**FAR1 and the G₁ Phase Specificity of Cell Cycle Arrest by Mating Factor in Saccharomyces cerevisiae**

JOHN D. MCKINNEY AND FREDERICK R. CROSS

The Rockefeller University, New York, New York 10021

Received 8 December 1994/Returned for modification 6 February 1995/Accepted 20 February 1995

Significant accumulation of Far1p is restricted to the G₁ phase of the *Saccharomyces cerevisiae* cell cycle. Here we demonstrate yeast cell cycle regulation of Far1p proteolysis. Deletions within the 50 N-terminal amino acids of Far1p increase stability and reduce cell cycle regulation of Far1p abundance. Whereas wild-type Far1p specifically and exclusively promotes G₁ phase arrest in response to mating factor, stabilized Far1p promoted arrest both during and after G₁. The loss of the G₁ specificity of Far1p action requires elimination of *FARI* transcriptional regulation (by means of the *GAL1* promoter) as well as N-terminal truncation. Thus, the cell cycle specificity of mating factor arrest may be largely due to cell cycle regulation of *FARI* transcription and protein stability.

The Start control point late in the G₁ stage of the budding *Saccharomyces cerevisiae* cell cycle commits cells to DNA replication and mitosis. Execution of Start is inhibited by mating factors, which stimulate exit from the cell cycle and entry into a differentiation pathway leading to conjugation (28, 33). Execution of a potentially comparable commitment step in mammalian cells (24) is inhibited by cell-cell contact and by transforming growth factor β (16).

Progression through both Start and mitosis is driven by cyclin-dependent protein kinases (CDKs) (7, 20, 31). CDKs are activated by cyclin binding (20, 31). In budding yeast cells, cyclins coded for by the *CLN* gene family are necessary for Start, while *CLB* family B-type cyclins are required for later cell cycle transitions (20).

Physiological G₁ arrest can result from interference with the formation or function of cyclin-CDK complexes necessary for entry into the S phase (6, 14). Cyclin-CDK inhibition may be mediated by CDK-binding proteins (21). Transforming growth factor β activates p27Kip1, which can inhibit cyclin-CDK complexes present in mammalian cells during G₁ (27). The α mating factor induces binding of the yeast Far1p to Cln-Cdc28 kinase complexes, inhibiting their activity (25, 26, 35). In many cases, such as transforming growth factor β treatment in mammalian cells or mating factor treatment of budding yeast cells, cell cycle arrest is specific to the G₁ interval, although CDKs are necessary not only for entry into S from G₁ but also for entry into mitosis from G₂. In budding yeast cells, levels of the binding protein Far1p rapidly decline as cells pass through Start because of transcriptional and posttranscriptional control of *FARI* expression (18). Since Far1p is necessary for cell cycle arrest by α-factor (2), the G₁ specificity of cell cycle arrest by α-factor might be due to the loss of Far1p at Start (1, 18). Here we test this hypothesis by means of mutations in *FARI* that interfere with cell cycle regulation of Far1p stability.

**MATERIALS AND METHODS**

**Yeast strains and plasmids.** Standard methods were used for strain constructions (9). All strains were isogenic with BF264-15DaU (18). pFC24 is the *GAL1::FARI* plasmid based on the original *FARI* sequence (3). We have named this cassette *GAL1::FARI::ΔN* for clarity. The integrating LEU2::GAL1::FARI::ΔN plasmid pM305 was constructed by ligating a *PvuI* fragment from pFC24 (3) into the *PvuI* sites of pRS305 (32). To make the integrating LEU2::GAL1::FARI::ΔN plasmid pM306, the first 893 bp of *FARI* coding sequences were amplified from pFC21 (2) (provided by F. Chang) by PCR. The PCR oligonucleotides were 5’-TTACGAGAATCTATATGAAGACAC CAA-3’ (sense strand) and 5’-CCTGTGAACCTTCCGCG-3’ (non-sense strand). The initiation codon and restriction sites are underlined. The sense strand oligo generated a new Xhol site. The non-sense strand PCR oligo spanned a HindIII site within the *FARI* coding sequences (2). The amplified fragment was cut with Xhol and HindIII and ligated into the Xhol-HindIII sites of pM305 to generate pM306. For integration at *leu2*, pM305 and pM306 were cut with Cfl.

The N-terminal deletion series in *GAL1::FARI* was constructed in the same manner as pM306, except that the sense strand oligos used for PCR corresponded to nucleotides (nt) 34 to 49, 64 to 79, 94 to 109, and 124 to 139 (numbering from the *FARI::ΔN* ATG initiation codon) in the sequence 3’ to the *FARI::ΔN* ATG (see above).

The Δ39 and Δ40 mutations were introduced into the endogenous *FARI* gene in the following manner. *FARI* was disrupted with the plasmid pM304 containing *URA3* between two HindIII sites, one 5’ of the *FARI::ΔN* ATG and the other in the *FARI* coding sequence. The *FARI* gene (Kpnl to BglII) was cloned in a plasmid containing the *LEU2* gene, producing YplM343. Oligonucleotides corresponding to the *FARI* sense strand from a HindIII site 5’ of the ATG to the ATG, then corresponding to nt 94 to 109 (Δ39) or nt 124 to 139 (Δ40), were used in a PCR together with the same 3’ oligonucleotide used for pM306, with pFC15 (from F. Chang) being used as the template. PCR mixtures were digested with HindIII and cloned into HindIII-cut YplM343 to make YplM346 (Δ39) and YplM347 (Δ40). YplM343, -346, and -347 were digested with SstI and used to transform 1630-9C-1 (*MATa far1::ura3*). Transformants of a *far1::ura3* disruptant with these plasmids were streaked on 5-Fluoroorotic acid to select popouts of *URA3*. Leu− popouts restored the *FARI::ΔN*, or *FARI::Δ40* coding sequence with the wild-type *FARI* promoter.

**DNA sequencing.** DNA sequencing of wild-type *FARI* in pFC21 was done with a Pharmacia T7 Sequencing Kit. The primer was 5’-ACTCTTCTAACATATAC CCAAATGTGGGCAT-3’, hybridizing to *FARI* nt 463 to 492 (2).

**Cell culture and cell cycle synchronization.** Cells were grown in yeast extract-peptone (YPE) medium with 2% glucose, 3% galactose, or 3% raffinose (Sigma). Synchronization by centrifugal elutriation, the cells were grown to an *A₅₅₀* of ~1.0 in 1.5 liters of YEP-galactose at 30°C, collected by filtration through 0.65-μm-pore-size filters (Millipore), resuspended in 200 ml of fresh YEP-galactose, sonicated to disperse clumps, and loaded at 30°C at a pump speed of 50 ml/min into a Beckman JE5.0 elutriator rotor running at 3,000 rpm. After being loaded, the cells were equilibrated in the chamber for 10 to 15 min with YEP-galactose, and cell fractions were stopped off by incremental increases in pump speed. Fractions with the highest percentage of un budded cells were reincubated into fresh YEP-galactose and incubated at 30°C.

The percentages of unbudded and budded cells were determined as described previously (18). The percentage of binucleate cells was determined by nuclear staining with 4,6-diamidino-2-phenylindole (DAPI) (Sigma) as described previously (18). The determination of the cellular DNA content was done by flow cytomtery as described previously (5).
α-Factor response assays. For an analysis of α-factor arrest on solid medium, cells were grown to mid-log phase (A₅₆₀ of 0.5 to 1.0 at 30°C) in YEP-galactose, sonicated to disperse clumps, and plated on YEP-galactose and YEP-glucose with and without 0.1 μM α-factor (Sigma). The plates were incubated at 30°C for 2.5 h (YEP-glucose) and 3.5 h (YEP-galactose) and examined microscopically. The difference in the incubation times for YEP-glucose and YEP-galactose reflects cell doubling times (~100 min on YEP-glucose and ~150 min on YEP-galactose).

For an analysis of α-factor arrest in liquid cultures, α-factor was added to early-log-phase (A₅₆₀ of ~0.3) YEP-galactose cultures, and incubation was continued for 2.5 h before the cells were fixed.

For the determination of the percentage of budded cells that divide during α-factor treatment, we assumed that essentially all cells that were unbudded at the time of the α-factor addition arrested without undergoing bud emergence. We assume this because the percentage of single unbudded cells did not change over time when GAL1::FAR1+N and GAL1::FAR1/ΔN cells were plated on α-factor (see Fig. 2) (data not shown). The fraction of budded cells in the initial population that divide and then arrest in the unbudded state after the α-factor challenge can be calculated as follows, assuming that a budded cell that divides will generate two arrested unbudded cells. With a starting population of 100 cells, the number of cells arresting in the unbudded state after α-factor treatment is Uᵦᵤᵦ + 2 · F · Bᵤᵦ, and the total number of cells after α-factor treatment (budded plus unbudded) is 100 + F · Bᵤᵦ, where Uᵦᵤᵦ is the percentage of unbudded cells before α-factor treatment, Bᵦᵤ is the percentage of budded cells before α-factor treatment, and F is the fraction of budded cells that divide during α-factor treatment. The percentage of unbudded cells after α-factor treatment (Uᵦᵦ) is therefore represented by Uᵦᵦ = 100(Uᵦᵤᵦ + 2 · F · Bᵤᵦ)/(100 + F · Bᵤᵦ) or F = 100(Uᵦᵦ − Uᵦᵦ)/(100 − Uᵦᵤ) (20).

Antibodies and Western blot (immunoblot) analysis. Anti-Far1p (51-830) rabbit polyclonal antibodies were raised against a peptide (amino acids 10 to 24 of Far1p) coupled to keyhole limpet hemocyanin (Immuno-Dynamics Inc., La Jolla, Calif.). The detection of proteins on Western blots following transfer to Immobilon (Millipore) was by alkaline phosphatase-5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium staining as described previously (18) or by chemiluminescence detection (Amerham) following the manufacturer’s instructions. Equal amounts of cell culture were extracted by examination of the intensity of nonspecific bands detected by the immunoblot procedure and by Auro-dye (Amerham) staining of a backup Immobilon strip (data not shown). In general, extraction of equal optical density units of cells resulted in only minor variations in the yields of the protein extracted (data not shown).

RESULTS

Correction of the FAR1 sequence: 50 additional amino acids at the N terminus include a cell cycle-regulated Far1p epitope. Independent sequencing of the FAR1 gene (reference 35a and data not shown) showed that the published FAR1 sequence (2) was missing 2 nt (GA) after position 371. The change extends the potential FAR1 open reading frame for another 150 nt upstream of the originally proposed translation initiation codon. The full-length FAR1 open reading frame (referred to here as FAR1/+N) and the N-terminally truncated FAR1 open reading frame (FAR1/ΔN) were expressed from the GAL1 promoter (Fig. 1A). Under electrophoresis on sodium dodecyl sulfate-polyacrylamide gels, wild-type Far1p and Far1p/+N (from GAL1::FAR1/+N) comigrated, but Far1p/ΔN migrated aberrantly (Fig. 1B). An antibody raised against Far1p amino acids 10 to 24 recognized wild-type Far1p and Far1p/+N but not Far1p/ΔN (Fig. 1B), confirming the revised sequence.

Phosphorylation of Far1p as cells pass through Start causes a reduction in Far1p gel mobility (17, 18). The epitope recognized by anti-Far1p (10–24) was cell cycle regulated, since at Start, as Far1p gel mobility decreased, recognition of (shifted-up) Far1p by anti-Far1p (10–24) but not by anti-Far1p (51–830) was lost (Fig. 1C).

Figure 1B also shows that GAL1::FAR1 cells moderately but detectably overexpress Far1p by comparison with G₁-arrested cells. Far1p levels in G₁-arrested GAL1::FAR1 strains do not differ markedly from the levels in cycling cells (see Fig. 6) (data not shown).

MKTPTRVSEPKIIHTGPSGDDAESRSPFKFRLGR... 
...SGKVEKTPEFKKQOM... (52-830)

FIG. 1. An additional 50 amino acids at the N terminus of Far1p. (A) The corrected FAR1 open reading frame predicts an additional 50 N-terminal amino acids. The underlined methionine residues are predicted to be coded for by the ATG codons in the diagram. The region recognized by anti-Far1p (10–24) is bracketed. Also shown are the structures of the GAL1 promoter-driven alleles of FAR1. FAR1/+N encodes the putative full-length Far1p (Far1p/+N), and FAR1/ΔN encodes the N-terminally truncated (Far1p/ΔN) mutant protein. The GAL1::FAR1/ΔN cassette used here was originally described as GAL1::FAR1 (3).

(B) Anti-Far1p (10–24) peptide antibody recognizes wild-type Far1p and Far1p/+N but not Far1p/ΔN. All strains were MATA barl. Lanes 1 and 2, far1::URA3 cln1 cln2 cln3 GAL1::CLN3 (1627-1D and 1631-108B); lanes 3 and 4, FAR1 cln1 cln2 cln3 GAL1::CLN3 (1631-1A and 1631-12A); lanes 5 and 6, far1::URA3 GAL1::FAR1/+N (1630-4C and 1630-5B); lanes 7 and 8, far1::URA3 GAL1::FAR1/ΔN (1629-3A and 1629-4B). Cells were grown to early log phase in YEP-galactose and were shifted into YEP-glucose for 3 h (lanes 1 to 4). The galactose-to-glucose shift shuts off expression of GAL1::CLN3 (the sole source of CLN function), arresting cells in the G₁ phase with maximal Far1p levels (18). Cells were grown to mid-log phase in YEP-galactose (lanes 5 to 8). The protein samples were Western blotted with anti-Far1p (51–830) (upper panel) and anti-Far1p (10–24) (lower panel). (C) Cells of genotype MATA bar1 cln1 cln2 cln3 GAL1::CLN3 GDP::FAR1/+N (1631-108-BM310) were synchronized by the cdc block-release protocol (19). The cell cycle-constitutive GDP promoter was used in this experiment because it gives a prominent Far1p mobility shift in this protocol (17). At 0, 12, 24, and 36 min (lanes 1, 2, 3, and 4) after the release from the G₁ block, protein samples were extracted and Western blotted with anti-Far1p (51–830) (upper panel) and anti-Far1p (10–24) (lower panel).

N-terminal truncation of Far1p increases its functional stability. We examined the ability of previously synthesized Far1p to arrest the cell cycle in response to α-factor in the absence of new Far1p synthesis (Fig. 2). Cells of genotypes MATA bar1 far1::URA3 GAL1::FAR1/+N and GAL1::FAR1/ΔN were grown to mid-log phase in YEP-galactose and plated on YEP-
When plated on glucose medium plus α-factor (blocking new Far1p synthesis), *GAL1::FAR1/+N* cells that were unbudded at the time of plating did not bud after plating, whereas most cells that were budded at time of plating produced multiple new buds after plating (Fig. 2B [shaded bars]). This result suggests that Far1p/+N present in unbudded (G1) cells at the time of plating was stable and functional for several hours after the cessation of new synthesis and that Far1p/+N present in budded cells (post-G1) at the time of plating was insufficiently abundant or stable to promote a delay or the arrest of cell cycle progression. Thus, when plated on glucose plus α-factor, unbudded cells arrested while budded cells continued to proliferate.

In contrast, *GAL1::FAR1/ΔN* cells plated on glucose plus α-factor medium arrested as a mixture of singlets and doublets without new bud emergence, just as when they were plated on galactose plus α-factor medium (Fig. 2D [shaded bars]). Thus, previously synthesized Far1p/ΔN could promote at least 2 h of cell cycle arrest in response to α-factor, without new synthesis, at cell cycle positions other than G1.

These results suggest that Far1p/+N is more stable in unbudded (G1) cells than at other points in the cell cycle and that Far1p/ΔN is relatively stable even in the budded interval of the cell cycle. Biochemical confirmation of this suggestion is presented below.

**N-terminal truncation of Far1p interferes with the G1 phase specificity of cell cycle arrest by α-factor.** *GAL1::FAR1/+N, GAL1::FAR1/ΔN,* and wild-type *FAR1* cells all showed similar dose responses for arrest by α-factor as measured by a halo assay (Fig. 3Q to T). However, in response to α-factor, a significant proportion of *GAL1::FAR1/ΔN* cells arrested in the budded state with a DNA content greater than 1 N (Table 1 and Fig. 3). *GAL1::FAR1/ΔN* cells that arrested in the budded state were almost all mononucleate (as revealed by DAPI staining of nuclei), indicating that budded cells had not yet executed nuclear division (data not shown). The abnormal arrest of *GAL1::FAR1/ΔN* cells in the budded post-G1 interval of the cell cycle was not due simply to overexpression of *FAR1* from the GAL1 promoter, since *GAL1::FAR1/+N* cells responded normally to α-factor by arresting as unbudded G1 cells (Table 1 and Fig. 3).

The budded-cell-arrest phenotype due to the integrated *GAL1::FAR1/ΔN* cassette was somewhat sensitive to copy number, with two copies giving a more severe and reproducible phenotype (data not shown). All experiments reported here use a two-copy integrant. In contrast, even multiple copies of *GAL1::FAR1/+N* gave exclusively G1 unbudded arrest (data not shown). It is important to note that even with two copies, the *GAL1::FAR1* cassettes gave only a moderate overexpression of Far1p by comparison with peak expression from the endogenous promoter (Fig. 1).

Budded cell arrest is not due to a failure of Far1p/ΔN to arrest the cell cycle in G1 before bud emergence for the following reasons. The budded-cell-arrest phenotype conferred by the *GAL1::FAR1/ΔN* allele was largely dominant over wild-type *FAR1* (Table 1), indicating that budded cell arrest was not due to a deficiency in the ability of truncated Far1p to promote normal G1 arrest. Consistent with this, budded cell arrest (even in *GAL1::FAR1/ΔN*/ΔN *far1::URA3* strains) required high concentrations of α-factor (\(10^{-7}\) M) (data not shown); at lower concentrations of α-factor (\(10^{-9}\) M), budded *GAL1::FAR1/ΔN* cells divided and then arrested efficiently in the succeeding unbudded G1 interval. This result indicates that truncated Far1p is fully able to promote G1 arrest. *GAL1::FAR1/ΔN* cells did not undergo new rounds of bud emergence when treated with α-factor (Fig. 2D), indicating that budded cell arrest is not

---

FIG. 2. Functional stabilization of Far1p by N-terminal truncation. All strains were *MATa bar1 far1::URA3* and either *GAL1::FAR1/+N* or *GAL1::FAR1/ΔN*. (A and B) *GAL1::FAR1/+N* (1630-4C); (C and D) *GAL1::FAR1/ΔN* (1629-3A). Cells were grown to mid-log phase in YEP-galactose, sonicated to disperse clumps, and plated on YEP-galactose (open bars) and YEP-glucose (shaded bars) solid media either without (A and C) or with (B and D) α-factor (0.1 μM). The plates were incubated at 30°C for 2.5 h (YEP-glucose) and 3.5 h (YEP-galactose) and examined microscopically. Microcolonies were scored (200 per plate) as having 1, 2, 3, 4, or >4 cell bodies (cells plus buds, which are indistinguishable in this assay). When plated, 43% of *GAL1::FAR1/+N* cells and 15% of *GAL1::FAR1/ΔN* cells were unbudded.
FIG. 3. Loss of cell cycle specificity of α-factor arrest caused by N-terminal truncation of Far1p. The strains were all MATa bar1 (A to D and Q) FAR1ΔN (1630-9C). (E to H and R) far1::URA3 (1630-4A). (I to L and S) far1::URA3 GAL1::FAR1ΔN (1630-4C). (M to P and T) far1::URA3 GAL1::FAR1ΔN (1629-3A). (A to P) Cell cycle arrest by α-factor. Cells were grown to early log phase in YEP-galactose at 30°C and incubated for another 2.5 h either without (A, B, E, F, I, J, M, and N) or with (C, D, G, H, K, L, O, and P) addition of α-factor at 0.5 μM. Cell morphology was examined by photomicroscopy (A, C, E, G, I, K, M, and O). The cells were analyzed for DNA content by flow cytometry (B, D, F, H, J, L, N, and P). (Q to T) Halo assays of sensitivity to cell cycle arrest by α-factor (9). Filter disks spotted with 10 μl of α-factor at 50, 100, 200, and 400 μM were placed on lawns of cells on YEP-galactose medium. The plates were incubated at 30°C for 2 days. The zones of inhibition indicate α-factor sensitivity.

due to a leak-through of cells that were in G1 but failed to arrest. GAL1::FAR1ΔN cells (as well as wild-type cells) were quantitatively arrested in G1 by concentrations of α-factor greater than or equal to 10^{-8} M (data not shown).

A subpopulation of budded GAL1::FAR1ΔN cells divided before arresting in α-factor as G1 cells. To determine if this heterogeneity reflected cell cycle position, we synchronized cultures by centrifugal elutriation. Division of budded GAL1::FAR1ΔN cells was blocked by α-factor treatment early in the budded interval but was not blocked later (Fig. 4), accounting for the ability of some GAL1::FAR1ΔN cells from an asynchronous culture to divide in α-factor before arresting in G1. In contrast, essentially all budded GAL1::FAR1ΔN+N cells divided in the presence of α-factor and then arrested as G1 unbudded cells, irrespective of cell cycle position at the time of α-factor challenge (Fig. 4). This result suggests that GAL1::FAR1ΔN cells are not uniformly sensitive to non-G1 arrest throughout the cell cycle but are more sensitive early in the budded interval. Thus, deletion of the Far1p N terminus results in the acquisition of a novel function: the ability to block division in cells that have already passed Start and initiated DNA replication and budding.

Far1pΔN specifically delays Start in the absence of mating factor. GAL1::FAR1ΔN+N cells were large (about 175% of wild-type volume by Coulter Channelizer) (data not shown) and exhibited a significant delay in G1 compared with wild-type cells (Fig. 3), while deletion of FAR1 caused a small but reproducible decrease in the proportion of unbudded G1 cells (Fig. 3 and Table 1). Thus, Far1p may delay the Start transition in a dose-dependent manner even in the absence of α-factor.

Specific delay in G1 by GAL1::FAR1 required the Far1p N terminus. GAL1::FAR1ΔN cells were about as large as GAL1::FAR1ΔN+N cells (about 150% of wild-type volume) (data not shown), and yet GAL1::FAR1ΔN cells showed a greater proportion of budded cells with 2 N (G1-M) DNA content compared with wild-type cells (Fig. 3 and Table 1).

This could reflect the ability of truncated Far1p to inhibit cell division as well as Start, as was observed in the presence of mating factor (see above). In the absence of mating factor, these effects of both GAL1::FAR1ΔN and GAL1::FAR1ΔN+N were only temporary delays (as opposed to the blocks seen with mating factor), since in the absence of mating factor, the doubling times of these strains were the same as the doubling time of wild-type cells (data not shown).

Deletion mapping within the Far1p N terminus. To begin to determine what subregions within the 50 Far1p N-terminal amino acids could control cell cycle specificity of mating factor arrest, we deleted the N-terminal 10, 20, 30, or 40 amino acids. We integrated these GAL1::FAR1 genes and tested transformants for cell cycle specificity of arrest after incubation in mating factor, scoring the percentage of budded cells and the percentage of mononucleate budded cells. (Bimucleate budded cells could arise from a block to cytokinesis without a block to nuclear division.) We observed a roughly progressive increase in the severity of the phenotype with the increasing length of

<table>
<thead>
<tr>
<th>Strain genotype</th>
<th>Unbudded cells without α-factor (%)</th>
<th>Unbudded cells with α-factor (%)</th>
<th>Budded cells that divide in α-factor (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAR1</td>
<td>43 ± 1</td>
<td>99 ± 1</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>far1::URA3</td>
<td>37 ± 1</td>
<td>60 ± 2</td>
<td>NA</td>
</tr>
<tr>
<td>far1::URA3</td>
<td>55 ± 1</td>
<td>99 ± 1</td>
<td>96 ± 1</td>
</tr>
<tr>
<td>GAL1::FAR1ΔN+N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FARI GAL1::FAR1ΔN+N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>far1::URA3 GAL1::FAR1ΔN+N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FARI GAL1::FAR1ΔN</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are the means ± the standard errors of the mean (n = 4). The strains were all MATa bar1, with other genotypes as indicated. Log-phase YEP-galactose cultures were incubated for 2.5 h with and without 0.5 μM α-factor at 30°C.

1 The percentages of budded cells that divide in α-factor were calculated as described in Materials and Methods. This calculation was not applicable (NA) to far1::URA3 cells, which fail to arrest in α-factor (2).
the deletion up to 30 amino acids (Fig. 5). DNA flow cytometry (data not shown) confirmed an approximate correlation between the number of budded cells and the number of cells with greater than G1 (at or near G2) DNA content.

**N-terminal truncation increases the stability of Far1p.** Wild-type Far1p accumulates efficiently only in G1 phase cells (18), and we were therefore surprised to find phenotypic evidence for the function of N-terminally truncated Far1p late in the cell cycle. We examined the stability of wild-type and N-terminally truncated Far1p by turning off synthesis of \( \text{GAL1}:\text{FAR1} \) by adding glucose (repressing the \( \text{GAL1} \) promoter) (Fig. 6A). \( \text{GAL1}:\text{FAR1} \) transcripts declined to very low levels within 15 min of the glucose addition (data not shown), allowing us to monitor decay of previously synthesized Far1p in the absence of new Far1p synthesis. We observed rapid decay of wild-type Far1p; this decay was dependent on Cdc28 function and/or progression out of G2, as blocking a \( \text{cdc}28-4 \) temperature-sensitive strain in G1 by incubation at 35°C stabilized Far1p (Fig. 6B). The N-terminal deletion series described above, except for Far1p\( \Delta10 \), displayed clearly increased Far1p stability; \( \text{FAR1}/\Delta10 \) displayed only a modest non-G1-arrest phenotype (Fig. 5). Far1p\( \Delta30 \) was the most stable of the series (Fig. 6 and data not shown). The stability of Far1p\( \Delta40 \) was increased in \( \text{cdc}28-4 \)-blocked cells by comparison with cells in cycling cultures (Fig. 6B), suggesting that Cdc28-cell cycle control of stability of this protein was reduced but not eliminated.

The increased stability of Far1p\( \Delta30 \) compared with Far1p\( \Delta40 \) suggests that the N-terminal deletions may not simply inactivate an autonomously acting instability determinant. This is consistent with the result that the 50 N-terminal amino acids of Far1p did not confer instability when fused to \( \beta \)-galactosidase (data not shown). Other regions of Far1p may also be required for instability.

Far1p\( \Delta N \) (with the first 50 amino acids deleted) displayed a stability similar to that of Far1p\( \Delta40 \) in this protocol (data not shown).

**Cell cycle regulation of N-terminally truncated Far1p under control of the endogenous \( \text{FARI} \) promoter.** We introduced the \( \text{FARI} \) and \( \text{FARI}/\Delta40 \) N-terminal coding sequence deletions into the \( \text{FARI} \) gene by precise gene replacement so that the mutant proteins would be under the control of the wild-type \( \text{FARI} \) promoter instead of the \( \text{GAL1} \) promoter (see Materials and Methods). Using these strains, we examined cell cycle...
FIG. 7. Cell cycle regulation of N-terminally truncated Far1p. Cells of the genotypes MATa cnl1 cnl2 cnl3 leu2::GAL1::CLN3 and either FAR1 (solid circles) or FAR1Δ30 (open circles) (note that the endogenous FAR1 promoter was driving FAR1 expression in these strains), as indicated, were synchronized by chn block-release as described previously (18), and protein samples were taken at 12-min intervals following the release from the G1 block. Far1p was assayed by Western blotting. The percentages of unbudded cells during the time course are indicated.

regulation of Far1pΔ30 and Far1pΔ40 abundance by chn block release (Fig. 7). As described previously (18), wild-type Far1p shows a sharp reduction in abundance at the time of cell cycle Start, shortly before bud emergence, declining to almost undetectable levels. Far1pΔ30 shows only a minor reduction at the time of Start (Fig. 7) (about a fourfold reduction by scanning densitometry [data not shown]), consistent with the demonstrated high stability of this protein. This reduction may be due to the drop in FAR1 transcription at Start (18). Far1pΔ40 was intermediate between wild-type Far1p and Far1pΔ30 in its periodicity (data not shown), possibly reflecting increased instability of Far1pΔ40 compared with Far1pΔ30 (see above).

Unlike strains expressing Far1pΔ30 or Far1pΔ40 from the GAL1 promoter, strains expressing these proteins from the endogenous FAR1 promoter exhibited only very minor differences from wild-type strains, arresting with ≥90% of unbudded cells upon α-factor treatment (data not shown). The relevant difference between the GAL1 promoter and the FAR1 promoter could be the moderate overexpression due to the GAL1 promoter (Fig. 1) or the cell cycle deregulation of the GAL1 promoter compared with the FAR1 promoter (18). Thus, effective elimination of G1 specificity may require elimination of both controls on FAR1 abundance reported previously (18); the control of Far1p abundance and transcriptional control of FAR1 expression.

**DISCUSSION**

**Regulated instability of Far1p.** Far1p is stable during the pre-Start G1 interval of the yeast cell cycle but becomes unstable as cells pass through Start and commit to cell cycle progression. Deletions within the first 50 amino acids generate stabilized proteins with a reduced cell cycle regulation of abundance.

Far1p blocks Start by binding to and inhibiting Start-promoting Cln-Cdc28 kinase complexes (25, 26, 35). N-terminally truncated Far1p may promote post-Start cell cycle arrest by similarly inhibiting B-type cyclin-Cdc28 kinase complexes functioning in post-Start cells (20). Alternatively, N-terminally truncated Far1p may exert its effects by blocking the action of Cln-Cdc28 kinases at points of the cell cycle after wild-type Far1p has been degraded (the peak of CLN1 and CLN2 expression follows the decline in Far1p levels [18]).

We cannot rule out the possibility that post-Start arrest of cells expressing N-terminally truncated Far1p is not solely due to the stabilization of Far1p but is due also to an aberrant function of the mutant protein. For example, the N-terminal deletions could reduce the specificity of Far1p inhibition such that Cln-Cdc28 kinases as well as Cln-Cdc28 kinases could be inhibited by α-factor-activated Far1p. To test this by the assay of Peter and Herskowitz (26) requires the introduction of N-terminal deletions into the FAR1-22 constitutively active mutant version of FAR1 so that the assay of bacterially produced protein (by bypassing the requirement for mating factor pathway activation [26]) would be allowed. For this reason, we have not yet tested this possibility biochemically. Although Peter and Herskowitz (26) failed to detect inhibition of a Clb2-Cdc28 kinase preparation in vitro with a recombinant full-length Far1p, this result cannot rule out the possibility that such inhibition could occur in vivo. We consider it likely (although still unproven) that the mechanism of post-Start arrest by truncated Far1p is similar to the mechanism of pre-Start arrest. Clearly, both mechanisms are still dependent on the mating factor pathway, as overexpression of neither full-length nor truncated Far1p has any significant effect on the doubling time in the absence of mating factor.

Mating in yeast cells is restricted to the G1 phase (29). Since Far1p has a role in mating distinct from its cell cycle arrest function (2), Far1p cell cycle regulation may also contribute to the block to mating outside of G1; however, our preliminary attempts to detect mating in GAL1::FAR1Δ40 cells outside of G1 have been unsuccessful (data not shown).

**Cdk-binding proteins and cell cycle specificity.** Far1p is one of a number of proteins that interact physically with and inhibit CDKs in mammalian and yeast cells (21). The p40-SIC1 inhibitor of B-type cyclin-Cdc28 activity in yeast cells (19, 22, 30) presents an interesting parallel to Far1p. Its levels are cell cycle regulated, being high from mid-mitosis until Start. It is degraded at Start, as is Far1p (4, 18, 30). A significant difference is that Sic1 functions to block Clb-Cdc28 kinases independent of external signals whereas Far1p inhibition of Cln-Cdc28 kinases is α-factor dependent (2).

Cell cycle-regulated expression might be important for the proper function of some mammalian CDK-binding proteins. Expression of Cdi1 (a mammalian CDK-binding protein) is cell cycle regulated, and constitutive expression of Cdi1 affects cell cycle progression (10). Cell cycle-regulated expression could contribute to restricting the function of CDK-binding proteins to appropriate cell cycle intervals in the cases of CDK-binding proteins like p21 and Cdi1, which can associate with CDKs functioning at both the G1-S and G2-M transitions (8, 11, 36). mRNA for p21 is cell cycle regulated (15) in a pattern similar to that for FAR1 (18); high in G1, low in S, and rising again in G2-M. p27Kip1 levels were reported to be constant during the cell cycle (34), but p27Kip1 may exhibit some specificity of inhibition or increased affinity for G1-S-acting cyclin-Cdk complexes (27, 34).

The efficiency and specificity of mating factor arrest of budding yeast cells are remarkably high, with essentially quantitative cell cycle arrest occurring exclusively in the first G1 phase following mating factor addition (28). Accurate cell cycle regulation of Far1p abundance (high in G1, low elsewhere) may be important for both efficiency and specificity; we showed previously that moderate reduction of Far1p peak abundance in G1 compromises the efficiency of arrest (18), and we show here that sufficient ectopic expression of Far1p outside of the G1 interval may compromise the G1 specificity of arrest (on the
assumption that the N-terminal mutations affect only Far1p stability rather than intrinsic specificity of action. Regulation of FAR1 abundance may provide a redundant control together with the block to mating factor signalling at cell cycle Start (23); these controls could act together to provide high G1 specificity of mating factor arrest.

ACKNOWLEDGMENTS

We thank G. Ammerer, F. Chang, R. Deshaies, and D. Lew for plasmids, strains, and antibodies; M. Hoek and S. Odinsky for excellent technical assistance; and N. Heintz, L. Oehlen, and E. Vallen for discussions and comments on the manuscript.

This work was supported by the Lucille P. Markey Charitable Trust, by the A. and M. Beckman Foundation, and by PHS grant GM49716. F.R.C. was a Scholar of the Markey Trust. J.D.M. was supported by a National Science Foundation Graduate Fellowship.

REFERENCES

35a. White, M. Personal communication.