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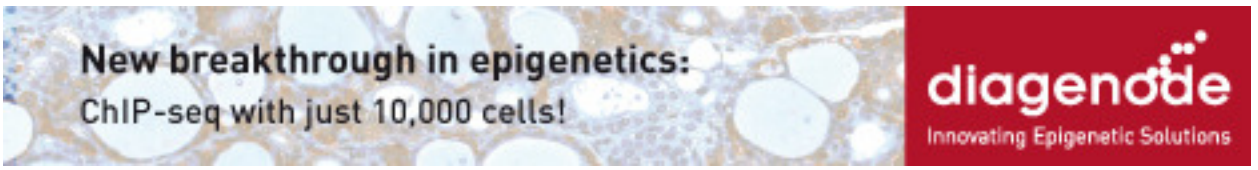

Negative regulation of *FAR1* at the Start of the yeast cell cycle

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In budding yeast, a switch between the mutually exclusive pathways of cell cycle progression and conjugation is controlled at Start in late G_1 phase. Mating pheromones promote conjugation by arresting cells in G_1 phase before Start. Pheromone-induced cell cycle arrest requires a functional *FAR1* gene. We have found that *FAR1* transcription and protein accumulation are regulated independently during the cell cycle. *FAR1* RNA and protein are highly expressed in early G_1, but decline sharply at Start. Far1 is phosphorylated just before it disappears at Start, suggesting that modification may target Far1 for degradation. Although *FAR1* mRNA levels rise again during late S or G_2 phase, reaccumulation of Far1 protein to functional levels is restricted until after nuclear division.

[*Key Words*: Yeast cell cycle; *FAR1*; α-factor; Start]

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During the haploid phase of the budding yeast life cycle, cells of mating types **a** and α secrete the diffusible mating pheromones **a**-factor and α-factor, respectively. Binding of **a**- or α- factor to cell-surface receptors displayed by cells of the opposite mating type generates an intracellular signal that elicits preparations for conjugation. Responses to pheromone include induced expression of genes that encode mating functions, changes in cell morphology, and arrest in the G_1 phase of the cell cycle (for review, see Cross et al. 1988; Marsh et al. 1991; Hirsch and Cross 1992). The action of mating pheromones may be analogous to that of negative growth factors that control differentiative responses in higher eukaryotes (Massague 1992). Cell cycle arrest in the G_1 phase is essential for conjugation, as cells blocked by drugs or mutations in other intervals of the cell cycle are not able to mate efficiently (Reid and Hartwell 1977). The restriction of mating to the G_1 interval ensures that the resulting **a**/α zygote will have the correct ploidy.

Commitment to cell cycle progression occurs at Start in the G_1 phase: Once past Start, cells will not arrest in response to mating pheromone until completion of the cell cycle in progress. Start thus functions as a switch between the mutually exclusive pathways of cell cycle progression and conjugation (for review, see Cross et al. 1988). Execution of Start requires the *CDC28* gene, which encodes the budding yeast homolog of the $p34^{cdc2}$ protein kinase (for review, see Cross et al. 1989), and at least one member of the *CLN* gene family (*CLN1*, *CLN2*, or *CLN3*), which encode products distantly related to mitotic cyclins (Richardson et al. 1989). The *CDC28* and *CLN* gene products interact physically and may function as a Start-promoting protein kinase complex (Wittenberg et al. 1990; Tyers et al. 1992).

Mating pheromone may inhibit Start by interfering with the function of the Cln/Cdc28 kinase. In support of this idea, dominant gain-of-function mutations in the *CLN* genes have been isolated, which confer partial or total resistance to pheromone-induced cell cycle arrest (Sudbery et al. 1980; Cross 1988; Nash et al. 1988; Hadwiger et al. 1989; Cross and Tinkelenberg 1991). Also, mutations in the *FAR1* and *FUS3* genes may identify pathways leading to inactivation of *CLN* function in response to mating pheromone. *FAR1* is thought to inhibit *CLN2* function, and *FUS3* to inhibit *CLN3* function, on the basis of genetic epistasis experiments (Chang and Herskowitz 1990; Elion et al. 1990). In the case of *FAR1*, it was shown that whereas *far1* mutants failed to arrest in response to mating pheromone, *far1 cln2* double mutants arrested efficiently (Chang and Herskowitz 1990). *far1* mutations do not eliminate pheromone induction of gene expression and morphological changes. These observations suggest that *FAR1* function is involved specifically in cell cycle arrest and not in general pheromone signal transduction (Chang and Herskowitz 1990). The mechanism of *FAR1* function is not known.

Here, we examine cell cycle regulation of *FAR1* gene expression. Complex controls restrict significant Far1 accumulation to the pre-Start G_1 interval, the time of Far1 function.

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Results

Far1 protein accumulation is depressed by Cln3-2 activation of Cdc28

The *CLN3-2* allele of *CLN3* (previously called *DAF1-1*) confers resistance to cell cycle arrest by mating pheromone via an unknown mechanism (Cross 1988). Because *FAR1* function is required for mating pheromone arrest, we examined whether Far1 protein accumulation was normal in *CLN3-2* strains by Western blot analysis of cell extracts using anti-Far1 antibody. *CLN3-2* strongly reduced the level of Far1 protein both in asynchronous culture and in α-factor-treated cultures (Fig. 1). This effect was dependent on an active *CDC28* gene (Fig. 1). [Note that several slowly migrating species of Far1 resulting from phosphorylation were detected after α-factor treatment (Chang and Herskowitz 1992)]. These results suggest the possibility that Far1 down-regulation contributes to the α-factor resistance of *CLN3-2* strains. The epistasis of *cdc28* to *CLN3-2* suggests that activation of the Cdc28 kinase by Cln3-2 (Tyers et al. 1992) might regulate Far1 accumulation. Because one effect of *CLN3-2* expression is a very short G_1 interval (Cross 1988), a simple explanation for this result might be that Far1 accumulation is restricted to G_1 cells. This idea would also account for the epistasis of *cdc28* inactivation to *CLN3-2*, because *cdc28* inactivation results in G_1 arrest (even in a *CLN3-2* background) (Pringle and Hartwell 1981; Cross 1989). We therefore examined cell cycle regulation of *FAR1* RNA and protein accumulation.

Cell cycle regulation of FAR1 *transcript and protein levels*

To examine expression of the *FAR1* gene during the cell cycle, cells were synchronized using a *cln* block/release

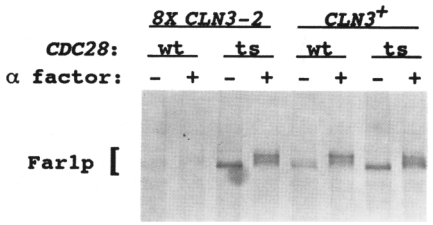

Figure 1. Far1 protein accumulation is depressed by Cln3-2 activation of Cdc28. All strains had the genotype *MAT***a** *bar1 CLN1*$^+$ *CLN2*$^+$ *CLN3*$^+$ and were congenic with 381G; additionally, strains were either *CDC28*$^+$ (wt lanes) or *cdc28-13*^{ts} (ts lanes) and contained (8× *CLN3-2* lanes) or did not contain (*CLN3*$^+$ lanes) eight copies of the dominant *CLN3-2* allele integrated at *CLN3* (Cross 1988). Cultures were grown to mid-log phase in YEP–glucose medium at 30°C and shifted to 37°C with or without the addition of α-factor to a final concentration of 0.1 μM, as indicated. After 2 hr at 37°C, protein samples were extracted from the cultures and analyzed by Western blot with anti-Far1 antibody. Aliquots of each culture were analyzed for budding index as an indicator of G_1 arrest; the proportions of unbudded cells accumulating were 29% (lane *1*); 36% (lane *2*); 85% (lane 3); 80% (lane *4*); 48% (lane 5); 97% (lane 6); 90% (lane *7*); 84% (lane *8*).

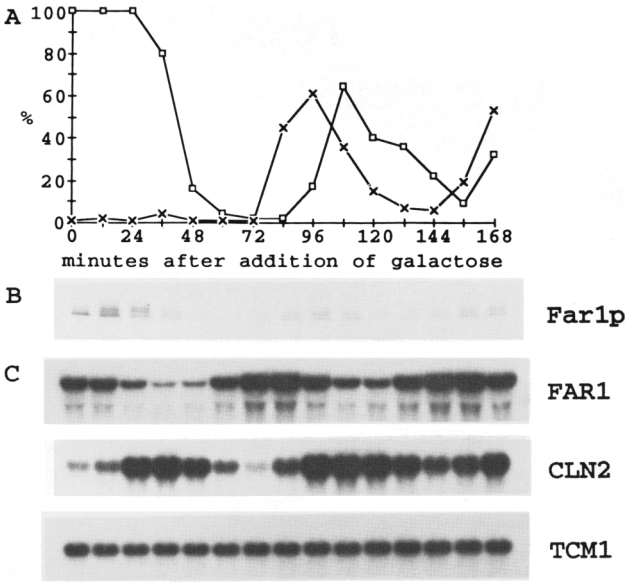

Figure 2. Cell cycle regulation of *FAR1* mRNA and protein levels. Cells of genotype *cln1 cln2 cln3 GAL1::CLN3* were synchronized in G_1 phase as described in Materials and methods: Cells were grown to log phase in YEP–galactose medium at 30°C, arrested in G_1 by an additional 2.5 hr of growth in YEP–raffinose, and galactose was added to 3% to restart the cell cycle. At the indicated time points following galactose addition, RNA and protein samples were extracted from the culture. RNA samples were analyzed by Northern blot hybridization with the indicated probes (*C*). Protein samples were analyzed by Western blot using anti-Far1 antibody (*B*). Aliquots of culture from each time point were analyzed for budding index [(□) percent unbudded cells] and percent binucleate cells (×) as morphological indicators of cell cycle position (*A*). Bud emergence roughly coincides with S-phase onset (data not shown), whereas binucleate cells accumulate just after nuclear division prior to cytokinesis. In this experiment, 50% of the cells executed Start (i.e., became resistant to α-factor inhibition of cell cycle progression, as judged by commitment to bud emergence) between 24 and 36 min after release from the G_1 block.

protocol, as described previously (Cross and Tinkelenberg 1991). *cln1 cln2 cln3 GAL1::CLN3* cells were arrested in G_1 by incubation in raffinose medium, which shuts off the *GAL1::CLN3* fusion gene, and then stimulated to re-enter synchronous cell cycles by the addition of galactose to turn on the *GAL1::CLN3* gene. Cells were harvested every 12 min for 3 hr and examined for cell cycle position by morphological criteria and for *FAR1* mRNA and protein levels (Fig. 2). In this protocol, cells execute Start between 24 and 36 min after galactose addition, complete S phase between 36 and 60 min, and complete nuclear division between 72 and 86 min (see legend to Fig. 2) (Epstein and Cross 1992).

FAR1 mRNA accumulation varied during the cell cycle: Levels were high during early G_1, low from Start to late S or G_2, and high in G_2 and M phases (Fig. 2C). The pattern of *CLN2* mRNA accumulation was virtually a mirror image of *FAR1* mRNA: *CLN2* mRNA levels were low in G_1-blocked cells, rose rapidly at about the time of

Start, and then fell again during S phase (Fig. 2C). [This pattern of *CLN2* expression was in agreement with previously published results using different methods of cell synchronization (Wittenberg et al. 1990)]. The contrasting patterns of *FAR1* and *CLN2* expression are interesting because *FAR1* and *CLN2* encode functional antagonists: *CLN2* promotes Start, whereas *FAR1* inhibits Start (Richardson et al. 1989; Chang and Herskowitz 1990). Note, however, that expression of *CLN2* during the cell cycle is not affected by a *far1* null mutation; also, *FAR1* function is not required for *FAR1* transcriptional regulation, as a *far1::URA3* null allele directs synthesis of a nonfunctional but normally regulated transcript (data not shown). The oscillation of *FAR1* mRNA levels during the cell cycle is probably entirely attributable to transcriptional control (see below).

Far1 protein levels also varied in the cell cycle, but with a different profile from the mRNA. Far1 protein was high only in early G_1 and was low from Start until the succeeding nuclear division (Fig. 2B). The G_1-specific accumulation of Far1 protein was confirmed using temperature-sensitive *cdc* mutants that block cell cycle progression at different points in the cell cycle (Pringle and Hartwell 1981). Far1 protein accumulated to high levels in G_1-arrested mutants (*cln1 cln2 cln3*, *cdc28*, *cdc34*, and *cdc4*), whereas only very low levels of Far1 protein accumulated in *cdc* mutants blocked in late G_1 (*cdc7*), S (*cdc8*), and G_2/M (*cdc13*, *cdc15*, and *cdc20*) phases (Fig. 3A; data not shown).

Cycling of *FAR1* mRNA and protein levels required synchronous cell cycle progression and was therefore not an artifact of the synchronization protocol used. Addition of galactose to an asynchronous population of raffinose-grown cells containing a functional *CLN2* gene in addition to the *GAL1::CLN3* fusion gene had little effect on *FAR1* mRNA and protein levels, presumably because the *CLN2* gene allowed cells to continue cycling in raffinose medium (data not shown). Also, the addition of α-factor at the time of release from G_1 arrest blocked cell cycle progression and kept *FAR1* mRNA and protein levels high (see Fig. 5, below).

Post-transcriptional cell cycle regulation of Far1 protein accumulation

Because the patterns of *FAR1* mRNA and protein differed in synchronized cultures (Fig. 2), we asked whether *FAR1* mRNA and protein levels are regulated independently during the cell cycle. Synchronous cell populations were prepared as described previously (see legend to Fig. 2), except that at the time of release from the G_1 block, the microtubule inhibitor nocodazole was added to prevent progression through mitosis (Fig. 4) (Jacobs et al. 1988). In the presence of nocodazole, *FAR1* mRNA levels declined at Start, rose again in late S or G_2, and remained high as cells arrested in mitosis. In contrast, after declining at Start, Far1 protein levels remained low as cells arrested in mitosis, even after several hours of incubation. Thus, mitotically blocked cells contain high levels of *FAR1* transcript but low levels of Far1 protein. Uncoupling of *FAR1* mRNA and protein accumulation was not a result of altered transcriptional initiation as determined by primer extension mapping of *FAR1* mRNA 5′ ends throughout the cell cycle and in nocodazole-arrested cells (Fig. 5C).

In higher eukaryotes, translation is inhibited during mitosis; we were therefore concerned that the failure of Far1 protein to accumulate in nocodazole-blocked cells might be attributable to nonspecific inhibition of trans-

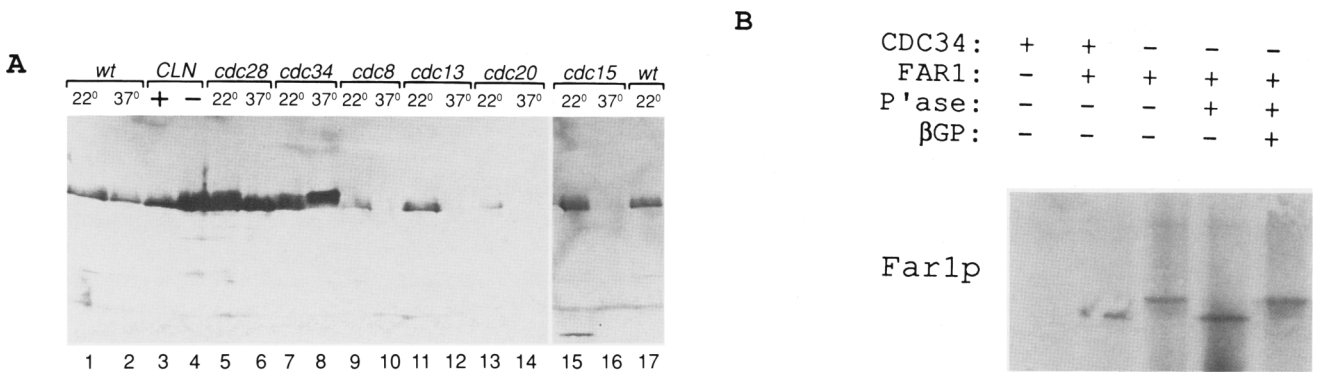

Figure 3. Cell cycle regulation of Far1 protein levels and phosphorylation state. (*A*) Cells were synchronized at the various *cdc* block points as described in Materials and methods. Cultures were grown to mid-log phase in YEP–glucose medium at 22°C and then split: Half of each culture was maintained at 22°C, and half was shifted to 37°C. After 3 hr of incubation at 22°C or 37°C, protein samples were extracted from the cultures and analyzed by Western blot using anti-Far1 antibody. The strains used had the indicated *cdc* genotypes. For lanes 3 and 4, cells of genotype *cln1 cln2 cln3 GAL1::CLN2* were grown to mid-log phase in YEP–galactose and then split: Half continued growth in YEP–galactose, and half were arrested in G_1 before Start by 3 hr of growth in YEP–glucose, which shuts off expression of the *GAL1::CLN2* allele. Protein samples were then extracted from the cultures and analyzed by Western blot using anti-Far1 antibody. (*B*) Cells of genotype *cdc34*^{ts} *FAR1*$^{+}$ and *cdc34*^{ts} *far1::URA3* were grown to mid-log phase in YEP–glucose at 22°C. The *FAR1*$^{+}$ culture was then split: Half was maintained at 22°C and half was shifted to 37°C, which causes cells carrying the temperature-sensitive *cdc34* mutation to arrest in late G_1 after Start. Incubation was continued for 3 hr, and then protein samples were extracted from the cultures. Aliquots of the protein samples were treated in vitro with calf intestinal phosphatase (P'ase) with or without the inclusion in the reaction of β-glycerophosphate (βGP, a phosphatase inhibitor). Samples were then analyzed by Western blot using anti-Far1 antibody.

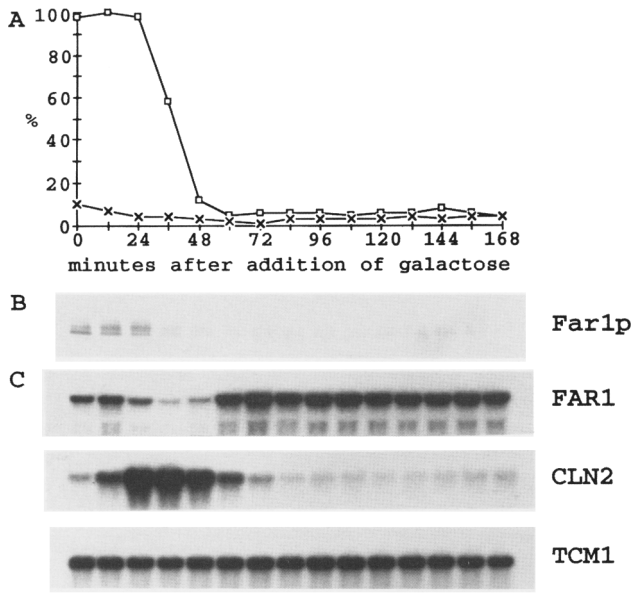

Figure 4. Accumulation of Far1 protein, but not *FAR1* mRNA, requires nuclear division. A cell-cycle time course identical to that of Figure 2 was performed, except that at the time of release from the G_1 block, the microtubule poison nocodazole was added to the culture at a final concentration of 15 μg/ml. In the presence of nocodazole, cells released from the G_1 block progressed normally through interphase but were unable to execute nuclear division. At the indicated time points following galactose addition, RNA and protein samples were extracted from the culture. RNA samples were analyzed by Northern blot hybridization with the indicated probes (*C*). Protein samples were analyzed by Western blot using anti-Far1 antibody (*B*). Aliquots of culture from each time point were analyzed for budding index [(□) percent unbudded cells] and percent binucleate cells (×) as morphological indicators of cell cycle position (*A*).

lation. Pulse-labeling of yeast cultures with [^{35}S]methionine, however, did not reveal any difference in label incorporation between asynchronous, *cln*-blocked, and nocodazole-blocked cells (data not shown), in agreement with previous work demonstrating that translation in yeast is not inhibited during mitosis (Johnston et al. 1977).

We next asked whether Far1 protein accumulation would be cell cycle regulated even in the absence of *FAR1* transcriptional regulation. While attempting to identify cell cycle regulatory sequences within the *FAR1* promoter, we found that truncation of the *FAR1* promoter to −350 bp (relative to the translational start codon) resulted in low-level constitutive expression of correctly initiated *FAR1* mRNA (data not shown). Although Far1 protein directed from the *Δ350FAR1* promoter accumulated at barely detectable levels, accumulation was still cell cycle regulated (data not shown). Fusion of the *CYC1* gene upstream activating sequence (UAS) to the *Δ350FAR1* promoter raised the level of transcription sufficiently to allow better detection of Far1 protein (Fig. 6). Transcription of *FAR1* from the unregulated *Δ350FAR1* and *UASΔ*350FAR1 promoters resulted in constitutive *FAR1* mRNA accumulation, whereas expression of *lacZ* from the full-length *FAR1* promoter was cell cycle regulated, suggesting that control of *FAR1* mRNA levels in the cell cycle may be entirely transcriptional. Making transcription of *FAR1* constitutive, however, did not abolish cell cycle regulation of Far1 protein accumulation (Fig. 6). These results demonstrate that the G_1-specific accumulation of Far1 protein is controlled by a post-transcriptional mechanism, probably cell cycle-specific protein degradation (J.D. McKinney and F. Cross, unpubl.).

Cell cycle regulation of Far1 protein phosphorylation

Cells arrested in G_1 by *cln* deprivation displayed predominantly a fast-migrating species of Far1 protein (Figs. 2B, 4B, and 5B). Upon release from the G_1 block, slow-migrating forms of Far1 protein appeared rapidly prior to the decline in total Far1 protein levels (Figs. 2B, 4B, and 5B). In subsequent cell cycles, we do not see Far1 reaccumulating as a purely fast-migrating species. This may be attributable to the decay of synchrony during subsequent cell cycles or may result from the presence of high constitutive levels of Cln3 after turn-on of the *GAL1::CLN3* gene. Slow-migrating forms of Far1 protein also accumulated in cells arrested by temperature-sensitive *cdc34* or *cdc4* mutations, which block cell cycle progression in G_1 after or within Start but before S phase (Fig. 3; data not shown) (Pringle and Hartwell 1981).

To test whether the retardation in Far1 protein mobility was attributable to phosphorylation, Far1 protein was immunoprecipitated from *cdc34* cells using anti-Far1 antibody, treated in vitro with calf intestinal phosphatase, and analyzed by Western blot (Fig. 3B). *cdc34* cells grown at the permissive temperature contained fast-migrating Far1 protein, which was shifted up to the slow-migrating forms after incubation of cells at the restrictive temperature (Fig. 3B, lanes 2,3). Incubation with phosphatase in vitro converted the slow-migrating Far1 protein to the fast-migrating form (Fig. 3B, lane 4). The increased mobility of Far1 protein by phosphatase was prevented by the inclusion in the reaction of β-glycerophosphate, a competitive inhibitor of phosphatase (Fig. 3B, lane 5), suggesting that the mobility retardation was attributable to phosphorylation of Far1.

Phosphorylated Far1 protein appears at about the time of Start, just prior to the decline in total Far1 protein levels, and Far1 protein accumulation is regulated independently of *FAR1* mRNA levels (Figs. 4 and 6). The timing of Far1 protein phosphorylation suggests that modification may target Far1 for destruction by a *CDC34*-dependent mechanism (see Discussion).

Regulation of FAR1 *expression is significant for Far1 function and the Start transition*

Accumulation of Far1 protein to high levels is restricted to the G_1 phase, which is the time when Far1 functions. Because *FAR1* is essential for cell cycle arrest in response to mating pheromone (Chang and Herskowitz 1990), we

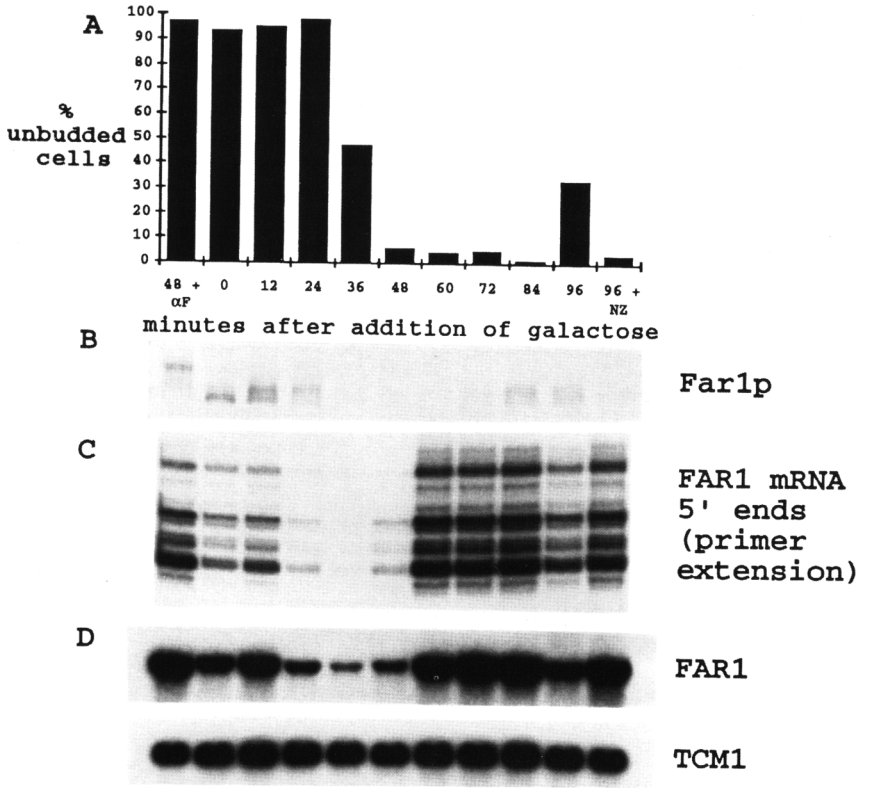

Figure 5. Uncoupling of *FAR1* mRNA and protein accumulation is not attributable to altered transcriptional initiation. Cells of genotype *cln1 cln2 cln3 GAL1::CLN3* were synchronized in G_1 phase as described in Materials and methods: Cells were grown to log phase in YEP–galactose medium at 30°C, arrested in G_1 by an additional 2.5 hr of growth in YEP–raffinose, and galactose was added to 3% to restart the cell cycle. At the time of galactose addition, aliquots of cells were removed, treated with either α-factor at 0.1 μM or nocodazole at 15 μg/ml, and then incubated at 30°C for another 48 min (α-factor) or 96 min (nocodazole). At the indicated time points following galactose addition, RNA and protein samples were extracted from the culture. RNA samples were analyzed by Northern blot hybridization with the indicated probes (*D*) or by primer extension with reverse transcriptase to determine the 5′ ends of the RNAs (*C*). Protein samples were analyzed by Western blot using anti-Far1 antibody (*B*). Aliquots of culture from each time point were analyzed for budding index as a morphological indicator of cell cycle position (*A*).

reasoned that the induction of Far1 levels in G_1 cells might be required for pheromone-induced cell cycle arrest. We therefore tested cells containing the *UASΔ350FAR1* allele for cell cycle arrest in response to mating pheromone, because these cells underexpress Far1 protein somewhat compared with wild type (Fig. 6).

The sensitivity of cells to α-factor arrest was determined by examining the accumulation of cells in the unbudded (G_1) state in response to a range of α-factor concentrations (see Chang and Herskowitz 1990). Remarkably, the *UASΔ350FAR1* cells failed to arrest even at the highest dose of pheromone tested [enough to saturate the recep-

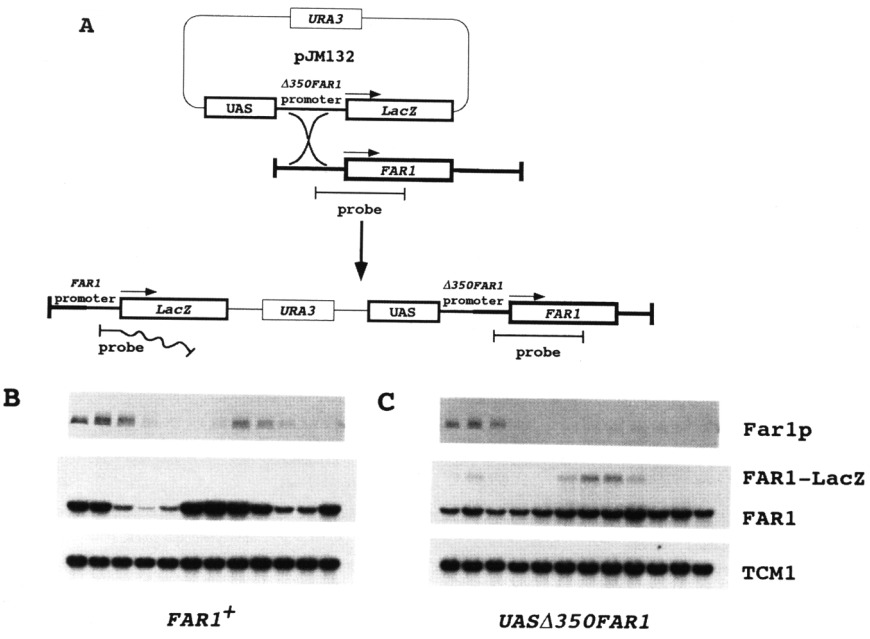

Figure 6. Far1 protein accumulation is cell cycle regulated independently of *FAR1* transcription. (*A*) Construction of the *UASΔ350FAR1* allele. pJM132 was constructed by insertion of a UAS cassette from the *CYC1* gene promoter upstream of *FAR1* promoter sequences from −350 to −1 (relative to the *FAR1* translational start codon) fused to the *lacZ* reporter gene (see Materials and methods). Integration of pJM132 at *FAR1* by homologous recombination places the *lacZ* gene under the control of the full-length *FAR1* promoter and places the *FAR1*-coding sequences under the control of the *UASΔ350FAR1* promoter provided by the plasmid. (*B,C*) Cells of genotype *cln1 cln2 cln3 GAL1::CLN3* and *FAR1*$^+$ (*B*) or *UASΔ350FAR1* (*C*) were synchronized, and RNA and protein were analyzed as described in the legend to Fig. 2. Note that time courses and blots for the *FAR1*$^+$ and *UASΔ350FAR1* strains were all performed in parallel and are therefore directly comparable.

tor (Jenness et al. 1983)], whereas the wild-type *FAR1*$^+$ controls arrested efficiently (Fig. 7A). The resistance of the *UASΔ*350FAR1 cells to cell cycle arrest by α-factor was confirmed by the halo assay (see Chang and Herskowitz 1990), in which a zone of growth inhibition around a source of α-factor indicates sensitivity. *far1* null mutant cells are not detectably inhibited in this assay, whereas wild-type *FAR1*$^+$ cells are strongly inhibited (Chang and Herskowitz 1990). The growth of *UASΔ*350FAR1 mutant cells was inhibited much less in this assay than wild type (*FAR1*$^+$), although *UASΔ*350FAR1 mutant cells were clearly more sensitive than *far1* null cells (data not shown). Also, in the genetic background tested (*cln1 CLN2*$^+$ *cln3 GAL1::CLN3*), resistance required *CLN3* expression, as the *UASΔ*350FAR1 mutant cells were largely α-factor sensitive in glucose medium (*GAL1::CLN3* off), whereas *far1* null mutants in the same genetic background were strongly α-factor resistant even on glucose medium. We have not yet tested the *UASΔ*350FAR1 in a wild-type *CLN* background, so the levels of Far1 required for efficient cell cycle arrest in wild-type cells cannot be determined directly from this experiment. Nevertheless, cell cycle arrest by Far1 appears strikingly dosage sensitive, which is presumably relevant to its cell cycle regulation.

Our comparison of levels of *FAR1* expression in *FAR1*$^+$ versus *UASΔ*350FAR1 cells was done in the absence of α-factor (Fig. 6), which moderately induces *FAR1*$^+$ transcription (Chang and Herskowitz 1990, 1992) but does not affect transcription of the *UASΔ*350FAR1 allele (data not shown). We therefore compared the levels of Far1 protein accumulation in

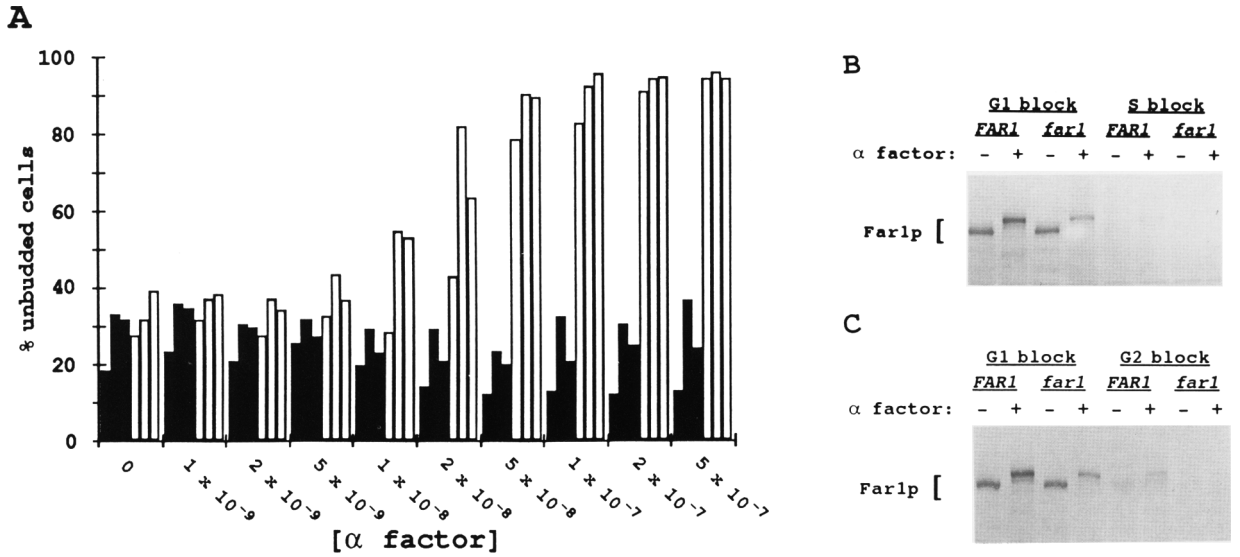

Figure 7. Functional significance of *FAR1* cell cycle regulation. (*A*) Isogenic strains of genotype *cln1 CLN2*$^+$ *cln3 GAL1::CLN3* and *FAR1*$^+$ (□) or *UASΔ*350FAR1(■) were constructed as described in Materials and methods. [Note that the *CLN2*$^+$ allele was included for this experiment, because the resistance of *far1* strains to pheromone-induced cell cycle arrest requires a functional *CLN2* gene (Chang and Herskowitz 1990).] *FAR1*$^+$ strains were each derived independently from a *UASΔ*350FAR1 progenitor by selection for Ura$^-$ revertants in which the pJM132 plasmid integrated at the *FAR1* locus had self-excised, regenerating the wild-type *FAR1* locus. Three independently isolated *UASΔ*350FAR1 strains and their *FAR1*$^+$ derivatives (presented in the same order in the graph) were assayed. Cells were grown in YEP–galactose medium at 30°C to mid-log phase, treated with α-factor at the indicated concentrations for an additional 3 hr at 30°C, and fixed and analyzed for budding index (see Materials and methods). Sensitivity to a given concentration of α-factor is indicated by the accumulation of unbudded cells above the level seen in the control (no α-factor) cultures. (*B*) Cells of genotype *cln1 cln2 cln3 GAL1::CLN3* and *FAR1*$^+$ (*FAR1*) or *UASΔ*350FAR1 (*far1*) were grown to mid-log phase in YEP–galactose medium at 30°C and then split: Half of each culture was shifted into YEP–raffinose medium (G_1 block), and half was treated with hydroxyurea at 0.2 M (S block). Incubation was continued for another 2.5 hr at 30°C to allow arrest in G_1 (YEP–raffinose cultures) or S (hydroxyurea-treated cultures), and the cultures were split again: Half of each culture was treated with α-factor at 0.2 μM (+ α-factor), and half was left untreated (− α-factor). After α-factor addition, incubation was continued for another 24 min at 30°C before protein samples were extracted from each culture and analyzed by Western blot using anti-Far1 antibody. Aliquots of each culture were analyzed for budding index as an indicator of the efficiency of cell cycle arrest: *cln*-blocked cultures accumulated ≥95% unbudded cells, whereas hydroxyurea-blocked cultures accumulated ≥93% budded cells. (*C*) Cells of genotype *cln1 cln2 cln3 GAL1::CLN3* and *FAR1*$^+$ (*FAR1*) or *UASΔ*350FAR1 (*far1*) were grown to mid-log phase in YEP–galactose medium at 30°C and then split: Half of each culture was shifted into YEP–raffinose medium (G_1 block) and half was treated with nocodazole at 15 μg/ml (G_2 block). Incubation was continued for another 2.5 hr at 30°C to allow arrest in G_1 (YEP–raffinose cultures) or G_2/M (nocodazole-treated cultures), and the cultures were split again: Half of each culture was treated with α-factor at 0.2 μM (+ α-factor) and half was left untreated (− α-factor). After α-factor addition, incubation was continued for another 24 min at 30°C before protein samples were extracted from each culture and analyzed by Western blot using anti-Far1 antibody. Aliquots of each culture were analyzed for budding index as an indicator of the efficiency of cell cycle arrest: *cln*-blocked cultures accumulated ≥95% unbudded cells, whereas nocodazole-blocked cultures accumulated ≥95% budded cells.

FAR1$^+$ and *UASΔ*350FAR1 cells arrested in G_1 phase (by *cln* deprivation), in S phase (by hydroxyurea), or in G_2 phase (by nocodazole) following α-factor treatment. We chose these three cell cycle blocks because the G_1 block corresponds to the cell cycle interval when Far1 protein accumulates to maximal levels and cells are competent to arrest in response to mating pheromone, whereas the S and G_2 blocks correspond to the interval when Far1 expression is lowest and cells are resistant to pheromone-induced arrest. At all three cell cycle blocks, α-factor treatment slightly induced Far1 protein levels in *FAR1*$^+$ cells but not in *UASΔ*350FAR1 cells (Fig. 7B,C). Even after α-factor treatment, however, the level of Far1 protein in G_1-arrested *UASΔ*350FAR1 cells remained higher than the level of Far1 in S- or G_2-arrested *FAR1*$^+$ cells (Fig. 7B,C).

The Start transition is defined by a switch from a mating pheromone-sensitive to a mating pheromone-resistant interval of the cell cycle. The time of acquisition of mating pheromone resistance correlates exactly with the down-regulation of Far1 to a level insufficient for cell cycle arrest. Thus, cell cycle regulation of Far1 accumulation may contribute significantly to the rapid acquisition of pheromone resistance at Start (Cross and McKinney 1992).

Discussion

FAR1 *regulation and the Start transition*

Start functions as a developmental switch between the mutually exclusive pathways of cell division and conjugation. Traversal of Start requires the *CDC28* and *CLN* gene products, whereas the inhibition of Start by mating pheromone requires the *FAR1* gene product, which may act by interfering with *CLN* gene function (Chang and Herskowitz 1990). We have shown that Far1 protein levels are cell cycle regulated, such that functional levels of Far1 accumulate only in pre-Start G_1 cells. The Start transition (and, perhaps, specifically activation of the Cdc28 protein kinase) results in rapid down-regulation of Far1 protein levels.

FAR1 regulation may be one example of a global mechanism for inhibition of the pheromone response pathway at Start, because *STE2* and *STE3* mRNAs (which encode the α-factor and a-factor receptors, respectively) also decline sharply at Start in parallel with *FAR1* mRNA (Zanolari and Riezman 1991; J.D. McKinney and F. Cross, unpubl.). It will be important to determine whether other components of the pheromone response machinery are regulated similarly. The down-regulation of just one component of the pheromone response machinery, *FAR1*, could account for the acquisition of pheromone resistance at Start. Elucidation of the mechanisms that control expression of *FAR1* and, perhaps, other key components of the pheromone response machinery, is therefore of considerable interest.

Multiple controls on Far1 accumulation

Cell cycle regulation of *FAR1* expression involves independent controls on *FAR1* transcription and protein accumulation. *FAR1* mRNA accumulates to high levels during the pre-Start G_1 interval, declines abruptly at about the time of Start, and rises again in late S or G_2 phase, remaining high through mitosis and into the succeeding G_1 interval. Regulation of *FAR1* mRNA levels is probably attributable entirely to transcriptional control (Fig. 6). In contrast, accumulation of Far1 protein to high levels is restricted to the G_1 interval; thus, cells in G_2/M contain high levels of *FAR1* mRNA but low levels of Far1 protein (Fig. 4). It is possible that the accumulation of *FAR1* mRNA during G_2/M is required to facilitate the rapid accumulation of Far1 protein when cells enter G_1 phase. This control may be important because of the asymmetry of cell division in budding yeast: One of the products of each cell division (the mother cell) is typically much larger than the other (the daughter cell); consequently, the large mother cell spends only a very short time in G_1 (Hartwell and Unger 1977). The accumulation of *FAR1* mRNA during the previous cell cycle may allow a rapid burst of Far1 protein accumulation when cells enter G_1 phase, ensuring that large mother cells will arrest efficiently in response to mating pheromone.

A clue to the mechanism controlling Far1 protein accumulation may be provided by our observation that Far1 is differentially phosphorylated during the cell cycle: At about the time of Start, highly phosphorylated forms of Far1 appear just prior to the decline in total Far1 protein levels (Figs. 2B, 4B, and 5B). The timing of Far1 phosphorylation suggests a role in targeting Far1 for degradation. A plausible candidate for the Far1-kinase is the *CDC28* gene product, which promotes Start. In support of this hypothesis, we have found that the phosphorylated forms of Far1, but not unphosphorylated Far1, can be specifically coimmunoprecipitated with Cdc28 (J.D. McKinney and F. Cross, unpubl.). Mapping of the phosphorylation sites within Far1 and mutagenic studies will be required to test this hypothesis. Intriguingly, we have found that highly phosphorylated forms of Far1 accumulate in cells arrested by a *cdc34* mutation (Fig. 3), which blocks cell cycle progression in late G_1 after Start (Pringle and Hartwell 1981). *CDC34* encodes a ubiquitin-conjugating enzyme (Goebl et al. 1988); in several systems, ubiquitination leads to protein degradation. These observations suggest the following speculative model for control of Far1 protein accumulation: At Start, Far1 is phosphorylated (perhaps by Cdc28), which targets Far1 for ubiquitination by Cdc34; ubiquitination then channels Far1 into a ubiquitin-dependent proteolytic pathway.

In response to α-factor, Far1 displays a phosphorylation-induced mobility retardation on SDS-PAGE that is distinct from the mobility retardation observed during the cell cycle (Fig. 5B) (Chang and Herskowitz 1992). Pheromone-induced phosphorylation does not require *CDC28* or *CLN* activity, because α-factor treatment of cells arrested by a *cdc28* temperature-sensitive mutation (Fig. 1 and data not shown) or by *cln* deprivation (data not shown) results in a similar gel mobility retardation. Because the cell cycle-regulated phosphorylation that we observe requires both *CLN* and *CDC28* function (Figs.

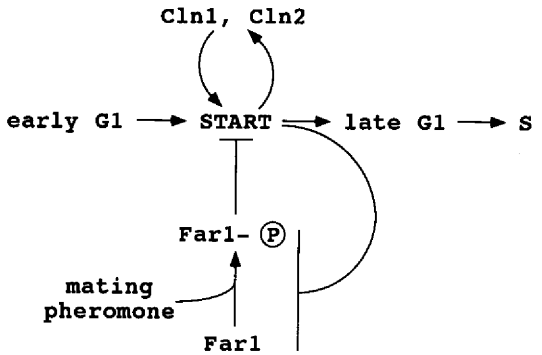

Figure 8. A potential role for *FAR1* cell cycle regulation in the Start transition. *FAR1* encodes a function essential for cell cycle arrest in response to mating pheromones. Cells are sensitive to pheromone arrest only during early G_1 phase before Start. Functional levels of Far1 protein accumulate only during the pheromone-sensitive (pre-Start G_1) period. Down-regulation of Far1 to nonfunctional levels at Start may therefore contribute to the transition from pheromone sensitivity (pre-Start) to pheromone resistance (post-Start). The Cln1 and Cln2 proteins, which promote Start, may stimulate their own synthesis via a positive feedback loop for *CLN1* and *CLN2* transcription. In response to mating pheromone, Far1 stimulates its own synthesis by arresting cells in the pre-Start G_1 interval, when *FAR1* expression is maximal. Passage through Start may be accelerated by the concurrent rise in levels of activators of Start (Cln1 and Cln2) and decline in the level of an inhibitor of Start (Far1).

2B, 3B, 4B, 5B, and 6; data not shown), the kinase involved is likely to be different.

Mutual inhibition and self-activation by Start regulators

Recently, evidence has accumulated that the Cln proteins may activate transcription of *CLN1* and *CLN2*, resulting in a positive feedback loop, which could accelerate the Start transition (Cross 1991; Cross and Tinkelenberg 1991; Dirick and Nasmyth 1991). Similarly, in response to mating pheromone, Far1 indirectly promotes its own accumulation by holding cells in G_1 before Start, when *FAR1* is maximally expressed. *FAR1* is thought to inhibit Start by interfering with the accumulation or activity of Cln2 (Chang and Herskowitz 1990); we present evidence that the converse may also be true, as passage through Start, which is promoted by Cln proteins, inhibits the accumulation of Far1 protein. The mutually inhibitory interactions between positive and negative regulators of Start, combined with the ability of these regulators to stimulate their own synthesis, may make Start a more efficient switch, as entry into either the cell cycle or conjugation pathway would be self-reinforcing and would inhibit entry into the alternative pathway (Fig. 8) (Cross and McKinney 1992).

Materials and methods

Yeast strains and plasmids

All strains used were isogenic with YH110 (Richardson et al. 1989) except strains used in the *cdc* mutant analysis (Fig. 3), which were congenic with A364A (Hartwell et al. 1974). All YH110-isogenic strains used were *bar1Δ* (provided by D. Lew, Research Institute of Scripps Clinic, La Jolla, CA). Mutant alleles created in the YH110 background were *cln1Δ* and *cln2Δ* (Cross and Tinkelenberg 1991), *cln1::TRP1* (Hadwiger et al. 1989), *cln3Δ* (Cross 1990), and *far1::URA3* (Chang and Herskowitz 1990). Triple-mutant *cln1 cln2 cln3* strains were constructed by inclusion of galactose-conditional *CLN3* constructs (Cross 1990), except that in these experiments the *GAL1::CLN3* cassette (Cross 1990) was integrated at *leu2* or *trp1*. For all cell cycle time-course experiments, the *leu2::LEU2::GAL1::CLN3* allele was used, as it gave the best synchrony. Standard methods of transformation and tetrad analysis (Guthrie and Fink 1991) were used for all strain constructions. For construction of the strains used in Figure 7A, strains carrying the *UASΔ350FAR1* allele (see below) were reverted to *FAR1*$^+$ by selection of Ura$^-$ popouts on medium containing fluoro-orotic acid (FOA) (Ausubel et al. 1987). FOA kills cells that contain a functional *URA3* gene but does not kill *ura3* mutants. Thus, reversion of *UASΔ350FAR1* to *FAR1*$^+$ by self-excision of the pJM132 plasmid (which contains the *URA3* marker; see Fig. 6A) allowed growth on FOA.

YEpLG178 (Guarente and Mason 1983) contains a minimal *CYC1* gene promoter driving expression of the *lacZ* reporter gene cloned into the vector YEp24, which carries the *URA3* selectable marker gene and the origin of replication from the yeast 2μ circle. YEpLG178 was converted to the integrating plasmid YIpLG178 by cutting with *Hin*dIII and religating to remove the 2μ circle *ori* sequences. The first 350 bp of the *FAR1* promoter was amplified from plasmid pFC15 (Chang and Herskowitz 1990) by use of the polymerase chain reaction (PCR) (*Taq* polymerase from Perkin-Elmer Cetus), with the generation of new *Xho*I and *Bam*HI sites at the 5′ and 3′ ends, respectively, of the amplified fragment. The sense-strand PCR oligonucleotide had the sequence TTATTACTCGAGGGTACCTTGGTTCACAGTATA, and the nonsense-strand PCR oligonucleotide had the sequence ATTATTGGATCCCTGTTGCTTCTTAAATTCTGG. The amplified *FAR1* promoter fragment was cut with *Xho*I and *Bam*HI and ligated into the *Xho*I–*Bam*HI sites of YIpLG178 to generate pJM131. Plasmid pJM132 was derived from pJM131 by the insertion of the 134-bp UAS from the *CYC1* gene promoter upstream of the *FAR1* promoter. The *CYC1* UAS was amplified from plasmid YEpLG312 (Guarente and Mason 1983) by use of PCR. The sense-strand PCR oligonucleotide had the sequence TTATTACTCGAGCCCGGGAGCAAGATCAAGATG and the nonsense-strand PCR oligonucleotide had the sequence ATGTGTCAGCACTAAAGTTGC. The amplified *CYC1* fragment was cut with *Xho*I and inserted (in the reverse orientation) into the *Xho*I site of pJM131 to generate pJM132.

For integration at *FAR1*, pJM131 and pJM132 were partially digested with *Hin*dIII (which cuts once within the *FAR1* promoter sequences and once within the YIpLG178 vector) and transformed into strain 1608-21C (genotype *MATa bar1 cln1::TRP1 cln2Δ cln3Δ leu2::LEU2::GAL1::CLN3 ura3 his2*) by the lithium acetate procedure (Ausubel et al. 1987). Transformants (Ura$^+$) were selected on Sc galactose–uracil medium (Ausubel et al. 1990). The correct integration of plasmids at *FAR1* was confirmed by Southern blot analysis of the transformed cell lines as described below.

Culture conditions and cell cycle synchronization protocols

Cells were grown in YEP medium (1% Difco yeast extract, 2% Difco Bacto-peptone) containing 2% glucose, 3% galactose, or 3% raffinose as carbon source (all sugars from Sigma). For growth on solid medium, 2% Bacto-agar (Difco) was added. To determine cell budding index, cells were fixed in PBS contain-

ing 10% formalin, sonicated, collected by centrifugation, resuspended in water, and examined microscopically. For DAPI staining of nuclei, aliquots of cells were fixed in 3 ml of 95% ethanol, 1 μl of a 1 mg/ml DAPI (Sigma) solution was added, and the stained cell suspension was allowed to incubate at room temperature for 5 min. Stained cells were then collected by centrifugation, resuspended in 3 ml of water, sonicated, again collected by centrifugation, resuspended in a small volume of water, and examined in a UV-fluorescence microscope (Nikon Microphot FX). The proportion of binucleate cells was scored as an indication of the completeness of nuclear division: A cell was considered binucleate if the mother and daughter compartments each contained a distinct nucleus with no DAPI staining in the neck of the bud.

For cell cycle time-course experiments (Figs. 2, 4, 5, and 6), cells of genotypes indicated in figure legends (also see Yeast strains and plasmids, above) were grown to an A_{660} of 0.4–0.8 in YEP–galactose medium at 30°C and collected by vacuum filtration on a 0.65-μm-pore-size filter (Millipore), washed on the filter once with 25 ml of YEP–raffinose, and resuspended in YEP–raffinose at an A_{660} of ~0.35. Cultures were maintained in YEP–raffinose at 30°C for 2.5 hr, during which time cells arrested uniformly in G_1 with a large, unbudded morphology. Cultures were adjusted to an A_{660} of 0.7 with YEP–raffinose and the time course was started by the addition of galactose to a final concentration of 3% from a 30% stock solution. Throughout the time course, cultures were fed with YEP–raffinose–galactose so as to maintain the A_{660} within the range of 0.7–0.9.

Nocodazole (Sigma) was used at a final concentration of 15 μg/ml from a stock solution of 10 mg/ml dissolved in dimethylsulfoxide (DMSO, Sigma). For experiments involving nocodazole, control cultures were treated in parallel with DMSO alone, which was found to have no significant effect on cell cycle progression and *FAR1* expression (data not shown). Hydroxyurea (Sigma) was added directly to a final concentration of 0.2 M. α-Factor (Sigma) was used at concentrations indicated in the figure legends from a stock solution of 1 mM in water.

For synchronization of temperature-sensitive *cdc* mutants, cell cultures were grown to an A_{660} of 0.5 in YEP–glucose medium at 22°C and then split: Half of each culture was maintained at 22°C, and half was shifted to 37°C for an additional 3 hr to allow arrest of the *cdc* mutants at their respective block points.

Mating pheromone dosage-response assay

For the analysis of pheromone arrest in liquid cultures (Fig. 7A), cells were grown to an A_{660} of 0.7 at 30°C in YEP–galactose medium and then diluted twofold into fresh YEP–galactose medium containing the indicated concentrations of α-factor (Sigma). Incubations were then continued at 30°C for 3 hr before cells were fixed and analyzed for budding index as described above.

Western blot analysis

Cells from 10 ml of culture at an A_{660} of 0.7–0.9 were poured over an equal volume of crushed ice in a glass culture tube and collected by centrifugation. Subsequent steps were performed in a 4°C cold room. The cell pellet was resuspended in 1 ml of ice-cold TE buffer, transferred to a 1.5-ml Eppendorf tube, and pelleted in a microcentrifuge. To the washed cell pellet were added 100 μl of extraction buffer (10 mM Tris-HCl at pH 7.2, 0.6% SDS, 10% aprotinin from Sigma, 10 μg/ml of leupeptin from Sigma, 10 μg/ml of pepstatin from Sigma, 0.5 mM PMSF from Sigma, and 1 mM sodium pyrophosphate from Sigma) and ~100 μl of glass beads (425–600 μm, acid-washed, from Sigma). Samples were vortexed in a Vortex Genie sleeve at top speed for 3 min at 4°C. One hundred microliters of 2× protein gel sample buffer (Ausubel et al. 1987) was added, and samples were incubated in a 90°C water bath for 3 min. Each sample (10 μl) was electrophoresed on a Hoefer Mighty-Small 1.5-mm minigel consisting of a 5% polyacrylamide stacking gel and 7% polyacrylamide resolving gel (Ausubel et al. 1987). Gels were electrotransferred to Immobilon P (Millipore) in a Hoefer TE50 Transfor electroblotter as described (Ausubel et al. 1987). Blots were blocked in PBS containing 0.5% Z3-14 detergent (Calbiochem) for at least 15 min at room temperature with agitation. Affinity-purified anti-Far1 antibody (Chang and Herskowitz 1992) was added in the same buffer and incubated overnight. To improve detection of Far1, an amplification procedure (suggested by R. Fuller) was employed involving sequential 1-hr incubations with goat anti-rabbit IgG and rabbit anti-goat IgG (both at 1 : 2000, from Cappel), followed by incubation for 1 hr with goat anti-rabbit IgG conjugated to alkaline phosphatase (1 : 2000, from Cappel). All antibody incubations were done at room temperature with agitation; after each incubation, blots were washed for 15 min with several changes of PBS buffer. Following antibody incubations, blots were washed once in veronal acetate buffer and developed for detection of alkaline phosphatase as described (Ausubel et al. 1987). The sequential antibody incubations were found to result in a 5- to 10-fold amplification of signal (data not shown).

Immunoprecipitation of Far1 protein and phosphatase treatment in vitro prior to Western blot analysis (Fig. 3B) were performed as described (Chang and Herskowitz 1992).

Northern and Southern blot analysis

For Northern blot analysis, yeast RNA was isolated from log-phase cells as follows. Ten milliliters of culture at an A_{660} of 0.7– 0.9 were poured over an equal volume of crushed ice in a glass culture tube and collected by centrifugation. Subsequent steps were performed in a 4°C cold room. The cell pellet was resuspended in 1 ml of ice-cold TE buffer, transferred to a 1.5-ml microtube, and pelleted in a microcentrifuge. To the washed cell pellet were added ~300 μl of glass beads (425–600 μm, acid-washed, from Sigma), 350 μl of phenol–chloroform–isoamyl alcohol (25 : 24 : 1), and 350 μl of NETS buffer (0.3 M NaCl, 1 mM EDTA, 10 mM Tris-HCl at pH 7.5, and 0.2% SDS). Samples were vortexed in a Vortex Genie sleeve at top speed for 10 min and then microcentrifuged for 5 min. The aqueous layer was transferred to a fresh Eppendorf tube and 1 ml of ice-cold ethanol was added. Samples were allowed to precipitate at 4°C for ≥1 hr and were then pelleted by microcentrifugation for 10 min at 4°C, washed once with ice-cold ethanol, and allowed to air-dry for 15 min. Sample pellets were then resuspended in ETS buffer (10 mM Tris-HCl at pH 7.9, 1 mM EDTA, 0.2% SDS) and stored at −80°C. Samples were prepared for electrophoresis and separated on denaturing formaldehyde–agarose gels as described (Ausubel et al. 1987) and transferred by capillary blotting to GeneScreen Plus nylon membrane (Du Pont) according to the manufacturer's protocol.

For Southern blot analysis, yeast genomic DNA was isolated (Holm et al. 1986), digested with *Xho*I and *Bam*HI (Boehringer Mannheim Biochemicals), and separated by agarose gel electrophoresis as described (Ausubel et al. 1987). Gels were tranferred by capillary blotting to GeneScreen Plus nylon membrane (Du Pont) according to the manufacturer's protocol.

After nucleic acid transfer, membranes were air-dried, UV-cross-linked using a Stratalinker UV box (Stratagene), and baked at 80°C for 2 hr in a vacuum oven. Membrane prehybridization

and hybridization were performed according to the manufacturer's protocol (Du Pont) in a solution consisting of 50% formamide, 1% SDS, 1 M NaCl, and 10% dextran sulfate at 42°C in a Hybaid MKII Mini Hybridization Oven. Probes were plasmid restriction fragments purified by electrophoresis in low-melting-point agarose (Boehringer Mannheim Biochemicals) and labeled by the random-primer method using a Prime-It Kit (Stratagene). Probe fragments used were *CLN2*, an 864-bp *Xho*I–*Hin*dIII fragment containing coding sequences for amino acids 86–378 (Hadwiger and Reed 1990); *TCM1*, an ~800-bp *Hpa*I–*Sal*I fragment from pAB309Δ (Schultz and Friesen 1983; provided by J. Hirsch); and *FAR1*, a 920- bp *Hin*dIII fragment from pFC21 (Chang and Herskowitz 1990).

Primer extension analysis of mRNA 5′ ends

The primer used for primer extension analysis of *FAR1* mRNA had the sequence 5′-ACTCTCTTCAATATACCCAAATGTGGGCAT-3′, hybridizing to *FAR1* sense-strand sequences from +30 to +1 relative to the translational start codon (Chang and Herskowitz 1990). Primer extension was performed essentially as described (Ausubel et al. 1987). Samples were resolved by electrophoresis on a 0.6% polyacrylamide/8 M urea sequencing gel and processed for autoradiography as described (Ausubel at al. 1987).

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