by mating pheromone. We show that *FAR1* is required for the negative regulation of *CLN2* transcript abundance in response to mating pheromone but is not required for cell cycle regulation of that transcript during vegetative growth. While *far1* mutants are also defective in regulation of *CLN1* transcript abundance by mating pheromone, this defect apparently occurs as a result of deregulation of *CLN2* expression, consistent with the coordinate regulation of these genes through a positive feedback mechanism. We provide evidence that *FAR1* does not act at the level of *CLN2* transcription but is instead required for the pheromone-induced loss of Cln2 protein. Our results suggest that this defect ultimately results in inactivation of the *CLN/CDC28*-dependent feedback mechanism required for maximal expression of *CLN1* and *CLN2*. We propose that the defect in posttranscriptional regulation of Cln2 is the primary cause of mating pheromone resistance in *far1* mutants.

MATERIALS AND METHODS

Strain construction and recombinant DNA manipulation. The strains used in this study are listed in Table 1. All strains are isogenic derivatives of BF264-150 (4). Replacement of chromosomal loci with mutant derivatives was done by one-step gene transplacement (37). Insertional mutations and deletions of *CLN1* and *CLN2* (17), *CLN3* (5), and *FAR1* (2) have been described previously.

An integrating plasmid (pHV104) containing the chimeric gene *CLN2^{3p}* was constructed as follows. Approximately 0.9 kb of the upstream untranslated region of the *CLN3* gene, extending from the naturally occurring *Sal*I site (5) to a *Bam*HI site which was introduced at position −13, was obtained by oligonucleotide-directed mutagenesis and polymerase chain reaction. This fragment was ligated to a

*Bam*HI fragment generated by polymerase chain reaction mutagenesis, containing the entire open reading frame of *CLN2* along with upstream sequences to −8 from the ATG and 56 nucleotides downstream from the translation termination site (17). A pUC18 plasmid was constructed that contained the yeast *HIS2* gene as a 1.35-kb *Eco*RI-*Sma*I fragment (23) and the $CLN2^{3P}$ chimera. Integration into the yeast genome was done by transformation with the plasmid which had been linearized at the *Hpa*I site of *HIS2* (38). All integrants were shown to be present in single copy at the *HIS2* locus by Southern blot analysis.

YEp*lac*112-*CLN1* and YCp*lac*33-*CLN1* contain the entire *CLN1* gene on the 3.1-kb *Bam*HI-*Hin*dIII fragment from pJHB1a (18) cloned into the polylinker of YEp*lac*112 or YCp*lac*33 (15), respectively. YEp*lac*112-*CLN3* contains the entire *CLN3* gene carried on a 3.2-kb *Sal*I-*Bam*HI fragment.

Northern (RNA blot) analysis. Total RNA was prepared from yeast cells by the method of Elder et al. (11a) and separated on 1% agarose gels containing formaldehyde. The RNA was then transferred to Magnagraph nylon membranes (Micron Separation, Inc.), and hybridization was performed as described previously (24). Probes were radiolabeled with [α-^{32}P]dCTP by random-primed labeling (Boehringer Mannheim) according to the manufacturer's instructions. The DNA probes used were as follows: for *CLN1*, the entire open reading frame on a 1.8-kb *Bam*HI fragment from the plasmid pUC19-*CLN1*BB; for *CLN2*, the entire open reading frame on a 1.8-kb *Bam*HI fragment from the plasmid pUC19-*CLN2*BB; for *CLN3*, the 1.0-kb *Hin*dIII-*Bam*HI fragment; for *FUS1*, the 1.0-kb *Pst*I-*Bam*HI fragment (41); and for *ACT1*, the 1.6-kb *Bam*HI-*Hin*dIII fragment containing the majority of the *ACT1* open reading frame (30).

Gel electrophoresis and immunoblotting. Polyacrylamide gel electrophoresis (PAGE) was performed with protein extracts prepared as described before (43). Protein sample quantities were normalized by A_{280} measurements, and 0.8 A_{280} unit of protein was loaded per lane. All protein gels were 6%/15% SDS polyacrylamide gradient gels. Immunoblotting was performed by electroblotting proteins to Magnagraph nylon membranes as described before (19). Membranes were blocked in 10% nonfat dry milk in Tris-buffered saline with 0.25% Tween 20, incubated overnight with affinity-purified anti-Cln2 serum (43) diluted 1:3,000 that had been incubated for 1 h in the presence of a total cell lysate of a *cln1*::*TRP1 cln2*::*LEU2* mutant strain (CWY230). This incubation was followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (1:7,500; Promega). Development was done with the chemiluminescent dye Lumiphos 530 (Boehringer Mannheim) according to the manufacturer's instructions. Membranes were exposed for 1 to 30 min.

Mating pheromone treatments, halo assays, and cell cycle synchronization. Mating pheromone arrest and arrest release synchrony were done as previously described (43) with the modifications noted in the figure legends.

Halo assays were performed as described previously (21). Briefly, approximately 10^5 cells were plated in 8 ml of molten nutrient agar on a plate of the same composition. Once solidified, 2 μl of α-factor at the designated concentrations was spotted onto the plate and allowed to diffuse during the growth period. All strains used were *bar1* mutants.

RESULTS

FAR1* is required for pheromone-induced repression of *CLN1* and *CLN2* transcription.** Mating pheromone leads to a decrease in the abundance of the *CLN1* and *CLN2* transcripts (43). We have analyzed the behavior of those transcripts in *far1* mutants following treatment with mating pheromone. *MATa** *bar1* mutant cells carrying either a wild-type *FAR1* gene (15Daub) or a *far1*::*URA3* insertional mutation (FC280; referred to herein as *far1*Δ) were exposed to the mating pheromone α-factor at 125 nM (200 ng/ml) for various times (Fig. 1), and their ability to arrest in the G_1 phase of the cell cycle was evaluated by determining the

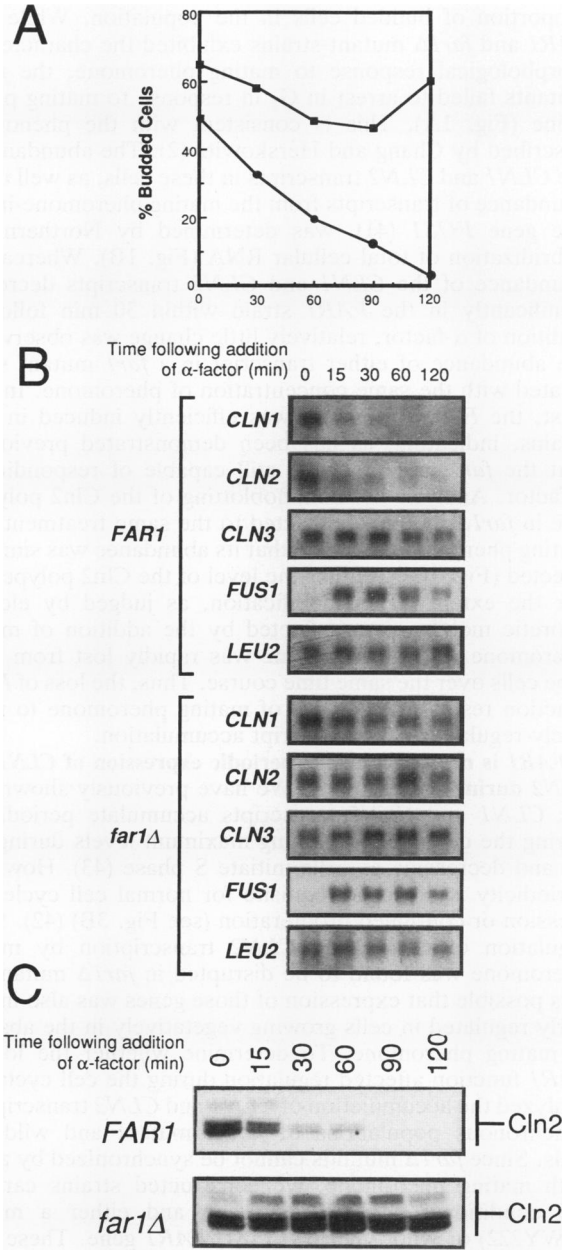

FIG. 1. Abundance of *CLN1* and *CLN2* transcripts and Cln2 protein in *FAR1* and *far1*Δ strains responding to mating pheromone. Wild-type cells (15Daub) (●) or cells carrying a *far1*Δ disruption (FC280) (■) were treated with 200 ng of α-factor per ml for the times (minutes) indicated. Cells were harvested and analyzed for percent budded cells (A), abundance of the indicated RNAs by Northern blot analysis (B), and abundance of the Cln2 polypeptide by immunoblotting (C).

proportion of budded cells in the population. While both *FAR1* and *far1*Δ mutant strains exhibited the characteristic morphological response to mating pheromone, the *far1*Δ mutants failed to arrest in G_1 in response to mating pheromone (Fig. 1A). This is consistent with the phenotypes described by Chang and Herskowitz (2). The abundance of the *CLN1* and *CLN2* transcripts in these cells, as well as the abundance of transcripts from the mating pheromone-inducible gene *FUS1* (41), was determined by Northern blot hybridization of total cellular RNA (Fig. 1B). Whereas the abundance of the *CLN1* and *CLN2* transcripts decreased significantly in the *FAR1* strain within 30 min following addition of α-factor, relatively little change was observed in the abundance of either transcript in a *far1* mutant strain treated with the same concentration of pheromone. In contrast, the *FUS1* transcript was efficiently induced in both strains, indicating, as has been demonstrated previously, that the *far1* mutants were still capable of responding to α-factor. Analysis by immunoblotting of the Cln2 polypeptide in *far1*Δ mutants subjected to the same treatment with mating pheromone revealed that its abundance was similarly affected (Fig. 1C). Neither the level of the Cln2 polypeptide nor the extent of its modification, as judged by electrophoretic mobility, was affected by the addition of mating pheromone. In contrast, Cln2 was rapidly lost from wild-type cells over the same time course. Thus, the loss of *FAR1* function results in a failure of mating pheromone to negatively regulate *CLN2* transcript accumulation.

***FAR1* is not required for periodic expression of *CLN1* and *CLN2* during the cell cycle.** We have previously shown that the *CLN1* and *CLN2* transcripts accumulate periodically during the cell cycle, attaining maximum levels during late G_1 and decreasing as cells initiate S phase (43). However, periodicity is not a prerequisite for normal cell cycle progression or continued proliferation (see Fig. 3B) (42). Since regulation of *CLN1* and *CLN2* transcription by mating pheromone was found to be disrupted in *far1*Δ mutants, it was possible that expression of those genes was also improperly regulated in cells growing vegetatively in the absence of mating pheromone. To determine whether the loss of *FAR1* function affected regulation during the cell cycle, we analyzed the accumulation of *CLN1* and *CLN2* transcripts in synchronous populations of *far1*Δ mutant and wild-type cells. Since *far1*Δ mutants cannot be synchronized by arrest with mating pheromone, we constructed strains carrying the conditional *cdc28-13* mutation and either a mutant (CWY222) or wild-type (CWY181) *FAR1* gene. These cells were arrested in G_1 phase by incubation at the restrictive temperature for the *cdc28* mutation (37°C) in the presence of α-factor and then allowed to synchronously reenter the cell cycle by being returned to fresh medium without α-factor at the permissive temperature (25°C) (Fig. 2A). Both the *far1*Δ mutant and wild-type strains initiated a new cell cycle, as evidenced by the appearance of budded cells approximately 75 min after the temperature shift.

Total RNA prepared from these cells was analyzed by Northern blotting to determine the abundance of the *CLN1* and *CLN2* transcripts (Fig. 2B). In both the *far1*Δ mutant and wild-type cells, the *CLN1* (not shown) and *CLN2* transcripts began to accumulate 60 min after the shift to 25°C, just prior to the appearance of budded cells. This pattern of expression is consistent with that observed in wild-type cells (43). Furthermore, accumulation of the transcript was periodic with respect to cell cycle position, decreasing as the cells become maximally budded and increasing again later in the time course. We assume that this

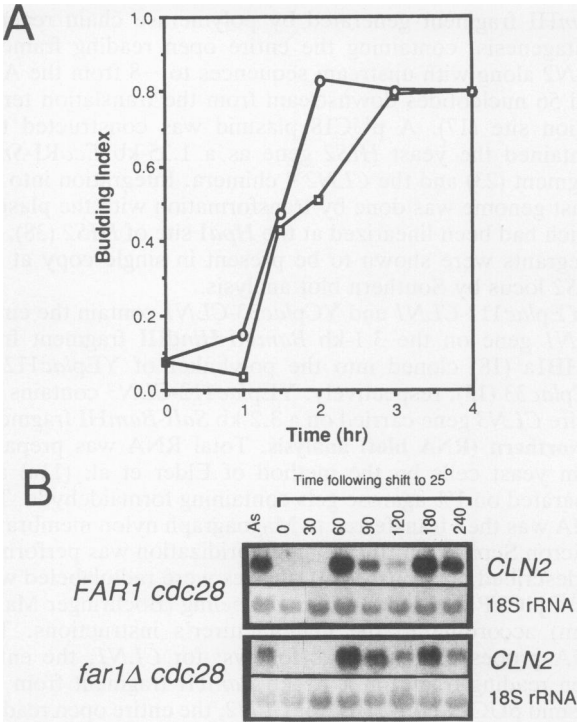

FIG. 2. Abundance of *CLN1* and *CLN2* transcripts in *FAR1* and *far1*Δ mutant cells in synchronous cultures. Cultures of *MAT***a** *FAR1 cdc28-13* cells (CWY181) (○) and *MAT***a** *far1*Δ *cdc28-13* cells (CWY222) (□) were arrested during G_1 by incubation at 37°C for 3 h in the presence of 100 ng of α-factor per ml, released by being shifted to fresh medium at 25°C without mating pheromone, and then incubated at 25°C. Cells were harvested at the times indicated and analyzed for budding index (A) and abundance of *CLN2* transcripts by Northern blot analysis (B). The lane labeled As contains RNA from an asynchronous population of the same cells. Times in panel B are shown in minutes.

increase is associated with cells that have reentered G_1 phase, as has been observed in our previous studies (43). It is unlikely that the pattern of expression observed in the second cell cycle following return to the permissive temperature is influenced by the inactivation of *CDC28*. The results of these studies demonstrate that cell cycle regulation of *CLN1* and *CLN2* transcript accumulation is unaffected in *far1*Δ mutants. From these results, it is unlikely that *FAR1* is involved in the mechanisms governing *CLN1* and *CLN2* transcription during vegetative growth; instead, its involvement is limited to regulation by mating pheromone.

Mating pheromone acts through a *FAR1*-dependent posttranscriptional mechanism to regulate accumulation of the Cln2 protein. The involvement of *FAR1* in the regulation of *CLN2* transcript abundance by mating pheromone could be explained by two alternative hypotheses. First, mating pheromone might act through *FAR1* to directly repress the transcription of the *CLN2* gene. Alternatively, *FAR1* may be required for posttranscriptional regulation of Cln2 activity, leading indirectly to a decrease in transcription of *CLN2* through disruption of the *CLN/CDC28*-dependent feedback pathway (7, 9, 22). The latter hypothesis was tested directly by separating transcriptional and posttranscriptional regulation of *CLN2* gene expression. Yeast strains were constructed in which the *CLN2* open reading frame was placed under control of the *CLN3* promoter (referred to as $CLN2^{3P}$;

see Materials and Methods). Accumulation of the *CLN3* transcript is constitutive with respect to cell cycle position and is modestly induced by pheromone treatment (8, 27, 43). Furthermore, the steady-state level of the *CLN3* transcript is similar to that of *CLN2*. A single copy of the *CLN2*^{3p} chimera was introduced into *FAR1 cln1*::*TRP1 cln2*::*LEU2* and *far1*Δ *cln1*::*TRP1 cln2*::*LEU2* strains by site-directed integration at the chromosomal *HIS2* locus. The resulting strains (KJY95 and KJY98, respectively) displayed no obvious defects in proliferation or growth under vegetative conditions. Furthermore, the chimeric gene was shown to act as a functional G_1 cyclin gene by its ability to rescue a Cln-deficient mutant (data not shown).

We first asked whether mating pheromone induces G_1 phase arrest in a strain carrying the *CLN2*^{3p} gene as its only source of *CLN2*. Pheromone sensitivity was evaluated during growth in liquid medium as well as by halo assays. Halo assays allow qualitative evaluation of sensitivity to a broad range of pheromone concentrations arising via diffusion from a point of application. Figure 3A shows that the α-factor sensitivity of a *FAR1 cln1*Δ *cln2*Δ strain carrying a single copy of the *CLN2*^{3p} gene (KJY95) was approximately equivalent to that of the parent carrying the wild-type *CLN2* gene (15Daub). This finding was confirmed by the decrease in the proportion of budded cells observed following exposure to α-factor (Fig. 3B). Furthermore, the pheromone sensitivity of cells carrying *CLN2* or *CLN2*^{3p} as the only source of *CLN* gene product was approximately equivalent (data not shown). Northern blot analysis of *CLN2* transcript abundance was used to confirm that the chimeric gene was expressed in the presence of pheromone (Fig. 3B). While the *CLN2* transcript in cells carrying a wild-type *CLN2* gene decreased rapidly following treatment with pheromone (Fig. 1B), levels of the same transcript decreased only modestly in cells carrying the *CLN2*^{3p} chimera. We conclude that pheromone induction of G_1 phase arrest is not dependent upon elimination of the *CLN2* transcript.

We analyzed the abundance of the Cln2 polypeptide during the same time course of pheromone treatment by immunoblotting with Cln2 antiserum (Fig. 3B). Surprisingly, the abundance of Cln2 protein decreased dramatically after mating pheromone treatment, decreasing to 20% of the initial level within 1 h and becoming barely detectable after 3 h of pheromone treatment. The decrease in Cln2 protein abundance significantly preceded any detectable decrease in the *CLN2* transcript level. While the kinetics of loss were slower than in cells carrying a wild-type *CLN2* gene (Fig. 1C), the decrease observed over the entire time course was approximately equivalent between these strains. This observation establishes the existence of a posttranscriptional mechanism that is sufficient to eliminate the Cln2 polypeptide in response to mating pheromone. Whether this effect is exerted at the translational or posttranslational level cannot be deduced from these experiments. However, the loss observed here is unlikely to be simply a result of cell cycle arrest, since Cln2 protein expressed from the *CLN2*^{3p} chimera persists in cells arrested during G_1 phase by a conditional *cdc28* mutation (42). Furthermore, exposure of those G_1-arrested cells to pheromone results in the loss of the Cln2 polypeptide (42). The difference between the kinetics of loss of Cln2 from cells carrying the wild-type gene and from cells carrying the *CLN2*^{3p} gene suggests that the transcriptional repression that takes place upon inactivation of the Cln2 protein makes a significant contribution to the rate of loss of that protein.

Having established the existence of a posttranscriptional mechanism for the elimination of Cln2 polypeptide in response to mating pheromone, we tested whether that mechanism was dependent upon *FAR1*. The first indication that this might be the case was the observation that a strain carrying *CLN2*^{3p} as its only source of *CLN2* and an inactivated *FAR1* gene (KJY95) was resistant to mating pheromone when assayed both by halo assay and in liquid culture

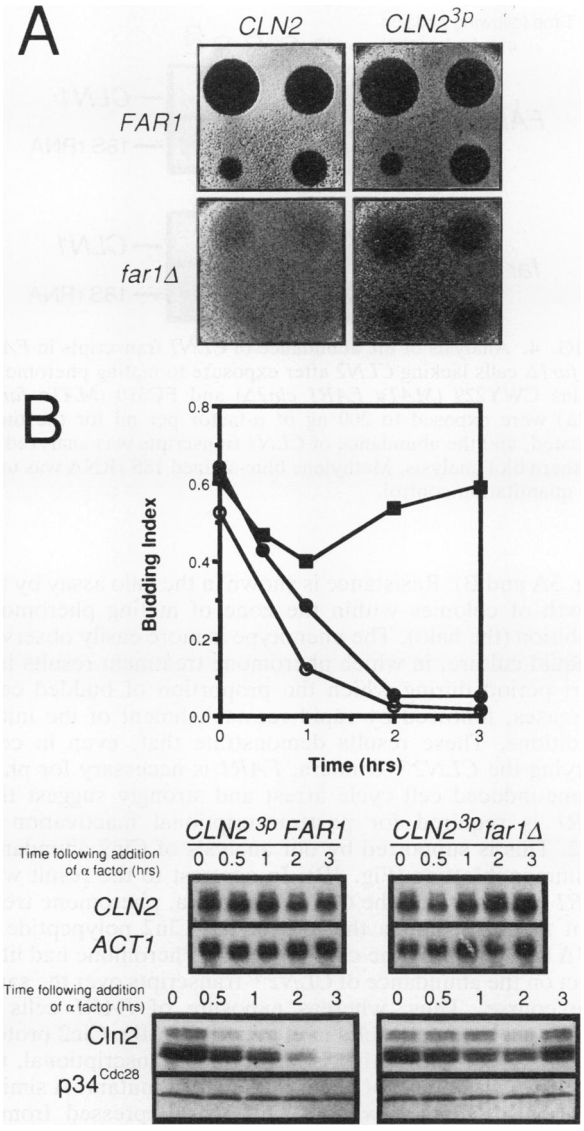

FIG. 3. *FAR1*-dependent posttranscriptional regulation of *CLN2* expression by mating pheromone in cells. (A) Halo assays were performed with 600, 200, 66, and 22 ng of α-factor (clockwise within each square, starting at upper left). The strains used were 15Daub (upper left), KJY95 (upper right), FC280 (lower left), and KJY98 (lower right). (B) Strains KJY95 (*MAT***a** *cln1*::*TRP1 cln2*::*LEU2 CLN2*^{3p} *FAR1*) (●) and KJY98 (*MAT***a** *cln1*::*TRP1 cln2*::*LEU2 CLN2*^{3p} *far1*Δ) (■) were treated with mating pheromone for the times indicated, and then aliquots of cells were taken and analyzed for the proportion of budded cells (results for the *MAT*α *CLN2 FAR1* strain 15Daub (○) are shown as a wild-type control), the abundance of the *CLN2* transcript was determined by Northern blot analysis, and the abundance of the Cln2 polypeptide was determined by immunoblotting. *ACT1* mRNA and the p34^{CDC28} polypeptide were used as quantitation controls.

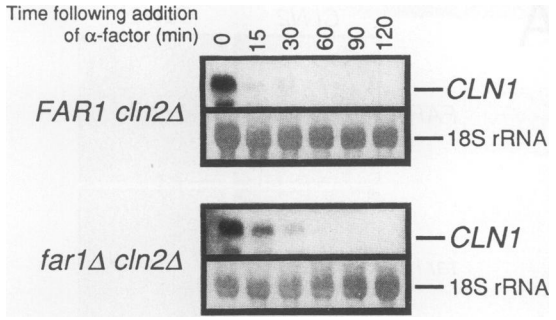

FIG. 4. Analysis of the abundance of *CLN1* transcripts in *FAR1* and *far1*Δ cells lacking *CLN2* after exposure to mating pheromone. Strains CWY229 (*MAT***a** *FAR1 cln2*Δ) and FC310 (*MAT***a** *far1*Δ *cln2*Δ) were exposed to 200 ng of α-factor per ml for the times indicated, and the abundance of *CLN1* transcripts was analyzed by Northern blot analysis. Methylene blue-stained 18S rRNA was used as a quantitation control.

(Fig. 3A and B). Resistance is shown in the halo assay by the growth of colonies within the zone of mating pheromone inhibition (the halo). The phenotype is more easily observed in liquid culture, in which pheromone treatment results in a short period during which the proportion of budded cells decreases, followed by rapid reestablishment of the initial conditions. These results demonstrate that, even in cells carrying the $CLN2^{3P}$ chimera, *FAR1* is necessary for pheromone-induced cell cycle arrest and strongly suggest that *FAR1* is required for posttranscriptional inactivation of Cln2. This is supported by our analysis of Cln2 abundance by immunoblotting (Fig. 3B). In contrast to the result with *FAR1* cells carrying the $CLN2^{3P}$ chimera, pheromone treatment failed to induce the loss of the Cln2 polypeptide in *far1*Δ cells carrying the chimeric gene. Pheromone had little effect on the abundance of $CLN2^{3P}$ transcripts over the same time course. Thus, whereas exposure of *FAR1* cells to mating pheromone results in elimination of the Cln2 protein through a mechanism that is largely posttranscriptional, the same response does not occur in the *far1*Δ mutant. A similar result was obtained when *CLN2* was expressed from a debilitated *GAL1* promoter, from which accumulation of Cln2 is approximately threefold higher than the wild-type level (data not shown). These results demonstrate that pheromone acts through a *FAR1*-dependent mechanism that leads to repression of Cln2 polypeptide accumulation and ultimately to G_1 arrest.

Persistence of *CLN1* transcripts in mating pheromone-treated *far1* mutants depends on a functional *CLN2* gene. The persistence of the *CLN1* transcript in pheromone-treated *far1*Δ mutants (Fig. 1) could result from a requirement for *FAR1* for pheromone repression of *CLN1* expression or, alternatively, could reflect an interaction between *CLN2* and *CLN1*. That is, *CLN2* could affect the expression of *CLN1* through its effect on the *CLN/CDC28*-dependent feedback stimulation (7, 9, 22). To determine whether this is the case, the effect of mating pheromone on *CLN1* transcript accumulation was analyzed in *far1*Δ mutant (FC310) and *FAR1* (CWY228) strains in which *CLN2* was inactivated (Fig. 4). Because they lack *CLN2*, these strains arrested in response to pheromone regardless of the state of *FAR1* (not shown). In both cases, the accumulation of the *CLN1* transcript was repressed following exposure to mating pheromone. A similar result was obtained with cells in which both the *CLN2* and *CLN3* genes were inactivated (data not shown). The simplest interpretation of these results is that the persistence of *CLN1* transcripts in the *far1*Δ strain occurs as a consequence of the failure to repress *CLN2* expression. Therefore, in the absence of *CLN2*, the regulation of *CLN1* gene expression by mating pheromone is independent of *FAR1*. However, that regulation must be overridden by the persistence of *CLN2* in the *far1*Δ mutant. The slower kinetics of loss of the *CLN1* transcript in the *far1*Δ *cln2*Δ mutant suggests that *FAR1* may in fact play a supplementary role in regulation of *CLN1* gene expression independent of its effect on *CLN2* (see Fig. 5).

***FAR1* plays a posttranscriptional role in the pheromone regulation of *CLN1* that is independent of *CLN2* and *CLN3*.** The experiments discussed above show that the *CLN1* transcript in *far1*Δ mutants is regulated normally by pheromone if *CLN2* is inactivated. However, the presence of extra copies of *CLN1* in the same cells reveals a requirement for *FAR1* that is not apparent when the gene is present in single copy. This is demonstrated by the pheromone response of *FAR1 cln2*Δ (CWY229) and *far1*Δ *cln2*Δ (FC310) strains carrying *CLN1* on the multicopy 2μm plasmid YEp*lac*112 (15), as evaluated by halo assays. The same plasmid either with no insert or carrying the *CLN3* gene was used as a control. The pheromone response of *FAR1 cln2*Δ cells carrying a plasmid with either the *CLN1* or *CLN3* gene was approximately equal to that of cells carrying the plasmid without an insert, as demonstrated by the formation of clear halos of comparable sizes (Fig. 5A). In contrast, *far1*Δ *cln2*Δ cells carrying *CLN1* on the multicopy plasmid were relatively insensitive to pheromone. Although a halo was observed in this assay, it was filled with colonies. This resistance was specific for *CLN1*, since cells carrying either the plasmid with no insert (YEp*lac*112) or one with the *CLN3* gene (YEp*lac*112-*CLN3*) remained sensitive to mating pheromone. From this experiment, we conclude that the *FAR1* gene is essential for the pheromone sensitivity of cells carrying multiple copies of the *CLN1* gene and that *FAR1* is required, at least under these circumstances, for pheromone-induced inactivation of *CLN1*. The persistence of *CLN1* activity in the *far1*Δ mutant was not dependent on *CLN3*, since the same result was obtained in the absence of a functional chromosomal copy of the *CLN3* gene.

Since *FAR1* is required for posttranscriptional regulation of Cln2 accumulation by pheromone, it seemed likely that it played a similar role in the regulation of *CLN1*. To determine whether pheromone regulation of *CLN1* transcript abundance in these cells was affected by *FAR1*, we analyzed its abundance in the presence and absence of pheromone. As expected, both the *far1*Δ and *FAR1* cells carrying the plasmid expressed elevated levels of *CLN1* (Fig. 5B). However, unexpectedly, expression in both strains was not repressed by exposure to mating pheromone. The reason for this failure is unclear. It is unlikely that it resulted from the loss of regulatory sequences, since the same *CLN1* gene is repressible by mating pheromone when present in single copy on a centromere plasmid (YCp*lac*33-*CLN1*; data not shown). Nevertheless, the fact that these cells arrested while continuing to express *CLN1* is consistent with a posttranscriptional effect of pheromone on Cln1 function. While we were unable to evaluate the level of the Cln1 polypeptide in these cells because of the lack of appropriate reagents, these results are consistent with the existence of a *FAR1*-dependent posttranscriptional mechanism for pheromone regulation of Cln1 abundance.

Mating pheromone represses feedback-independent expression of *CLN1* and *CLN2* through a *FAR1*-independent mech-

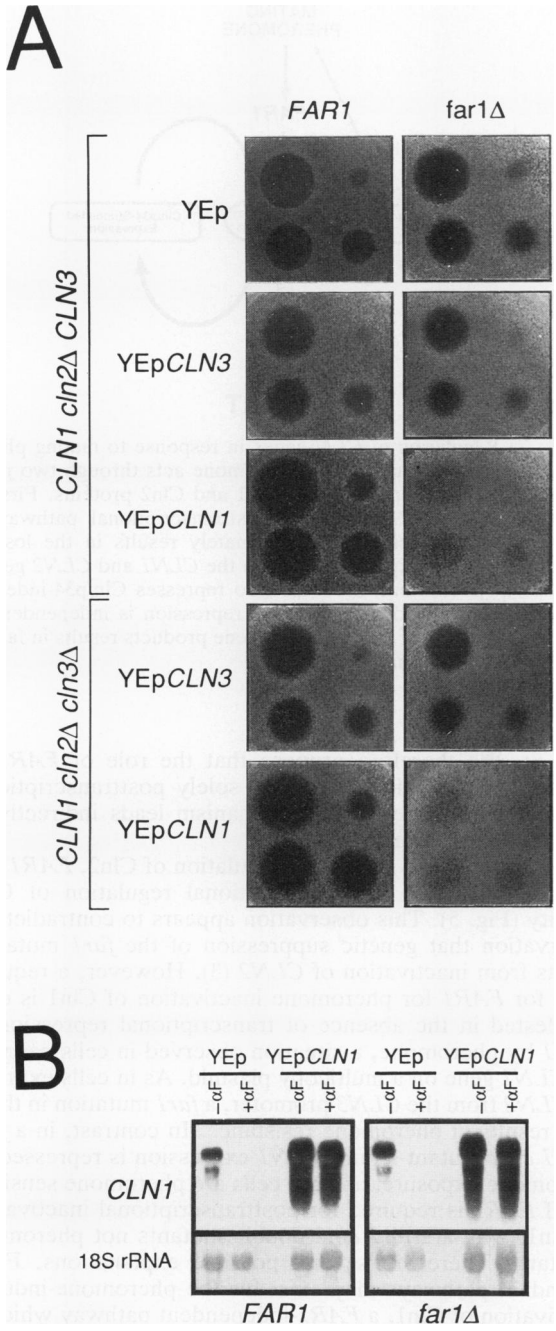

FIG. 5. Pheromone resistance of *far1Δ cln2Δ* and *far1Δ cln2Δ cln3Δ* mutant strains carrying *CLN1* on a multicopy plasmid. (A) Halo assays were performed with 600, 200, 66, and 22 ng of α-factor (clockwise within each square, starting at upper left). The strains used were CWY229 (top three squares in left column), FC310 (top three squares in right column), KJY47 (bottom two squares in left column), and FC322 (bottom two squares in right column). Strains contained either YEp*lac*112, YEp*lac*112-*CLN1*, or YEp*lac*112-*CLN3*. (B) Strains CWY229 (left) and FC310 (right) carrying YEp*lac*112 (YEp) or YEp*lac*112-*CLN1* (YEp*CLN1*) were untreated or treated with 200 ng of α-factor (αF) per ml for 3 h and analyzed for *CLN1* transcript abundance by Northern blot analysis. Methylene blue-stained 18S rRNA was used as a quantitation control.

anism. Maximal expression of *CLN1* and *CLN2* in late G_1 phase is dependent upon the function of the *CDC28* gene and at least one functional *CLN* gene (7, 9, 25). The simplest interpretation of this observation is that full induction of expression is dependent upon a feedback pathway that requires the activation of the $p34^{Cdc28}$ protein kinase by one of the *CLN* gene products. However, expression of *CLN1* and *CLN2* is detectable even in the absence of either of these activities. We refer to this level of expression as feedback-independent expression and to the maximal Cln/p34-dependent level of expression as feedback-stimulated expression. One consequence of feedback-stimulated expression is that the persistence of Cln1 and Cln2 proteins in pheromone-treated *far1Δ* mutants will result in feedback stimulation of *CLN1* and *CLN2* expression. This alone is sufficient to explain the failure of pheromone to regulate the *CLN1* and *CLN2* transcripts in *far1Δ* mutants. However, since earlier studies had suggested that, in addition to its effect on the feedback pathway, mating pheromone also represses feedback-independent expression of *CLN2* (7), it was possible that *FAR1* was also required for that regulation.

To assess the role of *FAR1* in the regulation of feedback-independent expression, it was first necessary to determine whether mating pheromone affects the accumulation of *CLN1* and *CLN2* transcripts in the absence of feedback stimulation. To do so, we used a strain in which all three of the endogenous *CLN* genes are disrupted but which carries the *CLN3* gene expressed under control of the inducible *GAL1* promoter (strain 1258-14B). These cells depend upon the galactose-inducible *CLN3* gene for continued growth as well as the induction of feedback-stimulated gene expression (6, 7, 36). Expression of *CLN2* can be studied in this strain by analyzing the accumulation of the transcript arising from the inactivated *cln2Δxs* gene. Galactose-grown cells were arrested by addition of 2% glucose. This treatment results in a dramatic decrease in the level of the *CLN3* transcript, so that the level after 3 h of growth in glucose is less than 10% of the wild-type asynchronous level (data not shown). The G_1-arrested cells were then treated for an additional 2 h with α-factor (200 ng/ml), and the abundance of the transcript derived from the *cln2Δxs* gene was determined by Northern blot analysis (Fig. 6).

While the abundance of *cln2Δxs* transcripts decreased noticeably upon repression of *CLN3* gene expression, as has been reported previously (7), that transcript was still easily detectable even after the cells had arrested during G_1 phase. Addition of pheromone to these G_1-arrested, *CLN*-deficient cells resulted in a further reduction in the level of the *cln2Δxs* transcript. That these cells respond to mating pheromone is shown by an increase in the pheromone-inducible *FUS1* transcript level. As expected, the abundance of the *cln2Δxs* transcript did not decrease when the cells were maintained in glucose for the same interval without the addition of mating pheromone. This experiment demonstrates that *CLN2* transcript accumulation remains pheromone sensitive even in the absence of feedback stimulation and shows that pheromone can repress *CLN2* expression independently of its effect on *CLN* abundance or activity. A similar result was obtained when feedback-stimulated expression was inhibited by inactivating the $p34^{CDC28}$ protein kinase catalytic subunit rather than by inactivating the G_1 cyclins. In that experiment, which was done with a temperature-sensitive *cdc28* mutant, feedback-independent expression of both the wild-type *CLN1* and wild-type *CLN2* genes was shown to be repressed by pheromone.

To evaluate whether *FAR1* was required for pheromone

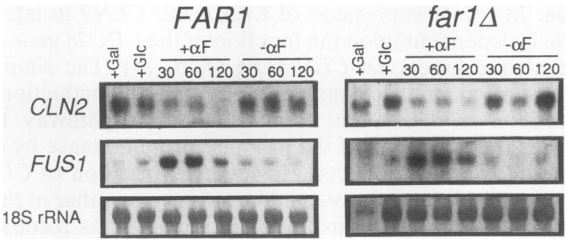

FIG. 6. Pheromone repression of *CLN1* and *CLN2* transcription in the absence of *CLN*-dependent expression. Strains 1258-14B (*MATa FAR1 cln1Δ cln2Δxs cln3Δ TRP1::GAL1-CLN3*) and DLY518 (*MATa far1::URA3 cln1Δ cln2Δxs cln3Δ TRP1::GAL1-CLN3*) were grown in galactose at 25°C (+Gal). The cultures were then adjusted to 2% glucose to repress *CLN3* expression and incubated for an additional 3 h to arrest the cells in G_1 phase (+Glc). The cultures were then split and either treated with α-factor (αF, 200 ng/ml) for the times indicated (in minutes) or maintained without further additions. Aliquots were taken at each time point, and the RNA transcripts from the inactivated *cln2Δxs* gene were analyzed by Northern blot analysis. *FUS1* mRNA was used as an indicator of the mating pheromone response. Methylene blue-stained 18S rRNA was used as a quantitation control.

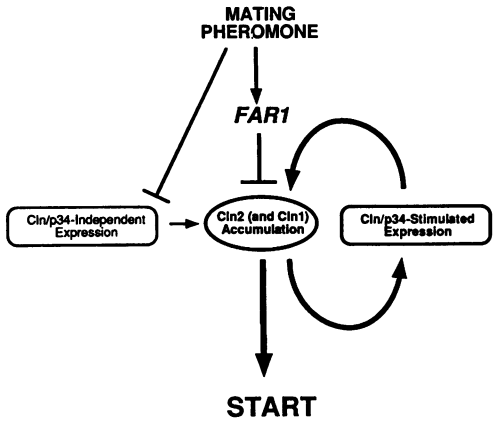

FIG. 7. Regulation of *CLN* genes in response to mating pheromone. We propose that mating pheromone acts through two pathways to repress accumulation of Cln1 and Cln2 proteins. First, it acts through a *FAR1*-dependent posttranscriptional pathway to inactivate both proteins, which ultimately results in the loss of Cln/p34-stimulated expression of both the *CLN1* and *CLN2* genes. In addition, pheromone exposure also represses Cln/p34-independent expression of both genes. That repression is independent of *FAR1*. Inactivation of all three *CLN* gene products results in failure of cells to progress through START.

regulation of feedback-independent expression of *CLN2*, we subjected a conditionally *CLN*-deficient *far1Δ* mutant strain to the regimen of glucose repression and pheromone treatment described above and then determined the abundance of *cln2Δxs* transcripts (Fig. 6). As observed with the congenic *FAR1* strain, the feedback-independent accumulation of *cln2* transcripts in the *far1Δ* mutant strain was repressed by mating pheromone. Since little, if any, difference in either the extent or kinetics of this decrease was observed between the two strains, we conclude that repression of the feedback-independent level of *CLN2* expression by pheromone does not require *FAR1*. Again, using a temperature-sensitive *cdc28* mutation to inactivate feedback-stimulated expression, we demonstrated a similar effect of pheromone on accumulation of the wild-type *CLN1* and *CLN2* transcripts (data not shown). Furthermore, if the expression observed here resulted from failure to completely inhibit feedback-stimulated expression, we would expect the effect of mating pheromone to be dependent upon *FAR1*. These results suggest that *FAR1* plays a role in pheromone regulation of *CLN2* expression that is unique to feedback-stimulated transcription.

DISCUSSION

Role of *FAR1*. This work demonstrates that mating pheromone acts through a *FAR1*-dependent posttranscriptional mechanism to repress the accumulation of the Cln2 polypeptide (Fig. 3). The failure of that mechanism results in the inability of *far1* mutants to arrest during G_1 in response to mating pheromone. In addition, the inability of pheromone to inactivate Cln2 can explain the persistence of the *CLN1* and *CLN2* transcripts observed in pheromone-treated *far1* mutants (Fig. 1) if it is assumed that the Cln2 protein acts to stimulate the expression of those genes through the Cln/p34-dependent feedback pathway (7, 9) (Fig. 7). Consistent with that assumption, we show that the persistence of the *CLN1* transcript in *far1* mutants depends upon a functional *CLN2* gene (Fig. 4). In contrast to its role in posttranscriptional regulation of Cln2, *FAR1* is not required for cell cycle regulation of *CLN2* expression (Fig. 2) or for pheromone repression of feedback-independent *CLN2* transcription (Fig. 6). We therefore propose that the role of *FAR1* in pheromone regulation of Cln2 is solely posttranscriptional and that the failure of that mechanism leads indirectly to deregulation of transcription.

In addition to its role in the regulation of Cln2, *FAR1* can also participate in posttranscriptional regulation of Cln1 activity (Fig. 5). This observation appears to contradict the observation that genetic suppression of the *far1* mutation results from inactivation of *CLN2* (3). However, a requirement for *FAR1* for pheromone inactivation of Cln1 is only manifested in the absence of transcriptional repression of *CLN1* by pheromone, a situation observed in cells carrying the *CLN1* gene on a multicopy plasmid. As in cells expressing *CLN2* from the *CLN3* promoter, a *far1* mutation in those cells results in pheromone resistance. In contrast, in a *far1 CLN1 cln2* mutant strain, *CLN1* expression is repressed by pheromone exposure, and the cells are pheromone sensitive (2). If *FAR1* is required for posttranscriptional inactivation of Cln1, why are *far1 cln2* double mutants not pheromone resistant? There are several possible explanations. First, redundant pathways may exist for the pheromone-induced inactivation of Cln1, a *FAR1*-independent pathway which is sufficient when *CLN1* is present in single copy and a *FAR1*-dependent pathway which becomes essential at higher levels of *CLN1* expression. However, it is also possible that, when the *CLN1* gene is present in a single copy, the Cln1 protein is insufficient to activate the *CLN1* gene via feedback, and consequently, inactivation of Cln1 may only require inactivation of feedback-independent expression. If that is the case, posttranscriptional regulation would not be necessary for the repression of single-copy *CLN1* by pheromone. The resolution of this apparent contradiction awaits further investigation.

While the results presented here support a role for *FAR1* in the posttranscriptional regulation of Cln2 in response to mating pheromone, the specific level at which it is required is unknown. The loss of Cln2 polypeptide after pheromone exposure could result from a decrease in the rate of Cln2

synthesis, from pheromone-induced destabilization of the Cln2 protein, or from a combination of these effects. Furthermore, both effects could result from influences at any of a number of steps in the process of Cln2 synthesis or degradation. In support of a posttranslational mechanism, we have recently obtained evidence that pheromone induces a posttranslational modification of Cln2, which is followed by a decrease in its abundance (42). Unfortunately, neither the predicted primary sequence nor the known properties of the *FAR1* gene product contribute to the resolution of this question. The putative product has no significant homology to proteins of known function, and while the *FAR1* product is known to be phosphorylated in response to mating pheromone (3), it is currently unknown whether that modification is essential for function.

Feedback-stimulated versus feedback-independent expression. The tripartite model of Chang and Herskowitz (2), while sufficient in its simplest form to explain the results of genetic studies, is insufficient to explain several observations. Closer examination of the behavior of the *CLN* transcripts and gene products in *fus3* and *far1* mutants suggests that both genes contribute significantly to pheromone regulation of more than one *CLN* gene. Evidence presented here supports a role for *FAR1* in the posttranscriptional regulation of Cln1. In addition, Elion et al. (12) have presented evidence that *FUS3* is required for proper regulation of all three G_1 cyclins in response to pheromone. That work showed that, although the cell cycle arrest defect of *fus3* mutants is suppressed by inactivation of *CLN3* (13), *fus3 cln3* mutants arrested in G_1 phase by pheromone continue to express both the *CLN1* and *CLN2* transcripts (12). The observation that *fus3 cln3* cells arrest in G_1 phase despite their continued expression of *CLN1* and *CLN2* transcripts can be explained if pheromone can inactivate both the Cln1 and Cln2 proteins through the *FAR1*-dependent posttranscriptional pathway revealed by this study (Fig. 7). However, this explanation appears to be inconsistent with the persistence of the *CLN* transcripts in those cells. This level of expression may be independent of the feedback pathway and may indicate a role for *FUS3* in pheromone regulation of feedback-independent expression of *CLN1* and *CLN2*. While there is evidence that *FAR1* and *FUS3* both affect pheromone regulation of Cln1, it is not known whether pheromone regulation requires other *CLN1*-specific regulatory elements, as suggested by Chang and Herskowitz (2).

The differential effect of *FAR1* on feedback-stimulated and feedback-independent expression indicates that the mechanisms governing these modes of expression are, in fact, distinct and suggests that feedback-independent expression does not simply reflect incomplete inactivation of the feedback pathway. Since feedback-independent expression of *CLN1*, like that of *CLN2*, is affected by pheromone (unpublished results), it is possible that the mechanism by which this regulation is exerted is conserved between these genes. Two genes, *SWI4* and *SWI6*, have been implicated as regulators of *CLN1* and *CLN2* gene expression. These genes, which were originally discovered as essential activators of the cell cycle-regulated *HO* gene (1, 28, 39), are required for maximal expression of *CLN1* and *CLN2* (29, 31). *SWI4* has been shown, by gel retardation assays, to interact with the *CLN2* promoter through the cell cycle box motif $CACGA_4$ (29). It has been suggested that these proteins mediate feedback-stimulated expression, perhaps through a requirement for an activating phosphorylation catalyzed by the Cln/p34 protein kinase. However, their involvement in feedback-independent expression has not yet been directly tested.

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