Identification and Characterization of *FAR3*, a Gene Required for Pheromone-Mediated G_1 Arrest in *Saccharomyces cerevisiae*

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ABSTRACT

In haploid *Saccharomyces cerevisiae* cells, mating pheromones activate a signal transduction pathway that leads to cell cycle arrest in the G_1 phase and to transcription induction of genes that promote conjugation. To identify genes that link the signal transduction pathway and the cell cycle machinery, we developed a selection strategy to isolate yeast mutants specifically defective for G_1 arrest. Several of these mutants identified previously known genes, including *CLN3, FUS3,* and *FAR1*. In addition, a new gene, *FAR3,* was identified and characterized. *FAR3* encodes a novel protein of 204 amino acid residues that is dispensable for viability. Northern blot experiments indicated that *FAR3* expression is constitutive with respect to cell type, pheromone treatment, and cell cycle position. As a first step toward elucidating the mechanism by which Far3 promotes pheromone-mediated G_1 arrest, we performed genetic and molecular experiments to test the possibility that Far3 participates in one of the heretofore characterized mechanisms, namely Fus3/Far1-mediated inhibition of Cdc28-Cln kinase activity, G_1 cyclin gene repression, and G_1 cyclin protein turnover. Our data indicate that Far3 effects G_1 arrest by a mechanism distinct from those previously known.

As a prelude to conjugation, haploid **a** and α cells communicate by secreting and responding to mating pheromones: **a** cells secrete **a**-factor and express the α-factor receptor, and α cells secrete α-factor and express the **a**-factor receptor. During this courtship period a number of physiological changes occur, including cell cycle arrest in the G_1 phase and transcription induction of genes that promote mating. These changes are dependent upon a signal transduction pathway that relays information from pheromone receptors at the cell surface to a transcription factor in the nucleus (reviewed in CROSS 1988a; SPRAGUE and THORNER 1992). The α- and **a**-factor receptors, encoded by the *STE2* and *STE3* genes, respectively, couple to a common signal transduction pathway (BENDER and SPRAGUE 1986; BENDER and SPRAGUE 1989) that begins with a heterotrimeric G-protein. Binding of pheromone to its cognate receptor presumably elicits a conformational change in the receptor that is sensed by the G-protein, stimulating dissociation of G_α from $G_{\beta\gamma}$. The $G_{\beta\gamma}$ complex, in turn, transmits the signal to the next step in the pathway, which is thought to be the Ste20 protein kinase (LEBERER *et al.* 1992; RAMER and DAVIS 1993). Below Ste20 is Ste5, a protein that serves as a scaffold to assemble elements of a mitogen-activated protein kinase cascade (CHOI *et al.* 1994; MARCUS *et al.* 1994; PRINTEN and SPRAGUE 1994) composed of protein kinases encoded by *STE11, STE7,* and either of the functionally redundant *FUS3* and *KSS1* pair. The signal flows down this cascade, from Ste11 to Ste7 (CAIRNS *et al.* 1992; STEVENSON *et al.* 1992; NEIMAN and HERSKOWITZ 1994), then from Ste7 to Fus3/Kss1 (ERREDE *et al.* 1993), ultimately leading to the phosphorylation and activation of a transcription factor, Ste12 (ELION *et al.* 1993). Ste12, in turn, activates transcription of genes such as *FUS1,* whose product is involved in fusion of the mating pair (MCCAFFREY *et al.* 1987; TRUEHEART *et al.* 1987).

The signal transduction pathway summarized above is also required for pheromone-mediated cell cycle arrest in the G_1 phase, before START (reviewed in PRINGLE and HARTWELL 1981). In the absence of pheromone, passage through START is catalyzed by the Cdc28 protein kinase, whose activity requires association with one of the functionally redundant G_1 cyclin proteins, Cln1, Cln2, or Cln3 (reviewed in NASMYTH 1993). The G_1 cyclins are unstable molecules and, in addition, the transcription of *CLN1* and *CLN2* oscillates throughout the cell cycle (WITTENBURG *et al.* 1990; TYERS *et al.* 1992, 1993; CROSS and BLAKE 1993). The net result is that significant levels of Cln proteins accumulate only late in the G_1 phase (WITTENBURG *et al.* 1990; TYERS *et al.* 1993). Since Cdc28 protein levels are invariant throughout the cell cycle (MENDENHALL *et al.* 1987; HADWIGER and REED 1988), the G_1 cyclins are considered to be the rate-limiting activators of Cdc28-Cln at START. In response to pheromone, *CLN1/CLN2* gene transcription, Cln1/Cln2 protein stability and Cdc28-Cln protein kinase activity are all reduced (MENDEN-

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HALL *et al.* 1987; WITTENBURG *et al.* 1990; TYERS *et al.* 1993), suggesting that the G_1 cyclins and Cdc28 are the direct targets of the signal transduction pathway.

To identify components that connect the signal transduction pathway to the cell cycle machinery, three research groups set out to isolate mutants specifically defective for G_1 arrest (CROSS 1988b; CHANG and HERSKOWITZ 1990; FUJIMURA 1990a). Each group developed a strategy to preclude or screen-out mutants defective for signal transduction *per se*. CROSS (1988b) selected for dominant α-factor resistant mutants using an **a**/**a** diploid strain. One of his mutants, *DAF1-1*, identified the *CLN3* gene and provided genetic evidence that the G_1 cyclins might be targets of the signal transduction pathway. CROSS's strategy precluded the isolation of recessive mutations in genes required for signal transduction, but it also precluded the isolation of recessive mutations in genes that might function specifically for G_1 arrest. To isolate such mutations CHANG and HERSKOWITZ (1990) and FUJIMURA (1990a) took independent, but similar, two-step approaches: they selected for α-factor-resistant mutants from haploid **a** strains and screened among these for mutants that were competent for signal transduction, as assessed by transcription induction of *FUS1* (CHANG and HERSKOWITZ 1990) or pheromone-induced shmoo formation (FUJIMURA 1990a). CHANG and HERSKOWITZ (1990) isolated seven *far1* alleles and one *far2* (*fus3*) allele at a frequency of ~1% of the total α-factor-resistant mutants, and FUJIMURA (1990a,b) isolated a single *dac2* (*fus3*) allele from more than 2000 α-factor-resistant mutants.

The discovery of *FUS3* and *FAR1* has indeed provided a link between the signal transduction pathway and the cell cycle machinery. Mating pheromone leads to the phosphorylation and activation of the MAP kinase Fus3 (GARTNER *et al.* 1992). In addition to its role in phosphorylating Ste12, a role shared with Kss1, Fus3 alone plays the role of phosphorylating Far1 (CHANG and HERSKOWITZ 1992; PETER *et al.* 1993; TYERS and FUTCHER 1993). Phosphorylated Far1, in turn, binds to Cdc28-Cln and directly inhibits its activity, leading to arrest in the G_1 phase (PETER *et al.* 1993; TYERS and FUTCHER 1993; PETER and HERSKOWITZ 1994).

In addition to *FUS3* and *FAR1*, there almost certainly are other genes that participate in pheromone-mediated G_1 arrest. This notion was made most apparent by the finding that Far1 is not required for pheromone-mediated repression of *CLN1* and *CLN2* transcription (VALDIVIESO *et al.* 1993), nor does it trigger Cln2 protein turnover (PETER *et al.* 1993). Although there is evidence that Fus3 plays a role in pheromone-mediated repression of *CLN1* and *CLN2* transcription (ELION *et al.* 1991) in addition to its role in signal transduction and Far1 activation, the specific proteins involved in transcription repression are not known. Thus the genes responsible for pheromone-mediated repression of *CLN1* and *CLN2* and for pheromone-mediated Cln1 and Cln2 protein turnover remain to be identified. Moreover, there may be additional, heretofore uncharacterized, mechanisms that effect pheromone-mediated G_1 arrest. To identify genes that link the signal transduction pathway to the cell cycle machinery, we developed a genetic strategy to select directly for haploid yeast mutants that were wild type for signal transduction, but defective for G_1 arrest. Our collection of Transduction$^+$ Arrest$^-$ strains harbored mutations in previously known genes, including *CLN3*, *FUS3*, and *FAR1*. In addition, the recessive mutations identified a new gene, *FAR3*, that was characterized in detail. The studies presented here indicate that *FAR3* effects pheromone-mediated G_1 arrest by a mechanism distinct from those previously known.

MATERIALS AND METHODS

Yeast strains and media: Standard media and yeast manipulations were as described by SHERMAN *et al.* (1982). S broth is SD medium lacking dextrose. Synthetic complete dextrose (SC) medium is SD medium supplemented with nutrients corresponding to strain auxotrophies. YEPGal and SGal are the same as YEPD and SC, respectively, except that galactose (2% w/v) replaced dextrose as the carbon source. Likewise, the carbon source in YEPRaf is raffinose (3% w/v). Aminotriazole and synthetic α-factor (both from Sigma) were spread from filter-sterilized stock solutions onto the surface of plate media.

Yeast strains are listed in Table 1. With the exception of strain 1608-21C and its derivative SY2619, all strains were derived from Sc252 (JIM HOPPER lab strain, provided by MALCOLM WHITEWAY); Sc252 was chosen because its response to the overexpression of *STE4* had been previously characterized (WHITEWAY *et al.* 1990). The *cln* block/release synchronization strain, 1608-21C, has been described previously (MCKINNEY *et al.* 1993). Genetic markers were introduced by one- and two-step gene replacement (ROTHSTEIN 1991) and by genetic crosses. Yeast were transformed by the lithium acetate method (SCHIESTL and GIETZ 1989). Gene replacements were confirmed by Southern blot analysis (SOUTHERN 1975).

To facilitate mutant isolation and characterization, strains harbored the *HIS3::pFUS1::HIS3*, *rad16::pGAL1::STE4*, and *mfa2-Δ1::pFUS1::lacZ* alleles integrated at the corresponding *HIS3*, *RAD16*, and *MFA2* loci. These alleles were introduced using *Nhe*I-digested pDH106, *Pvu*II-digested pDH15, and *Hin*dIII-digested pDH17, respectively. Mutant alleles of *LYS2*, *ARG4*, and *TRP1* were introduced using pCP7 (FOSS and STAHL 1995), *Sna*BI-digested YIp5:arg4-*Eco*RV → *Xho*I, and *Hin*dIII-digested pDH20, respectively. Two mutant alleles of *BAR1*, *bar1::LEU2* and *bar1Δc*, were introduced using *Bam*HI-*Hin*dIII-digested pZV77 and *Sph*I-digested pSL1872, respectively.

The *cln1::URA3*, *cln2::LEU2* and *cln3::LEU2* alleles were introduced using pcln1::URA3 (HADWIGER *et al.* 1989), *Hin*dIII-*Bam*HI-digested pDH22, and *Bam*HI-*Xho*I-digested pDH21, respectively. The *cln3::URA3::pGAL1::CLN3C* allele was introduced in two steps by first making strains *cln3::LEU2* and then evicting the *cln3::LEU2* allele by transformation with *URA3::pGAL1::CLN3C* DNA (TYERS *et al.* 1992). The *cln2Δxs* and *cdc28-13* alleles were introduced using pcln2Δxs (CROSS and TINKELENBERG 1991) and *Kpn*I-digested pHV112, respectively.

The two *far3* alleles, *far3Δh::LEU2* and *far3Δh::URA3*, were introduced using *Nhe*I-*Sst*I-digested pSL2310 and *Xho*I-*Sst*I-digested pSL2285, respectively. The *fus3-6::LEU2* and

TABLE 1

***Saccharomyces cerevisiae* strains**

Strain	Genotype	Notes
Sc252	*MAT***a** *ade1-1 leu2-2, 113 ura3-52*	*a*
YDH59	*MATα arg4*	*b*
YDH60	*MAT***a** *trp1*	*b*
YDH63	*MAT***a** *trp1 bar1::LEU2*	*b*
YDH222	*MAT***a** *trp1 bar1Δc hmlaΔ::LEU2*	*b*
SY3086	*MAT***a** *trp1 ste11::URA3*	*b*
60B1	*MAT***a** *trp1 CLN3-60B1*	*b, c*
60D4	*MAT***a** *trp1 far3-2*	*b, c*
60F3	*MAT***a** *trp1 far1-60F3*	*b, c*
59I1	*MATα arg4 far3-1*	*b, d*
SY2321	*MAT***a** *far3-1 bar1::LEU2 lys2*	*b*
SY2556	*MAT***a**/*MATα arg4*/+ *lys2*/+	*b*
SY2612	*MAT***a**/*MATα arg4*/+ *lys2*/+ *far3Δh::URA3*/+	*b*
YDH121	*MAT***a** *trp1 bar1Δc*	*b*
SY2610	*MAT***a** *trp1 bar1Δc farΔh::URA3*	*b*
SY2713	*MAT***a** *trp1 bar1Δc ste20::URA3*	*b*
SY1940	*MAT***a** *lys2 bar1::LEU2*	*b*
YDH28	*MATα lys2*	*b*
SY2673	*MAT***a** *trp1 bar1Δc far1Δhb*	*b*
SY2688	*MAT***a** *trp1 bar1Δc far1Δhb cln2Δxs*	*b*
SY2980	*MAT***a** *trp1 bar1Δc far1Δhb cln2Δxs far3Δh::URA3*	*b*
SY2977	*MAT***a** *trp1 bar1Δc fus3-6::LEU2*	*b*
SY2978	*MAT***a** *trp1 bar1Δc far1Δhb fus3::URA3*	*b*
SY2981	*MAT***a** *trp1 bar1Δc far3Δh::URA3 fus3-6::LEU2*	*b*
SY3072	*MAT***a** *trp1 bar1Δc far1Δhb fus3::URA3 far3Δh::LEU2*	*b*
SY2352	*MAT***a** *far3-1 bar1::LEU2 cln1::URA3 lys2*	*b*
SY2342	*MAT***a** *far3-1 bar1::LEU2 cln2Δxs*	*b*
SY2336	*MAT***a** *far3-1 bar1::LEU2 cln3::LEU2 lys2*	*b*
SY2350	*MAT***a** *far3-1 bar1::LEU2 cln2Δxs cln1::URA3*	*b*
SY2730	*MAT***a** *bar1Δc cdc28-13*	*e*
SY2731	*MAT***a** *bar1Δc cdc28-13 far3Δh::LEU2*	*e*
SY2781	*MAT***a** *bar1Δc*	*e*
SY2802	*MAT***a** *bar1Δc far3Δh::LEU2*	*e*
SY2831	*MAT***a** *bar1Δc cln3::URA3::pGAL1::CLN3C far3Δ::LEU2*	*e*
SY2832	*MAT***a** *bar1Δc cln3::URA3::pGAL1::CLN3C*	*e*
1608-21C	*MAT***a** *bar1 cln1::TRP1 cln2 cln3 leu2::LEU2::pGAL1::CLN3 ade1 his2 trp1 ura3*	*f*
SY2619	*MAT***a** *far3Δh::URA3*	*g*

[a] JIM HOPPER lab strain.
[b] Derived from Sc252; contains the following genetic markers: *HIS3::pFUS1::HIS3 mfa2-Δ1::pFUS1::lacZ rad16::pGAL1::STE4.*
[c] Spontaneous mutant derived from YDH60.
[d] Spontaneous mutant derived from YDH59.
[e] Derived from Sc252; contains the following genetic markers: *HIS3::pFUS1::HIS3 mfa2-Δ1::pFUS1::lacZ.*
[f] FRED CROSS lab strain.
[g] Derived from 1608-21C.

fus3::URA3 alleles were introduced using pYEE98 (ELION *et al.* 1990) and *Eco*RI-digested pGA1832, respectively. The *far1Δhb* allele was introduced using *Eco*RI-digested pSL2068. The *hmlαΔ::LEU2* allele was introduced using pJH455 (WHITE and HABER 1990). The *ste11::URA3* and *ste20::URA3* alleles were introduced using pSL1094 (STEVENSON *et al.* 1992) and pEL45 (LEBERER *et al.* 1992), respectively.

Plasmid constructions: Standard techniques were used for DNA and *E. coli* manipulations (SAMBROOK *et al.* 1989). pDH106 is a YIp5-based plasmid (STRUHL *et al.* 1979) that contains the *HIS3::pFUS1::HIS3* allele. In this allele, a 2.5-kb *Eco*RI-*Bst*BI fragment containing *HIS3* 5′-noncoding sequences flanks 1.2 kb of *FUS1* promoter sequences joined 22 bp upstream of the *HIS3* coding region. The junction between the *FUS1* promoter and the *HIS3* coding sequences is identical to that described by STEVENSON *et al.* (1992). Plasmid pDH15 contains the *rad16::pGAL1::STE4* allele and was created in two steps. First, the 1.8-kb *Eco*RI-*Sal*I *pGAL1::STE4* DNA fragment from pL19 (WHITEWAY *et al.* 1990) was subcloned into the same sites in pBLUESCRIPT (SK, M13+, Stratagene) to create pDH10. The 1.8-kb *Sma*I-*Sal*I *pGAL1::STE4* fragment from pDH10 was then ligated into pBM1499 (FLICK and JOHNSTON 1990) that had been digested with *Bam*HI, filled in with Klenow, and then digested with *Xho*I. Plasmid pDH17 is a pRS306-based plasmid (SIKORSKI and HIETER 1989) that contains the *mfa2-Δ1::pFUS1::lacZ* allele. This allele includes the 6.7-kb *Pst*I-*Sal*I fragment containing the *pFUS1::lacZ* reporter gene from pSL553 (HAGEN *et al.* 1991) flanked by 2.1 kb of

MFA2 5′-noncoding sequences that extend upstream of the *Sph*I site specified in pSM23 (MICHAELIS and HERSKOWITZ 1988) and a 550-bp *Bam*HI-*Eco*RI fragment containing *MFA2* 3′-noncoding sequences; the deletion endpoints of *mfa2-Δ1::pFUS1::lacZ* are the same as previously reported for the *mfa2-Δ1::URA3* allele (MICHAELIS and HERSKOWITZ 1988).

Plasmid YIp5:arg4-*Eco*RV → *Xho*I (provided by LARRY GILBERTSON) was created by subcloning an 8.0-kb *Bam*HI-*Xho*I fragment containing *ARG4* from pSPO13-1 (WANG *et al.* 1987) into YIp5. A linker insertion mutation was introduced within the *ARG4* open reading frame (ORF) of the resulting plasmid by digesting with *Eco*RV and then ligating shut in the presence of an 8-mer *Xho*I linker. Plasmid pDH20 was created in two steps. First, a linker insertion mutation was introduced within the *TRP1* ORF by digesting pJJ246 (JONES and PRAKASH 1990) with *Eco*RV and then ligating shut in the presence of a *Bam*HI 10-mer linker to create pDH18. Second, the 0.9-kb *Eco*RI-*Kpn*I fragment from pDH18 was inserted into the same sites of pRS306. Plasmid pZV77 (provided by VIVIAN MACKAY) was constructed by first subcloning the ~3.0-kb *Xho*I-*Hin*dIII *BAR1* fragment from pBAR2 (MACKAY *et al.* 1988) into the *Sal*I-*Hin*dIII sites of pUC13 (VIEIRA and MESSING 1982) to create pZV9. The *BAR1* ORF was then disrupted in pZV9 by inserting the 2.2-kb *Xho*I-*Sal*I *LEU2* fragment into the unique *Sal*I site. Plasmid pSL1872 was created by first subcloning the ~3.0-kb *Xho*I-*Hin*dIII *BAR1* fragment from pBAR2 into the same sites of pRS306. A 1.1-kb *Cla*I fragment corresponding to *BAR1* 5′-noncoding and ~1/3 of the *BAR1* ORF sequences was deleted from the resulting plasmid by digesting with *Cla*I and then ligating shut.

Plasmid pDH21 was created by first subcloning the 2.2-kb *Hpa*I-*Xho*I *DAF1-1* (*CLN3-2*) fragment from pFC101 (CROSS 1988b) into *Sma*I-*Xho*I-digested pBLUESCRIPT (KS, M13+), creating pDH100. The 1.2-kb *Eco*47III-*Hin*cII fragment of pDH100, corresponding to *CLN3* DNA specifying amino acids 2–441, was then replaced by the 2.1-kb *Hpa*I *LEU2* fragment from YEp13 (BROACH *et al.* 1979). Plasmid pDH22 was created by subcloning the 3.7-kb *Hin*dIII/*Sph*I *cln2::LEU2* fragment from pcln2::LEU2 (HADWIGER *et al.* 1989) into the same sites of pSP72 (Promega). pSL1994 is a *FAR1-URA3-CEN/ARS* plasmid created by subcloning the 5.2-kb *Cla*I-*Bgl*II *FAR1* fragment from pFC15 (CHANG and HERSKOWITZ 1990) into the *Cla*I-*Bam*HI sites of pRS316 (SIKORSKI and HIETER 1989).

The two *far3* gene replacement constructs were made as follows. First, the 1.8-kb *Sal*I-*Bgl*II *FAR3* fragment corresponding to the genomic DNA insert in pSL2252 (see Figure 2) was subcloned into the *Sal*I and *Bam*HI sites of pBLUESCRIPT (SK, M13+), creating pSL2262. pSL2310 and pSL2285 were then created by replacing the 0.35-kb *Hin*dIII fragment of pSL2262 with the 2.8-kb *Hin*dIII *LEU2* fragment from pJP23 (provided by JOHN PRINTEN) and the 1.2-kb *Hin*dIII *URA3* fragment of YEp24 (BOTSTEIN *et al.* 1979), respectively. Plasmid pGA1832 (provided by GUSTAV AMMERER) was constructed by first subcloning a 3.3-kb *Eco*RI fragment containing the *FUS3* gene (ELION *et al.* 1990) into the *Eco*RI site of pIC19R (MARSH *et al.* 1984). A *Xho*I fragment containing part of the *FUS3* ORF was then replaced with a *Sal*I-*Xho*I *URA3* fragment obtained from a plasmid containing the 1.2-kb *Hin*dIII *URA3* fragment (ROSE *et al.* 1984) subcloned into the *Hin*dIII site of pIC19R. Plasmid pSL2068 carries the *far1Δhb* allele and was created in two steps. First, the 5.2-kb *Cla*I-*Bgl*II *FAR1* fragment from pFC15 (CHANG and HERSKOWITZ 1990) was subcloned into the *Cla*I and *Bam*HI sites of pRS306. DNA corresponding to *FAR1* 5′-noncoding sequences and 75% of the *FAR1* ORF was then deleted from the resulting plasmid by digesting to completion with *Hin*dIII and *Bam*HI, filling in the ends with Klenow, and then ligating shut. Plasmid pJ4 is the yeast integration plasmid used to capture DNA harboring the *far3-1* and *far3-2* alleles; it is pRS306-based and carries a 2.7-kb *Eco*RV yeast genomic DNA fragment corresponding to a region 0.9–3.6 kb 5′ of the *FAR3* ORF. Plasmid pHV112 (provided by HENAR VALDIVIESO) is a *URA3*-marked yeast integrating plasmid that contains the *cdc28-13* allele.

Several plasmids were constructed to use as templates for the *in vitro* synthesis of riboprobes. Plasmid pSL2446 was created in two steps. First, a 0.8-kb fragment corresponding to *FAR3* 3′-noncoding sequences was removed from pSL2262 by digesting with *Nsi*I and *Not*I, filling in the ends with Klenow, and then ligating shut to create pSL2290. Second, 0.2 kb of *FAR3* 5′-noncoding sequences were removed from pSL2290 by digesting with *Sal*I and *Nhe*I, treating with Klenow to fill in the ends, and then ligating shut to create pSL2446. Plasmid pSL896 was made by subcloning a 1.8-kb *Acc*I (partial digest)-*Pvu*II fragment of *FUS1* DNA (MCCAFFREY *et al.* 1987) into the *Pst*I and *Sma*I sites of pSP65 (Promega). Plasmid pSL1708 was made by subcloning the 0.8-kb *Hin*dIII-*Xho*I *CLN2* fragment from plasmid pcln2::LEU2 (HADWIGER *et al.* 1989) into the *Hin*dIII and *Sal*I sites of pSP64 (Promega). Plasmid pSL1709 was made by subcloning the 1.0-kb *Eco*RI-*Sac*I *CLN1* fragment from plasmid pcln1::URA3 (HADWIGER *et al.* 1989) into the same sites of pSP65.

Mutant isolation and genetic analysis: Mutants were either spontaneously derived or induced with EMS from strains harboring the *rad16::pGAL1::STE4* construct; *STE4* encodes the G_β subunit of the heterotrimeric G protein, and its overexpression leads to pathway activation and G_1 arrest independent of pheromone treatment (COLE *et al.* 1990; NOMOTO *et al.* 1990; WHITEWAY *et al.* 1990). Spontaneous mutants in starting strains YDH59, YDH60, and YDH63 were obtained as follows. Each strain was streaked to YEPD plate medium to obtain 10 independent starting colonies. After 2 days growth, each colony was suspended in S broth and plated onto selective media. For YDH59 and YDH60, selective medium was SGal lacking histidine and containing aminotriazole at 3 mM (SGal −His + 3 mM AT). For YDH63, the *MAT***a** *bar1::LEU2* strain, selective medium was SD lacking histidine and containing aminotriazole at 3 mM plus α-factor at 5 μg/33 ml plate (SD −His + 3 mM AT + αF). After 3–5 days incubation, six of the mutant colonies that arose on each plate were picked and streak purified on YEPD. EMS-induced mutants were obtained in starting strain YDH222/pSL1994, which harbors the *hmlαΔ::LEU2* allele and carries an extra copy of *FAR1* on a *URA3-CEN/ARS* plasmid to preclude the isolation of *sir* and *far1* mutants. This strain was streaked to SC lacking uracil (SC −Ura) plate medium to obtain 10 independent starting colonies. After 2 days growth, colonies were used to inoculate overnight SC −Ura broth cultures. Cells were harvested the next day and treated with EMS according to AUSUBEL *et al.* (1987); 10% killing was achieved. After a 24-hr period of outgrowth in SC −Ura, cultures were plated on selective medium, which was SGal −His −Ura + 5 mM AT. Ten of the mutant colonies that arose on each plate after 3–5 days incubation were picked and streak purified on SC plates containing 5-fluoroorotic acid (BOEKE *et al.* 1984) to select for isolates that had lost pSL1994. All mutants isolated above were presumed to be defective for factor arrest (Far$^-$ phenotype).

For dominance testing and complementation analysis, mutants were crossed to tester strains that were either wild type or harbored known recessive mutations. The resulting diploids could not be scored for the Far phenotype, since the *MAT***a**/*MATα* cell type does not express the appropriate set of genes (JENSEN *et al.* 1983; MILLER *et al.* 1985). To meet the haploid cell-type requirement, the *MAT* homozygosity selection (MHS) was developed to convert **a**/α diploid cells into either **a**/**a** or α/α cells. Strains homozygous for the *HIS3::p-*

FUS1::HIS3 allele but heterozygous at *MAT* are His^- due to cell-type-specific repression of regulatory genes required for *FUS1* promoter activity. Because the *FUS1* promoter is active in mating type homozygous diploid cells (**a**/**a** and α/α) that are otherwise wild type for signal transduction, cells that spontaneously convert one or the other *MAT* alleles were recovered simply by selecting His^+ papillae from patches replica plated to SC −His medium. Typically, four independent His^+ papillae derived from an **a**/α diploid were streak purified and scored for *MAT* homozygosity by the pheromone production test (SPRAGUE 1991).

For dominance testing, mutants were crossed to wild-type cells of the opposite mating type. The resulting diploids were subjected to the MHS and then scored for the Far phenotype. A mutant was designated dominant if the mutant/wild-type hybrid was Far^-. Recessive mutants were subjected to complementation analysis where, in the first stage, they were crossed to either *far1* or *fus3* tester strains. Diploids were subjected to the MHS and then scored for the Far phenotype. A mutant was assigned to the same complementation group as a tester strain if the mutant/tester-strain hybrid was Far^-. In the second stage of complementation analysis, mutants scored as unique from the *far1* and *fus3* complementation groups were crossed among each other and analyzed for complementation as described above. Tetrad analysis was performed on *MAT***a**/*MAT*α diploids to test for linkage between mutations and various genetic markers. Since all four spore clones derived from each tetrad carried the *rad16::pGAL1::STE4* allele, the Far phenotype was conveniently assayed by replica plating the YEPD tetrad dissection plates to YEPGal medium and then scoring growth.

In toto, the genetic analysis led to the identification of the genes mutated in nearly 100 strains. Specifically, the genes and number of isolates (number of independent isolates) were as follows: *far1,* 76 (27); *fus3,* one (obtained as a *fus3 gal1::ste4* double mutant); *far3,* two (two); *ste20,* three (three); *ste2,* one (**a** cell-type specific); *sir3,* one; *mat*α2, one; $CLN3^{Dom}$, six (six); $STE3^{Dom}$, one (**a** cell-type specific).

Assays of the Far phenotype and pheromone response: The Far phenotype was scored by one or more of three tests that distinguish Far^+ and Far^- strains harboring the *pGAL1::STE4* construct: (1) growth of yeast patches replica plated from dextrose to galactose medium, (2) plating efficiency of yeast spotted from cell suspension or streaked to galactose medium, (3) microscopic examination of yeast that had been incubated on a galactose plate overnight. In some cases a plate halo assay similar to that described by SPRAGUE (1991) was used to measure resistance to α-factor-mediated G_1 arrest. Fresh *MAT***a** *bar1* cell suspensions were spread on the surface of YEPD plate medium and allowed to dry. A sterile solution of synthetic α-factor in water was applied either directly on top of the lawns or to sterile filter disks that had been placed on top of the lawns.

For β-galactosidase assays, *MAT***a** *pFUS1::lacZ bar1* strains were grown to stationary phase in YEPD broth and then stored as stable stock cultures at 4° for up to 1 week. Stock cultures were diluted (1:~2000) into fresh YEPD to give a culture density of $A_{600} \sim 0.5$ after overnight incubation at 30°, at which time the exponentially growing cultures were split and either treated or not treated with α-factor. After 30 min, cells were harvested and assayed. Units of β-galactosidase activity were calculated as previously described (JARVIS *et al.* 1988).

Other tests of mating-specific functions were performed essentially as described by SPRAGUE (1991).

DNA sequencing: DNA sequencing was by the dideoxy chain termination method (SANGER *et al.* 1977), using double-stranded DNA templates and the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical). The entire 1.8-kb sequence presented in Figure 2 was determined on both strands using T3, T7 (both purchased from Promega), and custom sequencing primers on DNA restriction fragments subcloned into pBLUESCRIPT or the pBLUESCRIPT-based shuttle vector, pRS316. The contiguous DNA sequence in Figure 2 was compiled using the University of Wisconsin Genetics Computer Group package (DEVEREUX *et al.* 1984). DNA and protein sequences were compared with those maintained in the National Center for Biotechnology Information database using the BLAST alignment program (ALTSCHUL *et al.* 1990).

To determine the DNA sequence for the *far3-1* and *far3-2* alleles, DNA was isolated from strains 59I1 and 60D4, respectively, by the allele rescue technique (ROTHSTEIN 1991). To accomplish this, plasmid pJ4 was digested with *Nru*I to target integration 5′ of the *far3* locus in each strain. Genomic DNA was then prepared (NASMYTH and REED 1980) and digested with *Bam*HI to release vector sequences coupled to *far3* DNA. Custom sequencing primers were used to determine the nucleotide sequence on at least one strand of DNA corresponding to ~50 bp 5′ of the *FAR3* start codon to ~160 bp 3′ of the *FAR3* stop codon.

Northern blot analysis: Cell culture conditions for the Northern blot experiments are described within the figure legends. Total yeast RNA was extracted and processed for Northern blot analysis as described by MCKINNEY *et al.* (1993), except that formaldehyde was omitted from the 1% agarose/MOPS gel as suggested by LIU and CHOU (1990). Gel lanes were loaded with 10 μg total RNA, as determined by A_{260}. RNA was visualized by immersing the blots in methylene blue stain (0.03% methylene blue, 0.3 M NaOAc pH 5.2) for 5 min with gentle agitation, followed by rinsing with deionized water. Membranes were prehybridized for 1–3 hr at 65° in JoeHyb (0.25 M NaPhosphate pH 7.5, 7% SDS, 1 mM EDTA, modified from CHURCH and GILBERT 1984). The prehybridization buffer was then exchanged for fresh hybridization solution (1 ml/10 cm^2 membrane), composed of 9 vol of JoeHyb, 1 vol of a 50% (w/v) dextran sulfate solution, ^{32}P-labeled probe (~3 × 10^6 cpm/ml), and heat-denatured, sonicated salmon sperm DNA (10 μg/ml). Hybridizations were carried out overnight at 65°. Membranes were washed three times at 65° for 15 min in 0.2× SSC, 0.5% SDS, and then exposed to X-ray film. ^{32}P-labeled probes were generated using random-primed DNA labeling (Boehringer Mannheim) and Riboprobe (Promega) kits.

Western blot analysis: Cell culture conditions for the Western blot experiments are described within the figure legends. The cells in 10 ml of culture fluid (A_{600} 0.8) were harvested and prepared as TE-washed cell pellets as described by MCKINNEY *et al.* (1993). Samples were then immediately frozen on crushed dry ice and stored at −80°. Protein extracts were prepared as described by PETER *et al.* (1993), except that Breakage Buffer (20 mM Tris pH 8.0, 50 mM NH_4OAc, 2 mM EDTA) replaced buffer A. Control experiments indicated that the addition of protease inhibitors was unnecessary (data not shown). If not used immediately, extracts were frozen on crushed dry ice and stored at −80°. Proteins were separated by SDS polyacrylamide gel electrophoresis on 8% gels and electroblotted to nitrocellulose membranes (Schleicher and Schuell) using the BioRad Minigel system as recommended by the manufacturer. Membranes were blocked for 1 hr in TTBS (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Tween-20) containing 3% (w/v) nonfat milk, and then incubated for 1 hr with the primary antibody in the same solution. Blots were washed three times for 10 min in TTBS, and then incubated for 1 hr in TTBS/3% milk containing the secondary antibody, which was HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.) used at 1:2500. Finally, membranes were washed four times for 10 min in TTBS be-

fore development with the ECL chemiluminescent detection system (Amersham).

RESULTS

Rationale and mutant isolation: Pheromone-responsive signal transduction leads to G_1 arrest and to the transcription induction of genes that promote mating. Previous studies demonstrated that the vast majority of mutants selected for resistance to pheromone-meditated G_1 arrest were simultaneously defective for transcription induction or other responses dependent upon it, such as mating and shmoo formation (HARTWELL 1980; FUJIMURA 1989; CHANG and HERSKOWITZ 1990; FUJIMURA 1990a). The bias toward obtaining Transduction$^-$ Arrest$^-$ mutants probably reflects the large number of mutable genes that function in the signal transduction pathway to control both processes. To focus on identifying genes required specifically for G_1 arrest, we developed a strategy for selecting Transduction$^+$ Arrest$^-$ mutants. To accomplish this, we took advantage of *FUS1,* a gene whose expression is absolutely dependent on an intact signal transduction pathway (HAGEN *et al.* 1991). In particular, we used yeast strains carrying the *HIS3::pFUS1::HIS3* allele; by demanding histidine prototrophy, this construct ensures that the pathway leading to transcription induction (*i.e.,* up to the Ste12 step, HAGEN *et al.* 1991) is intact. To facilitate the isolation and genetic analysis of mutants, strains carried an integrated *pGAL1::STE4* construct in addition to the wild-type *STE4* gene; *STE4* encodes the G_β subunit of the heterotrimeric G protein, and its overexpression leads to pathway activation and G_1 arrest independently of pheromone treatment (COLE *et al.* 1990; NOMOTO *et al.* 1990; WHITEWAY *et al.* 1990). Thus, our strategy was to select for mutants that could grow on galactose media lacking histidine and containing aminotriazole; aminotriazole is a competitive inhibitor of His3 (KLOPOTOWSKI and WIATER 1965) and was included in the media to select for induced levels of *pFUS1::HIS3* expression. The ability of this system to select for growth of Transduction$^+$ Arrest$^-$ strains is illustrated in Figure 1. According to the nomenclature set forth by CHANG and HERSKOWITZ (1990), new genes identified by this mutant hunt would be designated *far,* for their role in mating factor arrest.

The Transduction$^+$ Arrest$^-$ strains in our collection harbored mutations in previously identified genes, including *CLN3, FUS3,* and *FAR1.* In addition, two of the recessive mutants, *far3-1* and *far3-2,* identified a distinct complementation and linkage group. Genetic mapping studies demonstrated that *FAR3* was unique from the many genes known to be involved in either signal transduction or cell cycle control (HORECKA 1995a). This report focuses on the characterization of *FAR3* and its role in pheromone-mediated G_1 arrest.

Nucleotide sequence of the *FAR3* gene: The *FAR3* gene was cloned based on its genetic map position (HORECKA 1995a). Figure 2 shows the sequence of the

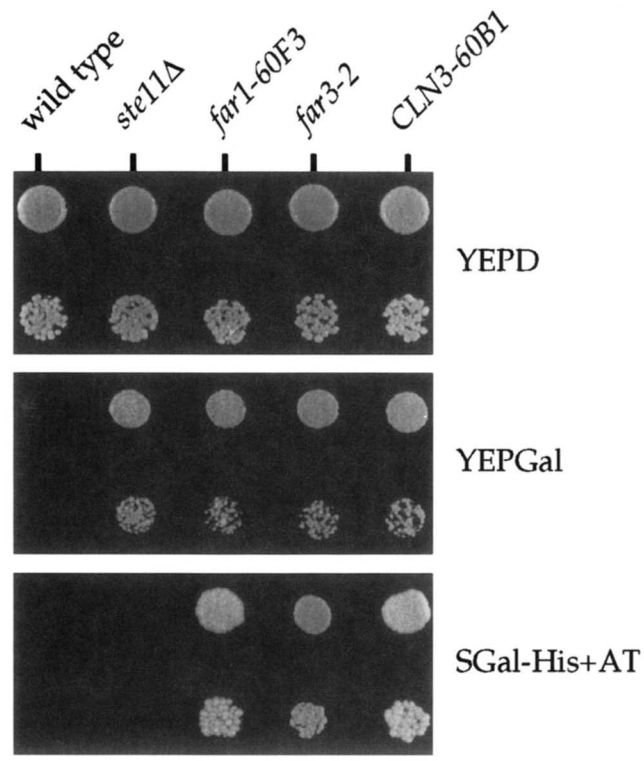

FIGURE 1.—Selective growth of Transduction$^+$ Arrest$^-$ strains on SGal −His + AT medium. Single colonies of the indicated strains were picked from a YEPD plate, suspended in S broth, diluted, and then applied to plate media at 500 and 50 cells per 5 μl spot (top and bottom rows, respectively, in each panel). Aminotriazole was present in the SGal −His + AT medium at 3 mM. The wild-type strain does not grow on galactose media due to G_1 arrest induced by *pGAL1::STE4* expression. The *ste11*Δ strain does not respond to *pGAL1::STE4* expression and consequently does not express *pFUS1::HIS3,* leading to cell division on rich galactose medium (YEP Galactose), but histidine auxotrophy on SGal −His + AT medium. The last three strains are Transduction$^+$ Arrest$^-$ mutants isolated in this study. Strains were as follows: wild type (YDH60), *ste11*Δ (SY3086), *far1-60F3* (60F3), *far3-2* (60D4), and *CLN3-60B1* (60B1).

1.8-kb *Sal*I-*Bgl*II genomic DNA fragment carried on plasmid pSL2252, which was determined in the original molecular cloning experiments to harbor the minimal *far3-1*-complementing fragment (HORECKA 1995a). DNA sequence analysis and homology searches of the National Center for Biotechnology Information (NCBI) database revealed that the 1.8-kb segment contains three genes: a $tRNA^{Trp}$, a 204-amino acid (aa) ORF, and a 242-aa incomplete ORF (Figure 2). Since each of these genes could potentially identify *FAR3,* we tested subclones derived from the 1.8-kb segment and found that the complete 204-aa ORF were necessary and sufficient for *far3-1* complementation (data not shown). The gene encoding the 204-aa ORF gene was therefore designated *FAR3.* A BLAST homology search (ALTSCHUL *et al.* 1990) of the NCBI database failed to yield any protein with significant homology to the predicted Far3 aa sequence. Hence, *FAR3* encodes a novel protein required for pheromone-mediated cell cycle arrest.

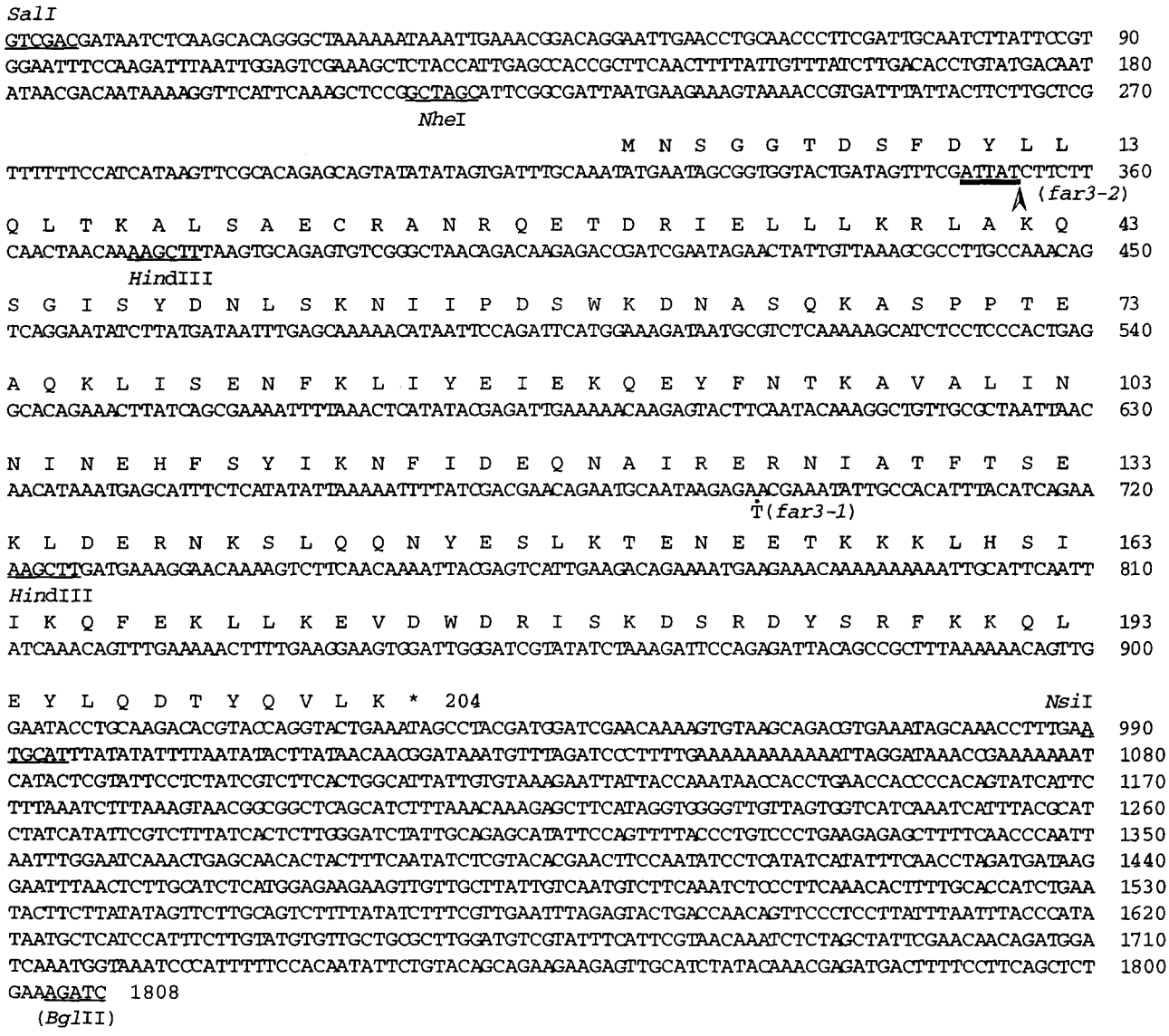

FIGURE 2.—Nucleotide sequence (GenBank accession number U35609) of the 1.8-kb yeast genomic DNA insert in the *far3-1* complementing plasmid pSL2252. The nucleotide sequence was determined for both strands as described in MATERIALS AND METHODS; the *FAR3* coding strand is shown and the 204-codon *FAR3* ORF is translated in one-letter amino acid code above the nucleotide sequence. Relevant restriction sites are underlined. The *far3-1* and *far3-2* alleles are indicated below the wild-type sequence. The arrowhead and bold underline mark the Ty*1* insertion site and the 5-bp repeated sequence flanking the Ty*1* insertion, respectively, in the *far3-2* allele. Coordinates of the $tRNA^{Trp}$ and incomplete ORF are nucleotides 39–144 (complement) and nucleotides 1080–1808 (complement), respectively.

***FAR3* is not an essential gene:** Although strains harboring the spontaneously derived *far3-1* and *far3-2* mutations grew normally, the possibility remained that *FAR3* was essential for viability. The cloned *FAR3* gene was used to construct two gene replacement alleles *in vitro*. In the *far3Δh::URA3* and *far3Δh::LEU2* alleles (also referred to as *far3Δ*), *URA3* and *LEU2* DNA replace the internal 0.35-kb *Hin*dIII fragment of *FAR3*, truncating the *FAR3* ORF after the 18th codon (Figure 2). A *far3Δh::URA3/+* diploid (SY2612) was constructed, sporulated, and subjected to tetrad dissection. The tetrads produced four viable spores that had no apparent differences from each other in terms of germination and vegetative growth (data not shown), indicating that *FAR3* is not an essential gene. Ura^+ sorted 2:2 and was always coupled with the $Far3^-$ phenotype, providing further evidence that this ORF encodes Far3.

DNA sequence analysis of the *far3-1* and *far3-2* alleles: The lesions in the spontaneously derived *far3-1* and *far3-2* alleles might identify specific amino acid residues or regions of the Far3 protein essential for its function. We used the allele rescue technique (ROTHSTEIN 1991) to recover *far3-1* and *far3-2* DNA and then determined the nucleotide sequence for both alleles over a region corresponding to ~50 bp 5′ of the *FAR3* start codon to ~160 bp 3′ of the *FAR3* stop codon. The

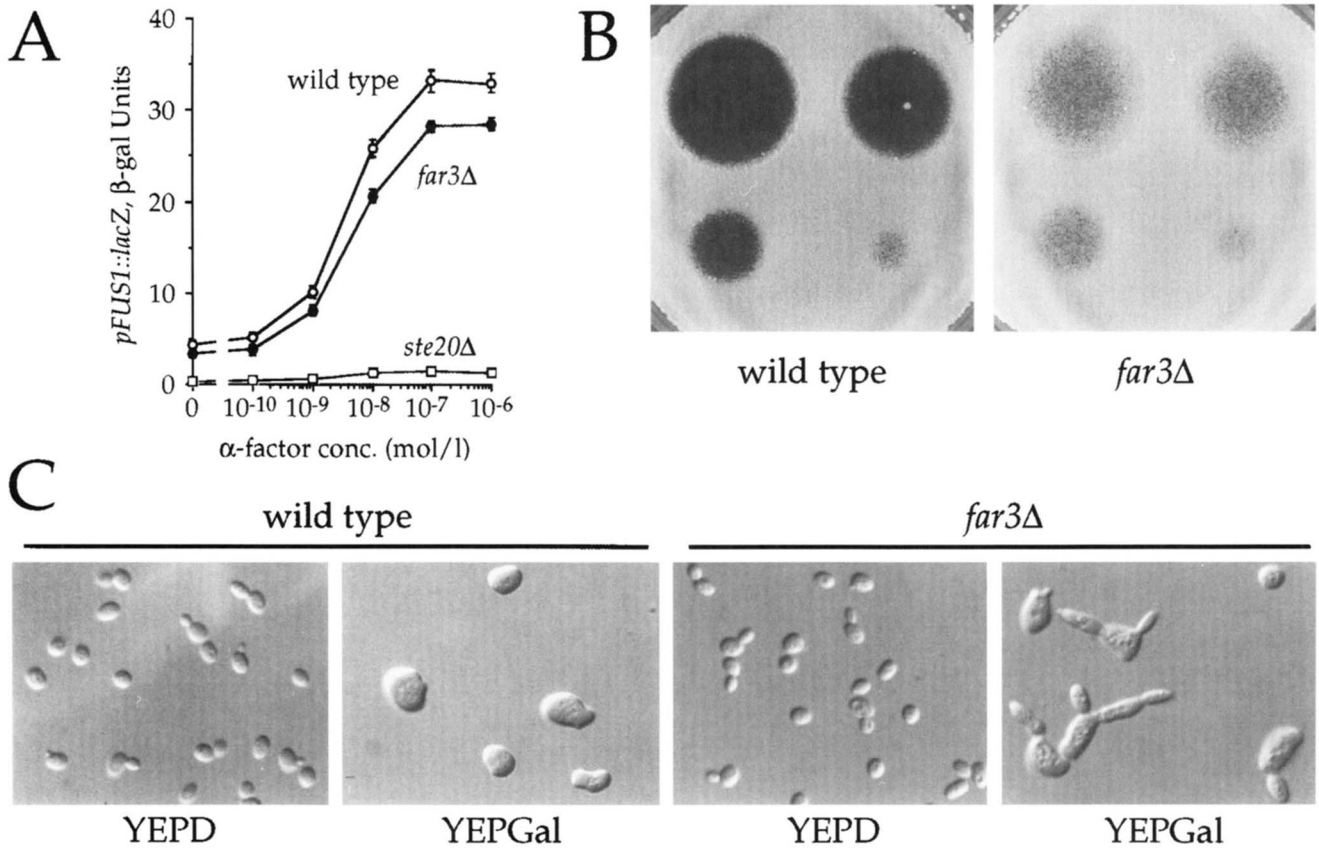

FIGURE 3.—Tests of signal transduction and cell cycle arrest in wild-type and *far3*Δ strains. (A) *MAT***a** *pFUS1::lacZ bar1* strains were either treated or not treated with α-factor at the indicated concentrations for 30 min and then assayed for β-galactosidase activity as described in MATERIALS AND METHODS. Assays were performed in triplicate on independent starting colonies; standard sample errors are indicated. (B) Halo assays were performed as described in MATERIALS AND METHODS by spotting α-factor (clockwise from upper left in each panel: 500, 250, 63, 125 ng) directly onto freshly applied lawns of *MAT***a** *bar1* strains. Halo assays were performed at least 100 times with similar results. (C) Cell morphology in response to *pGAL1::STE4* induction. Cells were grown at 30° in YEPD to exponential phase and a sample was harvested (left panels). The cells were then pelleted, washed once, and resuspended in prewarmed YEPGal. A second sample was harvested after 15 hr growth in YEPGal (right panels). Culture density was maintained at A_{600} ~0.8 throughout growth in YEPGal by adding prewarmed medium. Photomicrographs were shot at the same magnification. Strains were as follows: wild type (YDH121), *far3*Δ (SY2610), and *ste20*Δ (SY2713).

far3-1 nucleotide sequence differed from wild type by an A-to-T transversion at nucleotide 688, terminating the *FAR3* ORF after codon 122 (Figure 2). This lesion implies that the C-terminal 82 aa of Far3 are indispensable for Far3 function. For *far3-2*, restriction mapping indicated that the *FAR3* ORF in this allele was interrupted by a ~6-kb DNA segment. The exact site of insertion was determined by sequence analysis to be between nucleotides 348 and 349, disrupting the *FAR3* ORF after the ninth codon (Figure 2). The insertion is almost certainly a Ty*1* element because (1) it is the size of a Ty*1* element, (2) its ends are identical in sequence to δ elements, and (3) it is flanked by a 5-bp target site duplication (5′-GTTTC-3′; Figure 2) (FARABAUGH and FINK 1980; BOEKE and SANDEMEYER 1992). The presence of mutations in the *FAR3* gene in both *far3-1* and *far3-2* strains corroborates the designation of this ORF as *FAR3*.

Tests of pheromone-responsive signal transduction and G_1 arrest in *far3*Δ strains: Since the *far3-1* and *far3-2* strains were isolated by a technique designed to yield Transduction$^+$ Arrest$^-$ mutants, we characterized these two phenotypes in detail for strains harboring the *far3*Δ mutation. To characterize signal transduction, we measured expression of an integrated *pFUS1::lacZ* reporter construct. The data in Figure 3A show comparable basal and α-factor-induced *pFUS1::lacZ* expression for the wild-type control and *far3*Δ strains. In contrast, a *ste20*Δ control strain was severely defective for signal transduction. *pFUS1::lacZ* expression levels were also similar for wild-type and *far3*Δ strains when *pGAL1::STE4* expression was used to activate the signal transduction pathway (data not shown). Furthermore, Northern blot analysis of *FUS1* mRNA levels demonstrated that *far3*Δ strains were indistinguishable from the wild type (see Figure 9 and data not shown). The analysis of mating competency provides another measure of the signal transduction pathway's integrity, since strains defective for signal transduction are also defective for mating (HARTWELL 1980; HAGEN and SPRAGUE

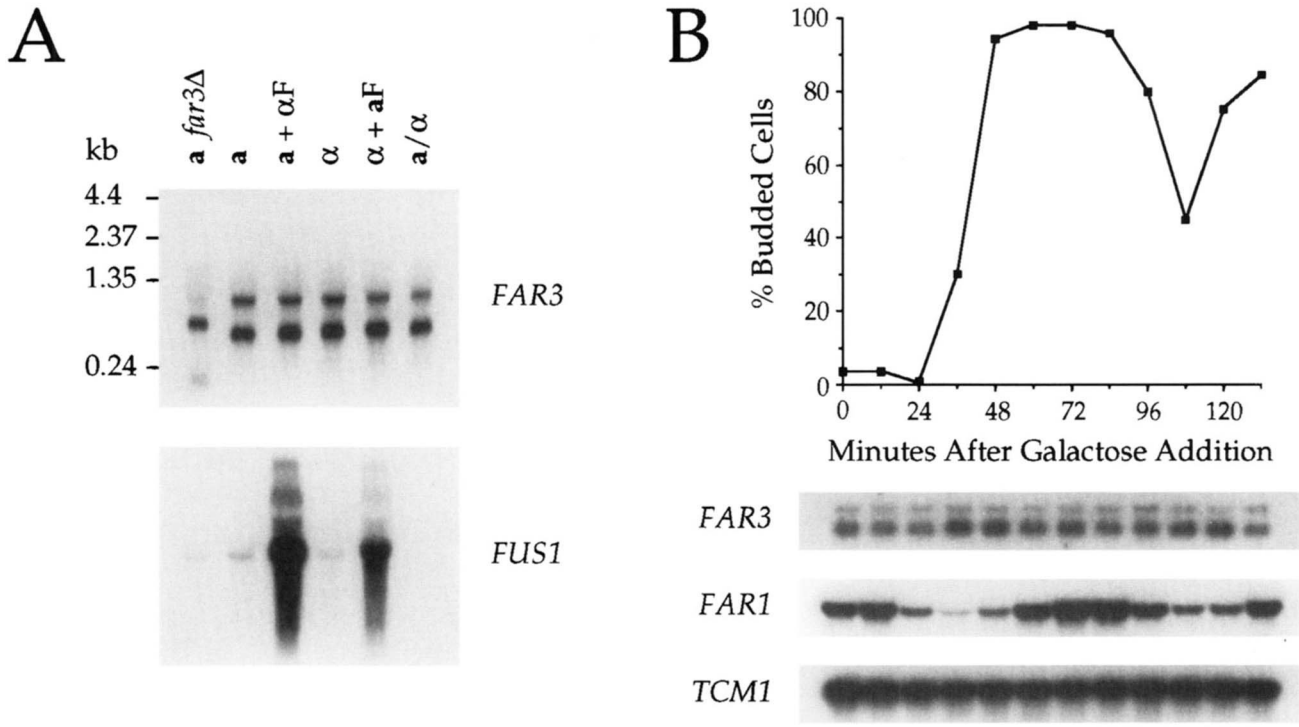

FIGURE 4.—Northern blot analysis of *FAR3* mRNA levels as a function of cell type, pheromone treatment, and cell cycle position. (A) Cultures were grown overnight to saturation in YEPD broth, diluted into fresh medium to A_{600} 0.2, and grown at 30° for one doubling. They were then either treated or not treated with pheromone and incubated for an additional 30 min before harvesting samples. *MAT***a** *bar1* cells were treated with 170 ng/ml synthetic α-factor; *MAT*α cells were treated with **a**-factor or mock **a**-factor prepared as described by DAVIS *et al.* (1993). Strains were as follows: **a** *far3*Δ (SY2610), **a** (SY1940), α (YDH28), and **a**/α (SY2556). (B) The *cln1 cln2 cln3 pGAL1::CLN3* strain 1608-21C was synchronized with a G_1 phase block by culturing to exponential phase in YEPGal at 30°, washing once with YEPRaf, resuspending in YEPRaf, and then incubating at 30° for 2.5 hr. Galactose was then added to 3% from a 30% stock solution to restart the cell cycle (zero timepoint), and samples were collected at 12-min intervals to score budding index and prepare RNA. Cells in the G_1 phase are unbudded, and bud emergence roughly corresponds to the onset of S phase (data not shown). RNA extraction and Northern blot analysis were as described in MATERIALS AND METHODS. Probes were as follows: *FAR3*, a riboprobe synthesized from *Xho*I-digested pSL2446 using T3 RNA polymerase; *FUS1*, a riboprobe synthesized from *Hin*dIII-digested pSL896 using SP6 RNA polymerase; *FAR1*, a random-primed 920-bp *Hin*dIII DNA fragment from pFC21 (CHANG and HERSKOWITZ 1990); *TCM1*, a random-primed ~800-bp *Hpa*I-*Sal*I DNA fragment from pAB309Δ (SCHULTZ and FRIESEN 1983).

1984). In a patch mating assay (SPRAGUE 1991) *far3*Δ strains mated efficiently to both wild-type and *far3*Δ partners, supporting the notion that they are wild type for signal transduction. In contrast, control strains defective for signal transduction mated poorly or not at all (data not shown).

Although *far3*Δ strains have an intact signal transduction pathway leading to *FUS1* transcriptional induction and mating, they are defective for G_1 arrest. In a standard halo assay (SPRAGUE 1991) *far3*Δ strains were resistant to α-factor-mediated cell cycle arrest, relative to the wild-type strain (Figure 3B). *far3*Δ cells are not wholly α-factor resistant in this assay, nor are *far1* cells (see Figure 6 and data not shown). We interpret this residual sensitivity to pheromone as an indication that *FAR3*-independent mechanisms are capable of impinging on cell cycle progression in the absence of *FAR3*. A second test of resistance to G_1 arrest was performed by examining the morphology of cells expressing the *pGAL1::STE4* construct (Figure 3C). When wild-type cells were cultured in galactose medium to induce *pGAL1::STE4* expression, they arrested in the G_1 phase as large, unbudded cells. In contrast, *far3*Δ cells continued to bud and divide under these conditions. *far3*Δ cells clearly responded to *STE4* overexpression, as indicated by their elongated, shmoo-like morphology. Taken together, these data indicate that *far3*Δ strains are normal for pheromone-responsive signal transduction, but defective for G_1 arrest.

Analysis of *FAR3* gene expression: Some yeast genes that are involved in the signal transduction pathway or cell cycle control exhibit transcriptional regulation that is sensitive to cell type, pheromone treatment, or position in the cell cycle. We therefore asked whether *FAR3* transcription was regulated by any of these conditions. As shown in Figure 4A, a strand-specific *FAR3* riboprobe detected two RNAs, ~0.8 and ~1.3 kb in size, that were both altered in the *far3*Δ*::URA3* strain. Transcript mapping experiments indicated that the two RNAs differed in their 3′ termini, reflecting alternative transcript termination sites or 3′ end processing (HORECKA 1995b). Both RNAs were constitutively expressed re-

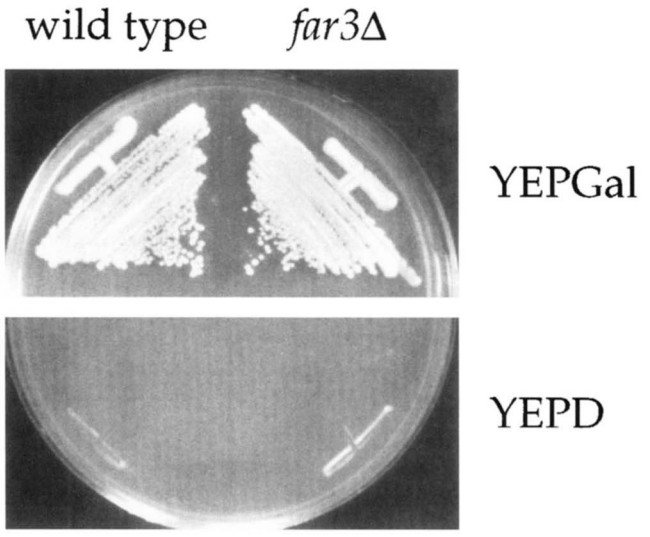

FIGURE 5.—Test of bypass G_1 cyclin function in *far3*Δ cells. *cln1 cln2 cln3 pGAL1::CLN3* strains grown on galactose medium were streaked to YEPGal (*pGAL1::CLN3* on) and YEPD (*pGAL1::CLN3* off) plate media and incubated at 30° for 3 and 7 days, respectively, before photography. Cells of both strains streaked to YEPD medium were examined microscopically and found to be uniformly arrested in the G_1 phase as large unbudded cells (data not shown). Strains were as follows: wild type (1608-21C) and *far3*Δ (SY2619).

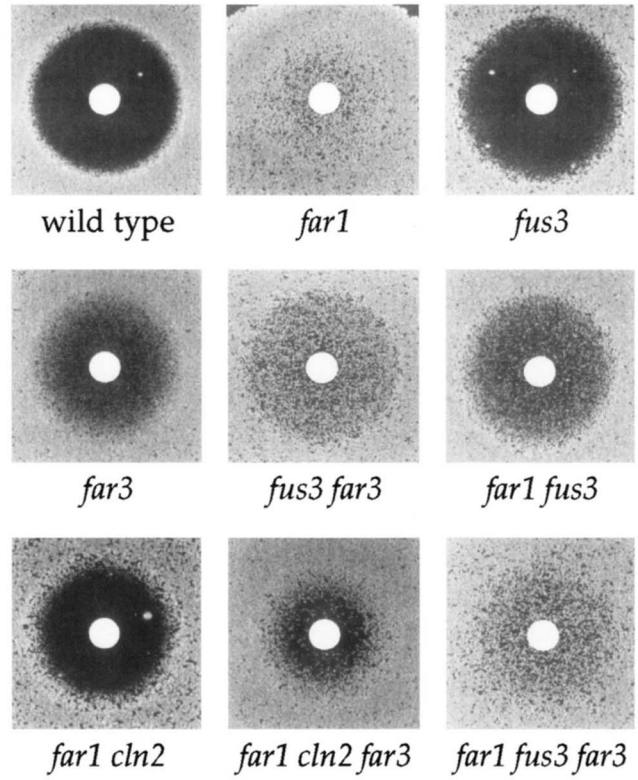

FIGURE 6.—Test of independence of Far3 from Fus3 and Far1. Halo assays were performed as described in MATERIALS AND METHODS using 2 μg α-factor applied to sterile filter disks placed on top of the indicated lawns. Halo assays were performed at least 10 times with similar results. Strains were as follows: wild type (YDH121), *far1* (SY2673), *fus3* (SY2977), *far3* (SY2610), *fus3 far3* (SY2981), *far1 fus3* (SY2978), *far1 cln2* (SY2688), *far1 cln2 far3* (SY2980), *far1 fus3 far3* (SY3072).

gardless of cell type and mating pheromone treatment. To control for experimental conditions, the blot was stripped and reprobed for *FUS1*, which showed the expected cell type-specific and pheromone-inducible transcription.

To determine whether *FAR3* was regulated as a function of cell cycle position, the *FAR3* riboprobe was hybridized to a Northern blot representing RNA samples prepared from a yeast culture synchronized with a *cln*-deficient G_1 phase block (MCKINNEY *et al.* 1993). Following release from the G_1 block, culture samples were harvested every 12 min and analyzed for budding index (a measure of cell cycle progression) and RNA levels (Figure 4B). Here, too, the levels of both RNAs detected by the *FAR3* riboprobe were constitutive. In contrast, *FAR1* RNA levels fluctuated throughout the cell cycle, as previously reported by MCKINNEY *et al.* (1993). Expression from the constitutive *TCM1* gene was invariant and served as a loading control.

***far3* mutants do not bypass G_1 cyclin function:** Based on the Transduction$^+$ Arrest$^-$ phenotypes associated with *far3* mutants, we assumed that Far3 functions as a link between the pheromone response pathway and the cell cycle machinery. However, it is also possible that Far3's role in G_1 arrest is indirect. This possibility arises from the finding that under certain conditions the activity of Cdc28-Clb5, an S-phase cyclin-dependent protein kinase, can affect G_1 arrest. Constitutive expression of *CLB5* leads to α-factor resistance in an otherwise wild-type strain (SCHWOB and NASMYTH 1993), and overexpression of the gene, even from its own promoter on a low-copy plasmid, can bypass the G_1 arrest associated with a *cln1 cln2 cln3* triple mutant (SCHWOB and NASMYTH 1993; ESPINOZA *et al.* 1994). Hence, mutations in other genes that lead to an increase in Clb5 activity are expected to display similar phenotypes. We tested whether *far3* mutants, like cells overexpressing *CLB5*, bypass G_1 cyclin function. Wild-type and *far3*Δ cells harboring *cln1 cln2 cln3 pGAL1::CLN3* alleles were constructed and propagated on galactose medium (*pGAL1::CLN3* on) and then tested for their ability to form colonies on glucose plating medium (*pGAL1::CLN3* off). *far3*Δ cells, like the wild type, could not bypass G_1 cyclin function (Figure 5), indicating that Far3 operates by a mechanism distinct from one affecting Clb5 or a similar activity.

Far3 can function independently of Fus3 and Far1: Three independent mechanisms have been proposed to bring about pheromone-mediated G_1 arrest: repression of *CLN1* and *CLN2* gene transcription (CROSS and TINKELENBERG 1991; VALDIVIESO *et al.* 1993), degradation of Cln protein (TYERS *et al.* 1992; VALDIVIESO *et al.* 1993), and the direct inhibition of Cdc28-Cln kinase activity by a mechanism involving Fus3 and Far1 (PETER *et al.* 1993; TYERS and FUTCHER 1993). We used a genetic test to address the possibility that Far3, in some way,

functioned together with Fus3 and Far1. Our analysis was based on the assumption that if two genes operated in independent pathways to promote G_1 arrest, then a strain mutated for both genes would be more resistant to G_1 arrest than the most-resistant single mutant. A plate halo assay was used to compare α-factor resistance among strains harboring various combinations of *fus3, far1,* and *far3* alleles (Figure 6).

As a first test, we compared *fus3, far1,* and *fus3 far1* strains to each other. Although Fus3 and Far1 were shown biochemically to function in the same pathway to inhibit Cdc28-Cln activity (PETER *et al.* 1993; TYERS and FUTCHER 1993), the *fus3* and *far1* single mutants did not exhibit equal levels of α-factor resistance, as might have been predicted. In fact, the *fus3* strain showed virtually no resistance [we note that other groups have reported greater resistance for *fus3*Δ strains, *e.g.,* TYERS and FUTCHER (1993)]. *fus3*Δ penetrance may depend on strain background, since we found slightly greater resistance in a *fus3*Δ strain derived from W303a; however, in this background we also found a similar, striking difference between *fus3*Δ and *far1*Δ). How can these two mutants, which are argued to function in the same pathway, exhibit such different arrest phenotypes? One possibility is that *fus3,* but not *far1,* mutants are partially defective for signal attenuation. Both mutants respond initially to α-factor, but then recover and resume budding to different degrees (Figure 6). Fus3 is a target of negative regulation by the Msg5 tyrosine phosphatase (DOI *et al.* 1994), suggesting that Fus3 activity is one of the control points for attenuation of the signal transduction pathway. Indeed, *fus3* mutants induce *pFUS1::lacZ* to higher levels than the wild type (J. HORECKA, J. PRINTEN and G. F. SPRAGUE, JR., data not shown). Thus, *fus3* and *far1* mutants may show different sensitivities to pheromone because of different capabilities at attenuating the pheromone signal. This line of reasoning also rationalizes the observation that the *fus3 far1* double mutant is more sensitive to pheromone than the *far1* mutant, as described below.

The level of α-factor resistance shown by the *fus3 far1* double mutant was intermediate to those shown by *fus3* and *far1* single mutants. The observation that the *fus3* strain is more sensitive to G_1 arrest than the *fus3 far1* strain suggests that Far1 can function in the absence of Fus3 activation (Figure 6, compare *fus3* and *fus3 far1*). How can Far1 function without phosphorylation by the Fus3 kinase? One possibility is that Kss1 has a limited ability to phosphorylate Far1 that has not been detected by the biochemical assays published thus far (PETER *et al.* 1993; TYERS and FUTCHER 1993). A second possibility emerges from the fact that *FAR1* transcription is inducible by pheromone. Perhaps increased levels of Far1 protein generated in response to α-factor can effect G_1 arrest in the absence of α-factor-induced posttranslational modification. Consistent with this notion, MCKINNEY and CROSS (1995) found that Far1 expressed at high levels from a *pGAL1::FAR1* construct could delay START in the absence of pheromone treatment. The phenotype of the *fus3 far1* double mutant also suggests that *far1* strains, which are almost wholly α-factor resistant, can be made more sensitive by eliminating Fus3-mediated signal attenuation (Figure 6, compare *far1* and *fus3 far1*). The salient result in the comparison of these three strains, however, is that the *fus3 far1* double mutant was no more α-factor resistant than the strongest single mutant (*far1*); thus the predicted outcome of this genetic test, based on the biochemically defined Fus3/Far1 mechanism, was fulfilled.

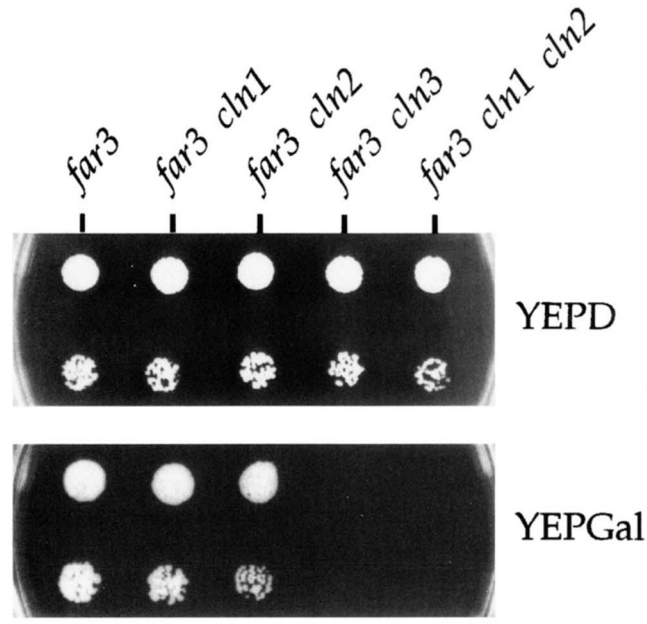

FIGURE 7.—Genetic interactions between *FAR3* and the G_1 cyclin genes. Plating efficiency is shown for conditions of repression and induction of the *pGAL1::STE4* construct. Single colonies from the indicated strains were picked from a YEPD plate, suspended in S broth, diluted, and then applied to plate media at ~500 and ~50 cells per 5-μl spot (top and bottom rows, respectively, in each panel). No growth on YEPGal (YEP Galactose) medium indicates suppression of the $Far3^-$ phenotype. Strains were as follows: *far3* (SY2321), *far3 cln1* (SY2352), *far3 cln2* (SY2342), *far3 cln3* (SY2336), *far3 cln1 cln2* (SY2350).

All strains that combined *far3* with the other mutations were more resistant to G_1 arrest than the *far3* single mutant and the corresponding *FAR3* strains (Figure 6). The *far3 fus3* double mutant was more resistant than *far3* and *fus3* single mutants. Likewise, a *far3 far1 cln2* mutant was more resistant than the *far3* or *far1 cln2* strains. In this latter comparison, a *far1 cln2* strain was used because the *far1* mutant alone was almost wholly resistant, and therefore any potential synergistic effects when combined with *far3* might not be distinguishable. These results suggest that Far3 can function by a mechanism independent of Fus3 and Far1. In support of this hypothesis, the strain defective for both mechanisms (*i.e.,* a *far3 fus3 far1* triple mutant) was

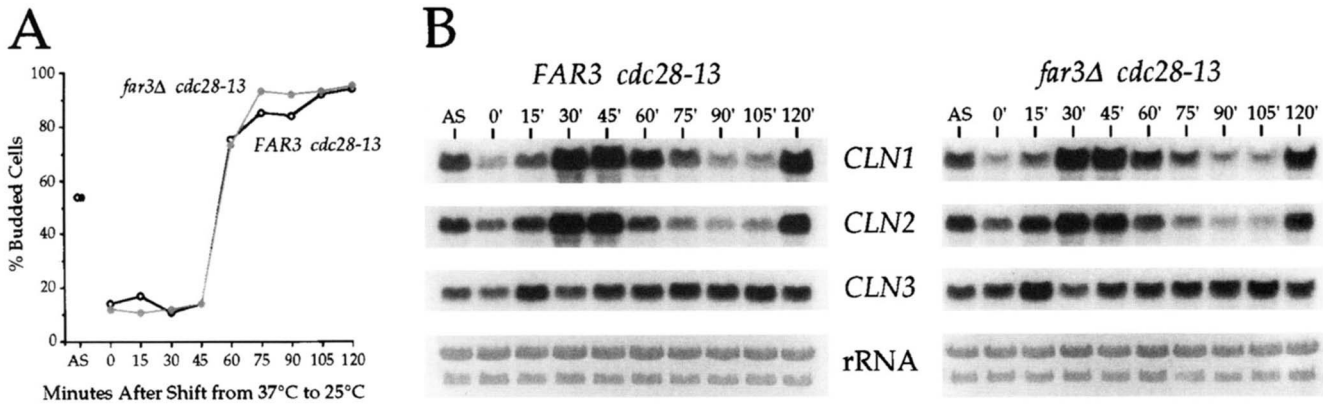

FIGURE 8.—*CLN* gene expression in cell-cycle-synchronized wild-type and *far3*Δ cultures. *cdc28-13* strains were inoculated from 4° stock cultures and grown overnight at 30° to A_{600} ~0.8 as described for β-galactosidase assays in MATERIALS AND METHODS. An asynchronous sample (AS) was taken immediately before shifting the cultures to 37° for 2 hr to arrest cells in the G_1 phase. Cultures were then shifted to 25° to release the *cdc28-13* block and restart the cell cycle (zero timepoint). Samples were harvested at the indicated timepoints to score budding index (A) and to prepare RNA for Northern blot analysis (B). Culture density was maintained at A_{600} 0.8–1.0 throughout the experiment by adding fresh YEPD at the appropriate temperature. Strains were as follows: *FAR3 cdc28-13* (SY2730) and *far3*Δ *cdc28-13* (SY2731). Riboprobes were as follows: *CLN1,* synthesized from *Bam*HI-digested pSL1709 using SP6 RNA polymerase; *CLN2,* synthesized from *Bam*HI-digested pSL1708 using SP6 RNA polymerase; *CLN3,* synthesized from *Hin*dIII-digested pDH100 using T7 RNA polymerase. Methylene blue-stained rRNA bands in the lower panels serve as controls for equivalent loading and transfer of RNA.

more α-factor resistant than strains defective for either mechanism alone (compare to *far3* and *fus3 far1*).

Genetic and functional interactions between *FAR3* and the G_1 cyclins: Since Far3 can function independently of the Fus3/Far1 mechanism, we examined the possibility that it acts by one of the other two proposed mechanisms, namely by affecting G_1 cyclin gene expression or protein stability. To begin this analysis we performed a genetic test to see if the *far3* mutant required a particular G_1 cyclin gene or combination of genes for resistance to G_1 arrest (CHANG and HERSKOWITZ 1990). We reasoned that if Far3 impinged on a particular cyclin, say ClnX, then *far3* mutants would be resistant to G_1 arrest because of unregulated ClnX activity. This implies that *far3 clnx* mutants should again be sensitive to G_1 arrest. We constructed and tested strains that were mutant for *FAR3* and at least one of the three G_1 cyclin genes. In this experiment the signal transduction pathway was activated by *pGAL1::STE4* expression. The results suggest that *FAR3* interacts genetically with all three *CLN* genes (Figure 7). Although *far3, far3 cln1,* and *far3 cln2* strains were resistant to G_1 arrest, the *far3 cln1 cln2* strain was sensitive, implying that the *far3* strain requires at least one of the *CLN1* and *CLN2* genes for resistance to G_1 arrest. The *far3 cln3* strain was also sensitive to G_1 arrest; interestingly, a halo assay showed that a *far3 cln3* strain is more sensitive than the *cln3* single mutant (data not shown).

To complement this genetic analysis, we directly compared G_1 cyclin mRNA and protein levels in wild-type and *far3*Δ strains. Two modes of regulation have been described for *CLN1* and *CLN2* gene expression: feedback-dependent and feedback-independent. Feedback-dependent regulation refers to a self-reinforcing, positive feedback loop that leads to a Cdc28-Cln-dependent burst in *CLN1* and *CLN2* transcription at START (CROSS and TINKELENBERG 1991; DIRICK and NASMYTH 1991). In a *cdc28* or *cln*-deficient mutant, feedback-dependent regulation is inoperative, abolishing most of the *CLN1* and *CLN2* transcription. The small amount of *CLN1* and *CLN2* transcription that persists at a *cdc28* or *cln*-deficient G_1 block is referred to as feedback-independent expression, and is subject to pheromone-mediated repression (CROSS and TINKELENBERG 1991; VALDIVIESO *et al.* 1993). We performed two experiments to address separately the possibilities that *FAR3* affects feedback-dependent and feedback-independent expression.

First, we compared wild-type and *far3*Δ strains for feedback-dependent gene expression. A *cdc28-13*^{ts} mutation was used to inactivate feedback-dependent expression and synchronize the cell cycle at a pre-START arrest point. In this protocol, when the *cdc28-13*^{ts} cells are shifted back to the permissive temperature, the feedback-dependent mechanism reactivates and a burst of *CLN1* and *CLN2* gene expression occurs as cells in the culture synchronously enter START. Using bud emergence as an indicator of cell cycle progression, we found that wild-type and *far3*Δ cells passed through START with indistinguishable kinetics following release from the *cdc28-13*^{ts} block (Figure 8). Likewise, Northern blot analyses yielded *CLN1* and *CLN2* expression profiles that were superimposable at START and throughout the cell cycle (Figure 8). *CLN3* mRNA levels were previously shown to be relatively invariant throughout the cell cycle (NASH *et al.* 1988; WITTENBURG *et al.* 1990; TYERS *et al.* 1993). We confirmed this result for the wild-type strain and found that *CLN3* expression is not aberrant in the *far3*Δ strain (Figure 8).

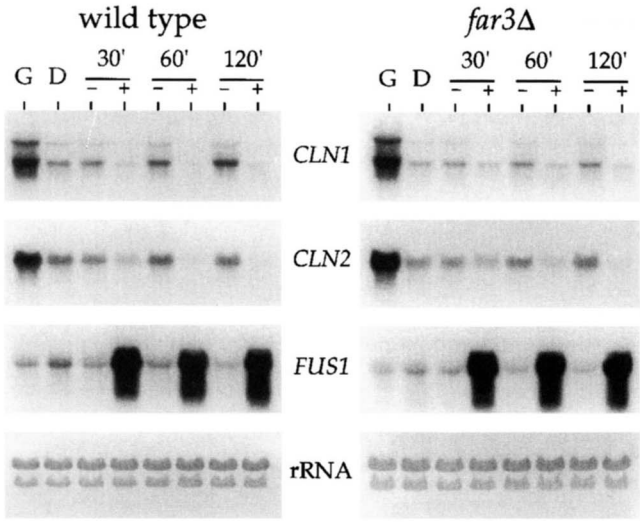

FIGURE 9.—Pheromone-mediated *CLN1* and *CLN2* mRNA repression in wild-type and *far3*Δ strains. *MAT***a** *bar1 cln1 cln2 cln3 pGAL1::CLN3* strains were grown overnight in YEPGal at 30° and then diluted into fresh YEPGal to a density of A_{600} ~0.25. The cultures were allowed to double once and a sample of the asynchronous YEPGal cultures was taken (G). Dextrose was then added to 3% from a 50% stock solution to repress *pGAL1::CLN3* transcription. After 3 hr, the cells were uniformly arrested in the G_1 phase (data not shown) and a sample was harvested (D). The cultures were then split into two, one of which received α-factor at 200 ng/ml, and samples were harvested at the indicated timepoints. Strains were as follows: *FAR3* (1608-21C) and *far3*Δ (SY2619). RNA extraction and Northern blot analysis were as described in MATERIALS AND METHODS. Riboprobes were as described in the legends for Figure 4 and Figure 8. Methylene blue-stained rRNA bands in the lower panels serve as controls for equivalent loading and transfer of RNA.

To examine pheromone-mediated repression of feedback-independent *CLN1* and *CLN2* expression, we used a protocol originally described by VALDIVIESO *et al.* (1993). The strains used for this protocol are disrupted for the endogenous *CLN* genes but are able to propagate on galactose medium because they harbor a *pGAL1::CLN3* construct. On glucose medium, *pGAL1::CLN3* expression is repressed and the cells arrest in the G_1 phase before START, inactivating feedback-dependent expression of *CLN1* and *CLN2*. These cells, which exhibit only feedback-independent transcription of *CLN1* and *CLN2*, were either treated or not treated with α-factor to test for pheromone-mediated repression. We found that both wild-type and *far3*Δ strains were effective for this repression, as revealed by the loss of the *cln1::TRP1* and *cln2*Δ*xs* transcripts (CROSS and TINKELENBERG 1991) (Figure 9). Taken together with the above results, these data indicate that *FAR3* does not operate by affecting either feedback-dependent or feedback-independent regulation of *CLN1* or *CLN2* mRNA levels.

Finally, we addressed the possibility that Far3 affects G_1 cyclin protein stability. The Cln1 and Cln2 proteins are similar in several respects; for example, they are ~57% identical (HADWIGER *et al.* 1989) and they are both destabilized in response to mating pheromone (WITTENBURG *et al.* 1990; TYERS *et al.* 1993). In contrast, Cln3 shares only 20–25% identity with Cln1 and Cln2 (HADWIGER *et al.* 1989), and although Cln3 is highly unstable (CROSS and BLAKE 1993), its stability is not further antagonized by pheromone treatment. We therefore performed two sets of experiments to examine the potential role of *FAR3* on the stability of these two sets of G_1 cyclins. To assay Cln protein levels, we took advantage of the available hemagglutinin (HA) epitope-tagged versions of Cln2 (PETER *et al.* 1993) and Cln3 (TYERS *et al.* 1992). In the first experiment, the fate of Cln2-HA protein was followed in wild-type and *far3*Δ cells subjected to an α-factor dose-response protocol. Both strains induced the turnover of Cln2-HA at α-factor concentrations of 10^{-7} M and higher (Figure 10A). Although the relative amounts of Cln2-HA protein were comparable between the two strains for each concentration of α-factor tested, the spectrum of polypeptides detected by the anti-HA monoclonal antibody appeared different. In particular, the cluster of bands detected in the *far3*Δ strain was biased toward the slower migrating forms. This qualitative difference was found to vary from experiment-to-experiment (M. PETERS, personal communication; our data not shown) and was unlinked to the *far3*Δ mutation (data not shown). Wild-type and *far3*Δ strains were also indistinguishable when Cln2-HA destruction was compared in two separate time-course experiments, using 10^{-7} M and 10^{-6} M α-factor (data not shown). Hence, deletion of *FAR3* does not affect pheromone-mediated turnover of Cln2 and, by inference, the structurally similar Cln1.

As mentioned above, Cln3 is an unstable protein that does not appear to be further destabilized by mating pheromone treatment. Nonetheless, dominant mutations that remove the C-terminal PEST instability determinants from Cln3 stabilize the protein and confer resistance to pheromone-mediated G_1 arrest (CROSS 1988b; NASH *et al.* 1988; TYERS *et al.* 1992; CROSS and BLAKE 1993). It was therefore possible that *far3*Δ strains might be resistant to G_1 arrest because they are defective for the constitutive destruction of Cln3 protein. To test this, we used a *pGAL1::CLN3-HA* construct, which has been used successfully to measure differences in stability between wild-type and truncated Cln3 proteins (TYERS *et al.* 1992). When cultured in galactose medium, Cln3-HA protein was present at similar steady-state levels in wild-type and *far3*Δ cells (Figure 10B). When dextrose was added to repress *pGAL1::CLN3-HA* expression, Cln3-HA was rapidly degraded in both strains, being undetectable at the shortest timepoint examined. Thus, as with Cln2 (and presumably Cln1), Cln3 stability is unaffected by the *far3*Δ mutation. Consistent with the Cln3 data, we found that a *far3*Δ *CLN3*^{Dom} double mutant was more resistant to G_1 arrest than either single mutant (data not shown), suggesting

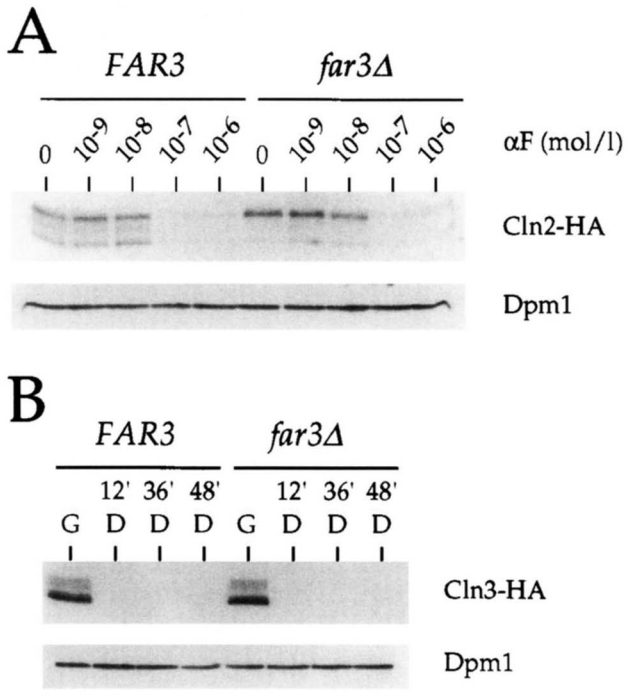

FIGURE 10.—Analysis of G_1 cyclin protein turnover in wild-type and *far3*Δ strains. (A) α-factor-induced Cln2 protein turnover. *MAT***a** *bar1* strains were transformed with plasmid pTP35, which carries the *CLN2* gene fused at the 3′ end of the ORF to DNA encoding a triple HA epitope tag (PETER *et al.* 1993). Cultures were grown at 30° in SC −Ura broth to a density of A^{600} ~0.4, split into five tubes, and either treated or untreated with α-factor at the indicated concentrations for 2 hr before harvesting samples. Host strains were as follows: *FAR3* (SY2781) and *far3*Δ (SY2802). (B) Cln3 protein stability. Strains carried as their only allele of *CLN3* a *pGAL1::CLN3C* construct, which is a fusion of *GAL1* regulatory sequences, the *CLN3* ORF, and DNA sequences encoding a triple HA epitope tag (TYERS *et al.* 1992). Cultures were grown at 30° to a density of A_{600} 0.5 in YEPGal broth to induce synthesis of Cln3-HA. A sample was harvested (G) and dextrose was added to 3% from a 50% stock solution to repress synthesis of Cln3-HA. Additional samples were then harvested at the indicated timepoints. Strains were as follows: *FAR3* (SY2832) and *far3*Δ (SY2831). Protein extraction and Western blot analysis were as described in MATERIALS AND METHODS. Primary mouse monoclonal antibodies were as follows: anti-HA, purchased from the Berkeley Antibody Company and used at 1:1000; anti-Dpm1p, obtained from the TOM STEVENS laboratory and used at 1:1000.

that the roles of Cln3 protein turnover and Far3 in G_1 arrest are independent.

DISCUSSION

We have identified and characterized a gene, *FAR3,* that is required for pheromone-mediated G_1 arrest but not for signal transduction. As a first step toward elucidating the mechanism by which Far3 promotes pheromone-mediated G_1 arrest, we performed genetic and molecular experiments to test the possibility that Far3 participates in one of the heretofore characterized mechanisms, which are Fus3/Far1-mediated inhibition of Cdc28-Cln kinase activity, G_1 cyclin gene repression, and G_1 cyclin protein turnover. Our data indicate that Far3 acts independently of these and therefore defines a new mechanism for pheromone-mediated G_1 arrest.

Since *FAR3* functions independently of the three known mechanisms, it is worth considering what other mechanisms might promote G_1 arrest. Pheromone treatment may, for example, decrease Cdc28-Cln kinase activity by posttranslational modifications independent of Fus3/Far1 inhibition and G_1 cyclin destabilization. One such mechanism can be inferred from the finding that, in addition to association with a cyclin molecule, Cdc28-like kinases require phosphorylation on Thr^{169} for activity (GOULD *et al.* 1991; FESQUET *et al.* 1993; POON *et al.* 1993; SOLOMON *et al.* 1993). Pheromone treatment might inhibit Cdc28-Cln by reducing Thr^{169} phosphorylation. Recent studies by PETER and HERSKOWITZ (1994) suggest, however, that pheromone-mediated posttranslational modifications of this type, if they do occur, do not lead to a detectable reduction in histone H1 kinase activity *in vitro.* In particular, they purified Far1-Cdc28-Cln2 protein complexes from α-factor-arrested yeast cells and found that Far1 could be dissociated from Cdc28-Cln2, restoring full kinase activity. Although these data tend to rule out covalent, inhibitory modifications of the Cdc28 and Cln2 polypeptides, it is still possible that the protein complexes assayed by PETER and HERSKOWITZ contained other inhibitory proteins that were dissociated together with Far1; Far3 might represent such a molecule. On the other hand, Far3 might affect Cdc28-Cln function indirectly, for example, by altering the subcellular localization of Cdc28-Cln or its affinity for *in vivo* substrates. Clearly, a number of potential mechanisms can be postulated.

Mutations that affect cell cycle components other than Cdc28-Cln might also lead to defects in pheromone-mediated G_1 arrest. This possibility was already discussed above with respect to the α-factor resistance phenotype associated with the overexpression of the S-phase cyclin Clb5 (see RESULTS). By a similar argument mutations that increase activity of the Pho85-Pcl kinase, another Cdk-cyclin, might also lead to α-factor resistance. This notion is inferred from three lines of evidence. First, two of the cyclins that associate with the Pho85 kinase, Pcl1 and Pcl2, play a role in promoting START in vegetative cells that partially overlaps that of Cln1 and Cln2 (OGAS *et al.* 1991; ESPINOZA *et al.* 1994; MEASDAY *et al.* 1994). Second, strains harboring a *pho85* deletion allele are supersensitive to pheromone-mediated G_1 arrest (MEASDAY *et al.* 1994). Third, in contrast to the repression of *CLN1* and *CLN2* transcription, *PCL2* expression is rapidly induced by pheromone treatment, and Pho85-Pcl2 kinase activity persists in α-factor-arrested cells (MEASDAY *et al.* 1994). Whether the overexpression of *PCL2,* like that of *CLB5,* leads to α-factor resistance has not been reported. Using genetic experi-

ments similar to those reported here to examine the relationship between *FAR3* and the *CLB5* and *CLN* genes, it would be interesting to examine the potential relationship between *FAR3* and the *PHO85* and *PCL* genes.

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