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 G1 phase. Mating pheromones promote conjugation by arresting cells in G1 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 G1, 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 G2 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 α-factor and α-factor, respectively. Binding of α- 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 G1 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 (Masague 1992). Cell cycle arrest in the G1 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 G1 interval ensures that the resulting a/α zygote will have the correct ploidy.

Commitment to cell cycle progression occurs at Start in the G1 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 p34cdc2 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; Haddon 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 G1 interval, the time of Far1 function.
Results

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

The CLN3-2 allele of CLN3 (previously called DAF1-I) 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 (Tycers et al. 1992) might regulate Far1 accumulation. Because one effect of CLN3-2 expression is a very short G1 interval [Cross 1988], a simple explanation for this result might be that Far1 accumulation is restricted to G1 cells. This idea would also account for the epistasis of cdc28 inactivation to CLN3-2, because cdc28 inactivation results in G1 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 protocol, as described previously [Cross and Tinkelenberg 1991]. cln1 cln2 cln3 GAL1::CLN3 cells were arrested in G1 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 G1, low from Start to late S or G2, and high in G2 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 G1-blocked cells, rose rapidly at about the time of

Figure 1. Far1 protein accumulation is depressed by Cln3-2 activation of Cdc28. All strains had the genotype MATa bar1 cln1 cln2 cln3 GAL1::CLN3 and were congenic with 831G; additionally, strains were either CDC28+ [wt lanes] or cdc28-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 G1 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].
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 G1 and was low from Start until the succeeding nuclear division (Fig. 2B). The G1-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 G1-arrested mutants [cln1 cln2 cln3, cdc28, cdc34, and cdc4], whereas only very low levels of Far1 protein accumulated in cdc mutants blocked in late G1 [cdc7], S [cdc8], and G2/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 G1 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 G1 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 G2, 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 S' ends throughout the cell cycle and in nocodazole-blocked 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-

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**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 G1 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+ FAR1+ and cdc34Δ 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 G1 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.
The presence of nocodazole, cells released from the G1 block proceed 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 [(% unbudded cells)] and percent binucleate cells (x) as morphological indicators of cell cycle position (A).

**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 G1 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 G1 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 [(% unbudded cells)] and percent binucleate cells (x) as morphological indicators of cell cycle position (A).
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 G1 phase as described in Materials and methods: Cells were grown to log phase in YEP-galactose medium at 30°C, arrested in G1 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-Farl 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 G1 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 un budded (G1) 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-
null mutant cells are not detectably inhibited in this assay, whereas wild-type FAR1+ cells are strongly inhibited. Our comparison of levels of FAR1 expression in FAR1+ versus UAS350FAR1 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 UAS350FAR1 allele (data not shown). We therefore compared the levels of Far1 protein accumulation in...
**FAR1** and UAS∆350FAR1 cells arrested in G₁ phase (by cln deprivation), in S phase (by hydroxyurea), or in G₂ phase (by nocodazole) following α-factor treatment. We chose these three cell cycle blocks because the G₁ 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 G₂ 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 UAS∆350FAR1 cells (Fig. 7B, C). Even after α-factor treatment, however, the level of Far1 protein in G₁-arrested UAS∆350FAR1 cells remained higher than the level of Far1 in S- or G₂-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 G₁ 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 α-factor receptors, respectively) also decline sharply at Start in parallel with **FAR1** mRNA (Zanolar 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 G₁ interval, declines abruptly at about the time of Start, and rises again in late S or G₂ phase, remaining high through mitosis and into the succeeding G₁ 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 G₁ interval; thus, cells in G₂/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 G₂/M is required to facilitate the rapid accumulation of Far1 protein when cells enter G₁ 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 G₁ [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 G₁ 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 G₁ 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.
ulators to stimulate their own synthesis, may make inhibitory interactions between positive and negative regulators, which would inhibit entry into the alternative pathway (Fig. 8). [52x175]McKinney et al. [1989] except strains used in the cdc mutant analysis [Fig. 3], which were congenic with A364A [Hartwell et al. 1974]. 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 UAS350FAR1 allele (see below) were reverted to FAR1+ by selection of Ura−/Ura+ on medium containing fluoroorotic acid (FOA) [Ausubel et al. 1987]. FOA kills cells that contain a functional URA3 gene but does not kill ura3 mutants. Thus, reversion of UAS350FAR1 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 lacZ reporter cloned into the vector Yepl24, which carries the URA3 selectable marker gene and the origin of replication from the yeast 2μ circle. YeplG178 was converted to the integrating plasmid YeplG178 by cutting with HindIII 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 XhoI and BamHI sites at the 5′ and 3′ ends, respectively, of the amplified fragment. The sense-strand PCR oligonucleotide had the sequence TTATTGCAGGTCATTGTTGCTCACAGTATA, and the nonsense-strand PCR oligonucleotide had the sequence ATTATTGAGTCCCTGTGTTCTTTAAAATTCG. The amplified FAR1 promoter fragment was cut with XhoI and BamHI and ligated into the XhoI–BamHI sites of YeplG178 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 TTATTATCGACCCCGGGAGACAGTCAAAGTC and the nonsense-strand PCR oligonucleotide had the sequence ATGGTGTCAGCCTAAAGTTCCGC. The amplified CYC1 fragment was cut with XhoI and inserted [in the reverse orientation] into the XhoI site of pJM131 to generate pJM132.

For integration at FAR1, pJM131 and pJM132 were partially digested with HindIII [which cuts once within the FAR1 promoter sequences and once within the YeplG178 vector] and transformed into strain 1608-21C [genotype MATa bar1Δ 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-

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**Materials and methods**

**Yeast strains and plasmids**

All strains 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].
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 G1, 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 each culture was 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 ~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-Cl 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-Cl at pH 7.9, 1 mM EDTA, 0.2% SDS) and stored at −80°C. Samples were prepared for electrophoresis and separated on a 7% non-denaturing polyacrylamide gel, and baked 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 Hybrid MKII Mini Hybridization Oven. Probes were plasmid restriction fragments purified by electrophoresis in low-melt- ing-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 XhoI-HindIII fragment containing coding sequences for amino acids 86–378 (Hadjigkou and Reed 1990), TCM1, an ~800-bp HpaI-Sall fragment from pAB309A (Schultz and Friesen 1983, provided by J. Hirsch), and FAR1, a 920-bp HindIII 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’-ACTCTCTTCATATACTGCATTAAGTTTGGGCAT-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 et al. 1987).

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