

FAR1 Is Required for Oriented Polarization of Yeast Cells in Response to Mating Pheromones

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Abstract. Cell polarization involves specifying an area on the cell surface and organizing the cytoskeleton towards that landmark. The mechanisms by which external signals are translated into internal landmarks for polarization are poorly understood. The yeast *Saccharomyces cerevisiae* exhibits polarized growth during mating: the actin cytoskeleton of each cell polarizes towards its partner, presumably to allow efficient cell fusion. The external signal which determines the landmark for polarization is thought to be a gradient of peptide pheromone released by the mating partner. Here we describe mutants that exhibit random polarization. Using two assays, including a direct microscope assay for orientation (Segall, J. 1993. *Proc. Natl. Acad. Sci. USA.* 90:8332–8337), we show that these mutants

cannot locate the source of a pheromone gradient although they are able to organize their cytoskeleton. These mutants appear to be defective in mating because they are unable to locate the mating partner. They carry mutations of the *FAR1* gene, denoted *far1-s*, and identify a new function for the Far1 protein. Its other known function is to promote cell cycle arrest during mating by inhibiting a cyclin-dependent kinase (Peter, M., and I. Herskowitz. 1994. *Science (Wash. DC)*. 265:1228–1232). The *far1-s* mutants exhibit normal cell cycle arrest in response to pheromone, which suggests that Far1 protein plays two distinct roles in mating: one in cell cycle arrest and the other in orientation towards the mating partner.

CELLULAR polarization is a critical feature of many eukaryotic cells. Polarization can be generated in response to an external signal, for example during chemotaxis. Dictyostelium cells polarize in a gradient of cAMP towards the source of the gradient and move in that direction (Devreotes and Zigmond, 1988); similarly, neutrophils polarize in and move towards a source of formylated peptides (Downey, 1994). Nonmotile cells can also exhibit polarization towards an external signal: helper T cells polarize towards their target antigen-presenting cell (Stowers et al., 1995). How external signals generate internal polarization remains poorly understood.

The budding yeast *Saccharomyces cerevisiae* exhibits polarized growth towards an external signal during mating, when each haploid cell polarizes towards the mating partner (for reviews see Madden et al., 1992; Chenevert, 1994). Polarization in yeast involves organizing the actin cytoskeleton and secretory apparatus towards a specific area of the cell surface (Ford and Pringle, 1986; Hasek et al., 1987; Gehrung and Snyder, 1990; Read et al., 1992). Microtubules are also oriented but are not required for po-

larized growth (Byers, 1981; Rose and Fink, 1987; Gehrung and Snyder, 1990; Meluh and Rose, 1990). During budding, the organization of the actin cytoskeleton and secretory apparatus towards the incipient bud site produces polarized growth at a small area of the cell surface, resulting in the growing bud. During mating, polarization towards the mating partner results in the local deposition of proteins needed for fusing the cell walls, plasma membranes, and nuclear membranes. The landmark for polarity during vegetative growth is specified by an internal signal which is determined genetically by the *BUD* genes (Bender and Pringle, 1989; Chant and Herskowitz, 1991; Chant et al., 1991; Fujita et al., 1994). During mating, the information to polarize towards the bud site is ignored. Instead, a mating cell reorients its actin cytoskeleton and secretory apparatus towards its mating partner (see Fig. 1).

During yeast mating, each haploid cell secretes a cell type-specific peptide pheromone: *a* cells secrete *a*-factor, which binds to a receptor on the surface of α cells, and α cells secrete α -factor, which binds to a receptor on the surface of *a* cells. Pheromone binding to its seven-transmembrane receptor triggers a signal transduction pathway which includes a heterotrimeric G protein, a MAP kinase cascade, and a transcription factor (Kurjan, 1992; Sprague and Thorner, 1992; Herskowitz, 1995). Three classes of events are induced by pheromone signaling: cell cycle ar-

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rest in G1, gene induction, and morphological changes including polarization towards the mating partner. This polarization can be visualized by adding α -factor to **a** cells, which causes the cells to form pear-shaped shmoo (Lipke et al., 1976; Tkacz and MacKay, 1979; Baba et al., 1989).

The signal which a cell uses to locate and polarize towards its mating partner is thought to be a gradient of pheromone (Kurjan, 1985; Michaelis and Herskowitz, 1988; Jackson and Hartwell, 1990a,b). Although this view has been widely accepted, it has only recently been shown directly that **a** cells exhibit polarized growth towards a source of pheromone when exposed to a gradient for several hours (Segall, 1993).

To understand how yeast cells polarize towards an extracellular signal, we have sought to find mutants that polarize and respond to pheromone normally but that are unable to locate its source. We expected such mutants to orient at random instead of towards their mating partners. A screen to identify such mutants yielded several candidates, including mutants with mutations (denoted *far1-s*) of the previously identified *FAR1* gene (Chenevert et al., 1994). The Far1 protein binds to and inhibits the CDC28/CLN1 and CDC28/CLN2 cyclin-dependent kinases to cause arrest of cells in G1 (Peter et al., 1993; Tyers and Futcher, 1993; Peter and Herskowitz, 1994). *FAR1* plays an additional role in mating because certain truncation alleles (such as *far1-c*, which lacks the COOH-terminal two-thirds of Far1) exhibit normal cell cycle arrest but are defective in mating (Chang and Herskowitz, 1990; Chang, 1991). The basis for this mating defect has been suggested to be due to the inability to orient towards the mating partner (Chang, 1991), but definitive tests have not been possible.

This paper further characterizes the *far1-s* mutants and demonstrates that they are defective in orientation. Here we show that *far1-s* mutants exhibit normal cell cycle arrest, gene induction, and morphological changes in re-

sponse to pheromone despite their defects in mating. Using two newly developed assays, the pheromone confusion assay (Dorer et al., 1995) and a direct microscope assay for orientation (Segall, 1993), we show that these mutants are unable to respond properly to a pheromone gradient and are defective in orientation. Instead of polarizing towards the mating partner, the *far1-s* mutants polarize towards their incipient bud site. We have analyzed the changes to the Far1 protein in the *far1-s* alleles, which indicates that two regions of the protein are involved in orientation, one in the amino terminus, the other in the carboxy terminus.

Materials and Methods

Yeast Strains, Plasmids, and Growth Conditions

Yeast strains are described in Table I. Standard yeast growth conditions and genetic manipulations were used as described (Rose et al., 1990).

Pheromone Response Assays

For cell cycle arrest (halo) assays, 10^3 – 10^4 cells from log phase **a** cultures were plated on YEPD plates. 1 μ g α -factor (Sigma Chem. Co., St. Louis, MO) in 10 μ l 0.01 M HCl was spotted on a sterile filter disk (Schleicher and Schuell, Keene, NH) and placed on the plates, which were then incubated for 2 d at 30°C.

Shmoo morphology was determined by the addition of 10^{-6} M α -factor to 3-ml log phase cultures for 2 h at 30°C. Cells were sonicated, fixed with formaldehyde to a final concentration of 3.7%, and viewed by differential interference contrast microscopy. An antibody raised against yeast actin (from D. Drubin) was used for indirect immunofluorescence according to standard protocols (Pringle et al., 1989).

To examine the induction of pheromone responsive genes, a plasmid carrying *FUS1::lacZ* (pSB286, a gift from J. Trueheart) was integrated at *FUS1*. Cells were treated with pheromone for 90 min and β -galactosidase activity was assayed as previously described (Chang and Herskowitz, 1990). Wild-type cells had 0.4 Miller units in the absence of pheromone and 96 in the presence of pheromone. *far1-s* mutants D1 and H7 had 92 and 80 Miller units in the presence of pheromone, 96 and 85% of wild-type levels, respectively.

Table I. Yeast Strains Used in This Study

Strain	Relevant genotype	Source
JC2-1B	<i>MATa HMLa HMRA bar1-1 met1 ade2-101 ura3-52</i>	Chenevert et al., 1994
	The following strains are all isogenic to JC2-1B	
NVY180	<i>MATa</i>	This study*
NVY80	<i>MATa far1-s(B4) bar1-1</i>	Chenevert et al., 1994
NVY162	<i>MATa far1-s(B4)</i>	This study*
NVY87	<i>MATa far1-s(D1) bar1-1</i>	Chenevert et al., 1994
NVY163	<i>MATa far1-s(D1)</i>	This study*
NVY98	<i>MATa far1-s(H7) bar1-1</i>	Chenevert et al., 1994
NVY164	<i>MATa far1-s(H7)</i>	This study*
NVY97	<i>MATa far1-s(G18) bar1-1</i>	Chenevert et al., 1994
NVY165	<i>MATa far1-s(G18)</i>	This study*
NVY145	<i>MATa M4</i>	This study [†]
NVY146	<i>MATa M5</i>	This study [†]
	Other strains	
IH1793	<i>MATa lys1</i>	IH collection
JC31-7D	<i>MATa far1-c lys1</i>	Chenevert et al., 1994
FC279	<i>MATa ura3Δ his2 ade1 trp1 leu2 bar1::LEU2</i>	Chang and Herskowitz, 1990
YMP18	<i>MATa far1Δ ura3Δ his2 ade1 trp1 leu2 bar1::LEU2</i>	Peter et al., 1993
K1989	<i>MATa cdc28-4 trp1-1 leu2-3,112, his3-11,15 ura3</i>	Peter et al., 1993
YMP188	<i>MATa cdc28-4 far1Δ trp1-1 leu2-3,112, his3-11,15 ura3</i>	This study

*The original strains were made *BAR1*⁻ after two-step gene replacement with pNV28, which contains a 2.8-kb *BAR1* fragment cloned into pRS306 (Sikorski and Hieter, 1989).

[†]The parent strains for the mutants M4 and M5 are *BAR1*⁺ derivatives of mutants E15 and I11, respectively (Chenevert et al., 1994).

Mating Assays

Patches of **a** cells were replica plated to lawns of wild-type α strains on restrictive SD minimal medium on which only diploids can grow and incubate for 2 d at 30°C.

For quantitative mating assays, $\sim 3 \times 10^6$ cells from log phase cultures of **a** and α strains were mixed and filtered onto 0.45- μ m nitrocellulose filters (Millipore Corp., Bedford, MA). The filters were placed on permissive YEPD plates and grown for 4 h at 30°C to allow mating. Cells were resuspended in 5 ml minimal SD medium by vigorous vortexing for 30 s, sonicated for 3 s to disrupt cell clumps, and plated on permissive YEPD plates to determine total colony-forming units and on selective SD plates to determine total diploids. Mating efficiency was calculated as the percentage of diploid cells.

Pheromone Confusion Assay

For patch mating, YEPD plates were spread with 0.5 mg α -factor (in 1 ml 0.1 M HCl) or no α -factor, allowed to dry for several hours, and then spread with a lawn of wild-type α cells. Patches of **a** *far1-s* mutants and wild-type **a** strains were replica plated onto these permissive YEPD plates (+ and - α -factor) and allowed to mate for 5 h at 30°C. The mating reactions were then replica plated to minimal SD plates and incubated for 2 d at 30°C to allow diploids to grow.

For quantitation of the pheromone confusion assay, 3.4×10^6 **a** cells and 8×10^6 α cells were mated in rolling cultures of 1.9 ml YEPD with 0 or 26 μ M α -factor for 5 h at 30°C. Cells were plated and quantitated as described above. A general reduction in mating efficiency was seen, apparently because cells were mated in rolling cultures instead of on plates. For example, wild-type **a** and α cells mate on plates (Table II) at 66.8% efficiency but in rolling cultures at 10.5% (Table III).

Orientation Assay

Orientation was assayed essentially as described (Segall, 1993). Log phase cultures were grown at 30°C in YEPD and lightly sonicated to disrupt cell clumps. Glass coverslips were treated with 1 mg/ml Con A (Molecular Probes, Inc., Eugene, OR) and allowed to dry. A 10- μ l suspension of cells was allowed to settle on the coverslip for 10 min before placing the coverslip in a microscope chamber; the chamber was then filled with YEPD and warmed to 35°C by a regulated water bath (Fisher Scientific, Pittsburgh, PA). Micropipettes were pulled from glass capillary tubing on a Flaming/Brown micropipette puller (Sutter Instruments Co., Novato, CA).

α -Factor was diluted to 325 nM in YEPD, filtered through a 0.45- μ m filter (Nalgene, Rochester, NY), and centrifuged for 20 min at 12,000 g. Pheromone was loaded into the micropipette, which was then placed under constant pressure of 10.3 kPa using an Eppendorf microinjector. The micropipette was lowered to within 10 μ m of the surface of the coverslip, and the surface of the medium was covered with light mineral oil (Sigma) to prevent evaporation. Cells were observed using a 40 \times objective lens over 5–7 h. (Experiments were carried out at 35°C instead of the standard 30°C because growth is faster at elevated temperatures. The *far1-s* mutants exhibited no temperature sensitivity for growth at 37°C.)

Images were recorded using a Cohu camera connected to a Panasonic optical disc recorder. For data analysis, cell outlines were traced on the video screen from live images because they allow a more accurate determination of cell orientation due to the three-dimensional nature of the polarized cells: the entire cell outline rarely was visible in one plane of view. We did not analyze obscured cells, which were cells with an adjacent cell

Table II. Mating Defects of *far1-s* Mutants

a cell*	Mating to α FAR1		<i>far1-c</i> FAR1
	<i>Mating to α far1-c</i>		
	%	%	
WT	66.0	6.2	0.093
B4	1.7	0.03	0.020
D1	0.83	0.002	0.002
G18	33.0	0.56	0.017
H7	3.6	0.01	0.003

*The **a** strains used were as follows: WT (NVY180), B4 (NVY162), D1 (NVY163), G18 (NVY165), and H7 (NVY164). The α strains were wild type (IH1783) and *far1-c* (JC31-7D).

in the direction of the needle. To quantitate the orientation assay, we determined the angle between the direction of the needle and the direction of the shmoo tip. Another method for analyzing orientation data determines the average cosine of these angles for a set of cells; perfectly oriented cells with an angle of 0° yielded a cos of 1; a randomly oriented population of cells predicts a cos of 0 (Segall, 1993). For orientation of wild-type cells, 81 cells analyzed in three experiments gave an average cosine of 0.51. 50 H7 mutant cells were analyzed in three experiments with an average cosine of -0.04, as predicted for a mutant unable to orient towards the source of pheromone.

Western Blot Analysis of *Far1p*

Log phase cultures were grown in YEPD at 30°C and treated where indicated with α -factor at 1 μ g/ml for 1 h. Cells were then pelleted and extracts were prepared as described (Peter et al., 1993). Proteins were separated by SDS-PAGE and electroblotted to nitrocellulose (Schleicher and Schuell) using the Minigel system (BioRad Labs, Hercules, CA). Blots were probed with affinity-purified Far1 antibodies as described (Peter et al., 1993) and developed using epichemiluminescence (Amersham Corp., Arlington Heights, IL).

Cloning and Sequencing of *far1-s* Alleles

To isolate the mutant alleles, plasmid pSL2287 was first integrated into each *far1-s* mutant, as previously described (Guthrie and Fink, 1991). This

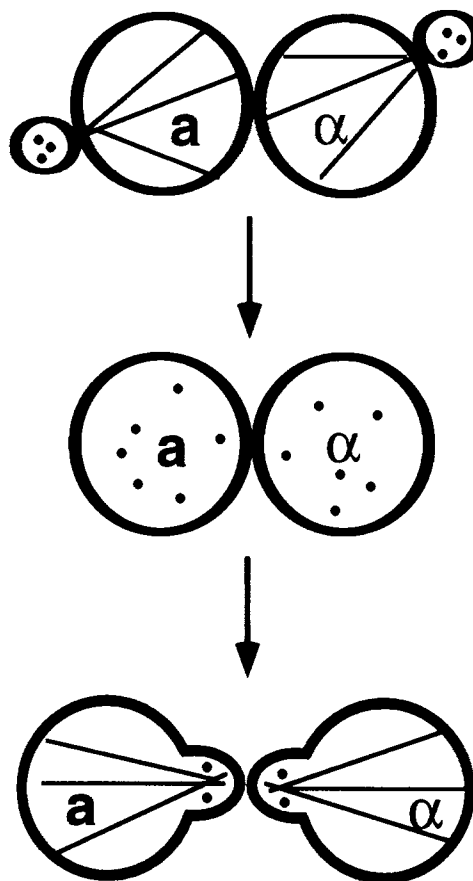


Figure 1. Orientation during mating. When two mating cells come in contact, they are each initially polarized towards the growing bud (top panel; Chant, 1994). The actin cables and patches are indicated in each mating partner. Before mating, each cell finishes budding and arrests in G1. The newly arrested cells lack polarization (middle panel; Madden and Snyder, 1992; Lew and Reed, 1993) and reorient their actin cytoskeleton and secretory apparatus towards the mating partner (bottom panel; Ford and Pringle, 1986).

plasmid (kindly provided by J. Horecka) carries sequences flanking *FAR1* but lacks most of the *FAR1* coding sequence. To recover the mutant alleles, chromosomal DNA was isolated (Nasmyth and Reed, 1980), digested with *Cla*I, religated, and transformed into DH5 α . Mutations responsible for the mating phenotype of *far1-s* were then mapped by further subcloning and complementation analysis. Fragments carrying the mutation were sequenced on both strands using a Sequenase kit as recommended by the manufacturer (United States Biochem. Corp., Cleveland, OH).

Determining the Bud Site in Zygotes

To determine the position of bud scars in zygotes, 10^7 **a** cells were stained with 4 μ g Calcofluor (Sigma) for 5 min at 30°C, washed twice with 50 ml YEPD, and mated with 10^7 α cells on filters for 3 h. Cells were fixed and the position of bud scars was quantitated as described (Madden and Snyder, 1992).

Results

Mating Defects of *far1-s* Cells

The *far1-s* mutants were isolated in a screen to identify mutants with mating defects that were not due to blocks in signal transduction (Chenevert et al., 1994). Mutations of

FAR1 were expected from this screen because it was known that certain *FAR1* mutants mate at reduced efficiency (Chang and Herskowitz, 1990; Chang, 1991). A centromeric plasmid carrying a wild-type copy of the *FAR1* gene complemented four mutants, B4, D1, G18, and H7, which were denoted the *far1-s* mutants (Chenevert et al., 1994).

Quantitative matings were used to characterize further the mating defect of each *far1-s* mutant (Table II). The *far1-s* mutants mated at reduced levels to a wild-type partner and exhibited a range of defects: G18 mated half as well as a wild-type **a** cell, while the mating efficiency of D1 was reduced nearly 100-fold.

One possible explanation for the mating defect of *FAR1* mutants is that Far1p is required to find the mating partner. A prediction for such an orientation mutant is that it should have a dramatic reduction in mating efficiency when both mating partners carry the same mutation, a “bilateral” mating defect. This enhanced defect is expected because mating between two such mutants would occur only when both partners orient towards each other by

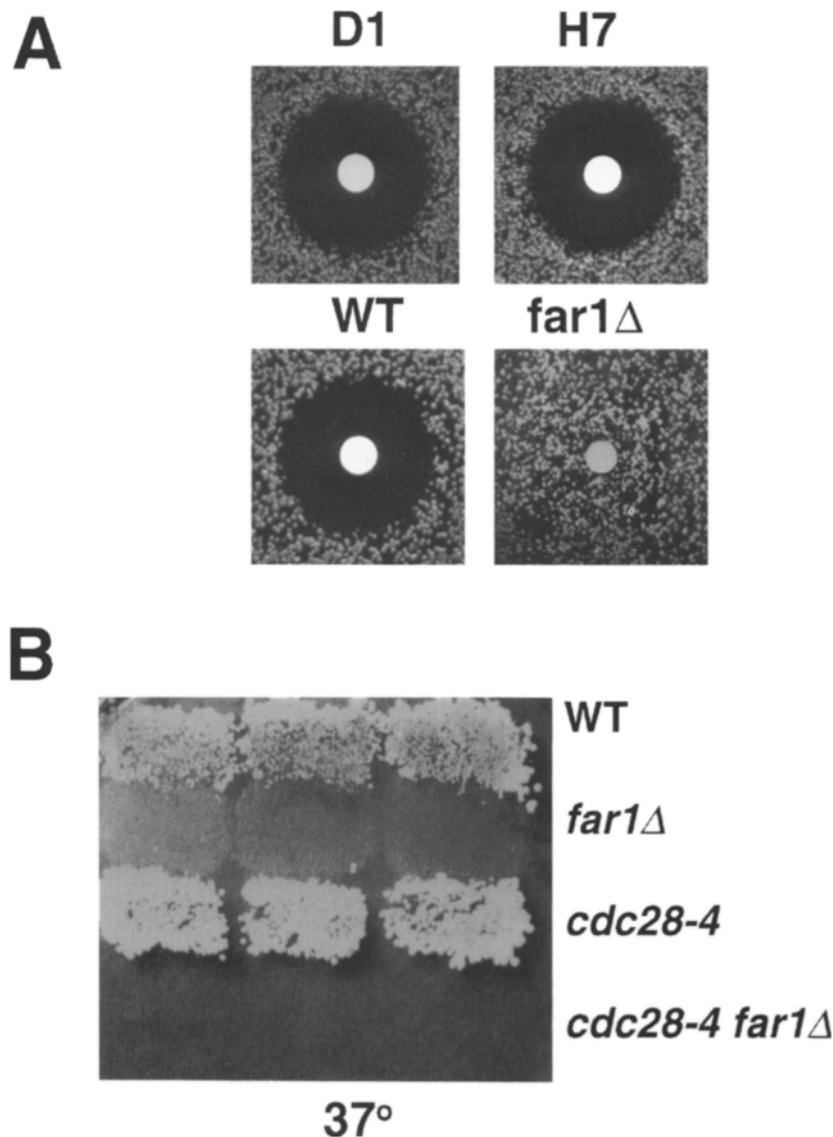


Figure 2. *FAR1* is required for a mating function independent of its role in cell cycle arrest. (A) *far1-s* mutants do not exhibit a defect in cell cycle arrest. Pheromone spotted on a filter disk arrests cell division of a wild-type strain near the disk. The size of the halos around the *far1-s* mutants D1 and H7 indicates that these cells arrest at the same concentration of pheromone as wild type. A *far1Δ* strain fails to arrest even at very high concentrations of pheromone adjacent to the disk. (B) Cells arrested at Start by a *cdc28-ts* mutation require *FAR1* for efficient mating. At 37°C, wild-type and *cdc28-4* strains mate well (lines 1 and 3). When *FAR1* is deleted, no mating is seen (line 2), even in the *cdc28-4* mutant (line 4). This strain arrests at Start due to its lack of Cdc28 activity, which indicates that *FAR1* is required for another function in addition to its role in cell cycle arrest during mating. Strains used were as follows (see Table I): **a** wild type (FC279), **a** *far1Δ* (YMP18), **a** *cdc28-4* (K1989), **a** *far1Δ cdc28-4* (YMP188), α wild type (IH1793).

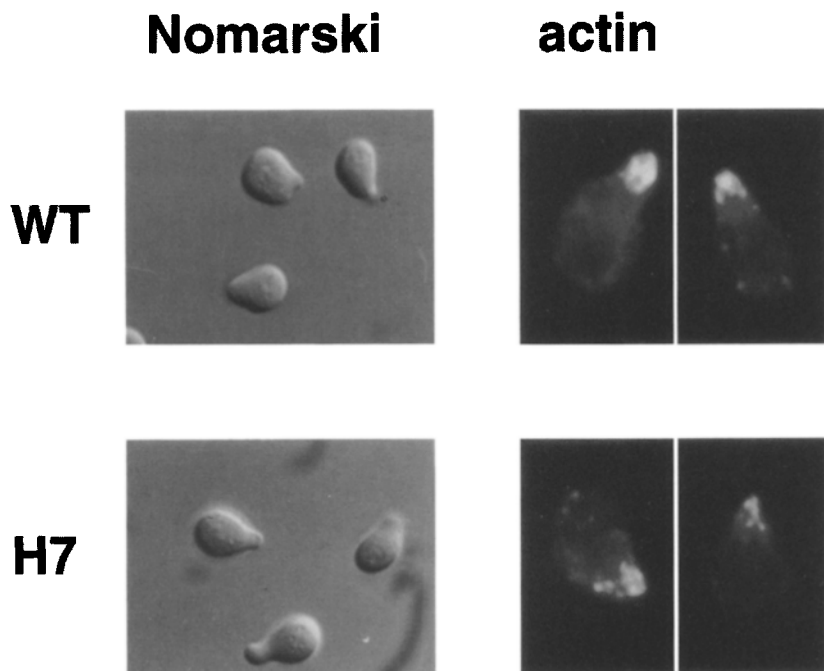


Figure 3. Polarization of a *far1-s* mutant in the presence of α -factor. The shmoo morphologies (*left*) of a *FAR1*⁺ (JC2-1B) and a *far1-s* strain H7 (NVY98) treated with α -factor for 2 h at 30°C are indistinguishable. Immunofluorescence (*right*) shows the actin distribution in *far1-s* and wild-type shmoos is directed towards the shmoo tip, as expected for cells which exhibit a normal shmoo morphology.

chance. Consistent with this hypothesis, the *far1-c* mutant (which carries a COOH-terminal truncation of *FAR1*) was previously found to be more defective in mating to another *far1-c* mutant than to wild-type cells (Chang, 1991).

The *far1-s* mutants also exhibited bilateral mating defects when mated to a *far1-c* strain (Table II). Wild-type cells had a tenfold decrease in mating frequency when mated to an α *far1-c* mutant. In contrast, the *far1-s* mutants all displayed a greater reduction in mating efficiency than wild-type cells. For example, the *far1-s* mutant H7 mated to wild type at a frequency of 3.6% but to *far1-c* at only 0.01%, almost a 300-fold decrease in mating efficiency. The other *far1-s* mutants displayed similar bilateral mating defects.

***FAR1* Plays a Role in Mating Independent of its Role in Cell Cycle Arrest**

The best characterized role of *FAR1* during mating is to inhibit cell cycle progression by functioning as a cyclin-dependent kinase inhibitor (CKI): Far1p binds to and inhibits the CDK complex of Cdc28p and Cln1p or Cln2p (Peter et al., 1993; Tyers and Futcher, 1993; Peter and Herskowitz, 1994). The *far1-s* mutants exhibited normal cell cycle arrest in response to α -factor (Fig. 2 A; Chang, 1991). This observation suggests that *FAR1* has a second function in mating which is not related to its role in cell cycle arrest.

We wanted to determine by another method if *FAR1* is required during mating for a function other than cell cycle arrest. For this purpose, we have used a temperature-sensitive *cdc28* mutant (*cdc28-4*) to arrest cells at Start and determined if *FAR1* was still required for efficient mating. A strain deleted for *FAR1* does not mate (Fig. 2 B); it cannot arrest the cell cycle and may be missing another function as well. When a *cdc28-4* strain is grown at 37°C, the cells arrest because Cdc28p is not present to drive the cells through the cell cycle; these cells are competent to mate (Fig. 2 B), as has been observed previously (Reid and

Hartwell, 1977). When *FAR1* is deleted in a *cdc28-4* strain, however, the cell loses its ability to mate at 37°C. This loss of mating cannot be due solely to *FAR1*'s function in cell cycle arrest, because the cells are arrested at Start via the *cdc28* mutation. We conclude that *FAR1* plays a role in mating independent of its role in cell cycle arrest. Furthermore, this function is likely independent of binding to CDC28/CLN1,2 because it is observed in the *cdc28-4* mutant under nonpermissive conditions.

Polarization of *far1-s* Cells

To address the basis of the mating defect of *far1-s* mutants, we determined whether two other responses to pheromone were affected in these mutants. Gene induction in response to pheromone was normal as determined by the production of pheromone (data not shown) and the induction of a pheromone-responsive reporter construct, *FUS1::lacZ* (see Materials and Methods). In addition, induction of *FAR1* was normal in the *far1-s* mutants, as indicated by Western blot analysis (Fig. 7 A).

The ability of *far1-s* mutants to polarize in response to pheromone was also characterized. The "shmoo" morphology of *far1-s* cells exposed to α -factor was indistinguishable from the morphology of wild-type cells (Fig. 3). Furthermore, the *far1-s* shmoos exhibited actin correctly polarized towards the shmoo tip. Taken together, these data indicate that their mating defect does not reflect inability to polarize in the presence of pheromone. Because *far1-s* mutants are able to polarize and arrest the cell cycle normally, we reasoned that they may be unable to polarize in the correct direction.

Pheromone Confusion Assay

The external signal which establishes the landmark for polarization is thought to be a gradient of pheromone created by the mating partner, with actin polymerization and

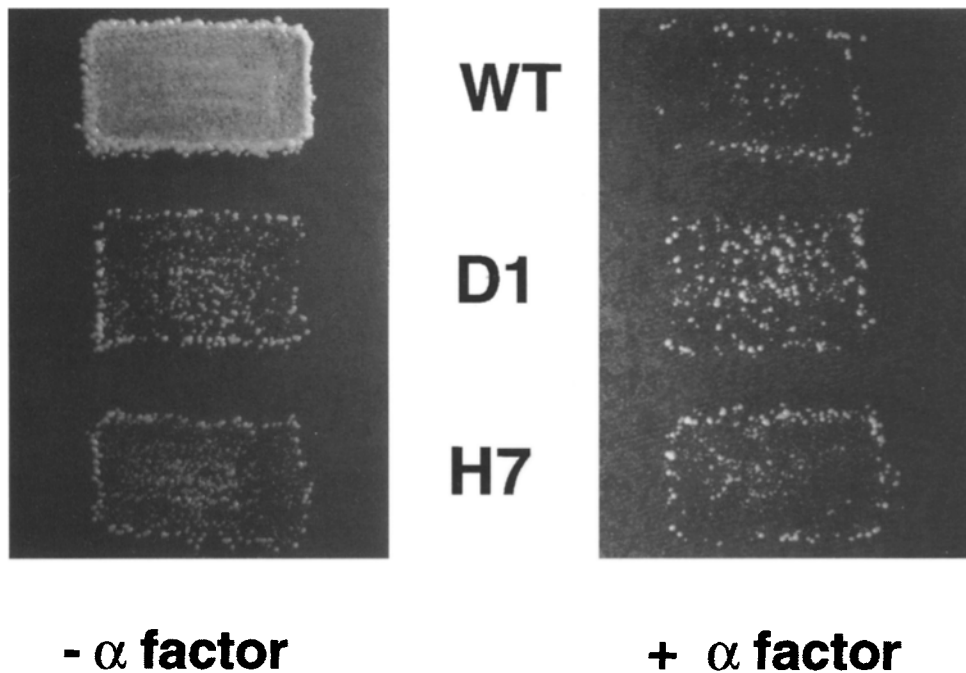


Figure 4. Patch mating of wild-type and *far1-s* mutants in the absence (left panel) and presence (right panel) of exogenous α -factor. The **a** strains were wild type (NVY180), D1 (NVY163), H7 (NVY164); the α strain was IH1793 (see Table I).

secretion directed to the area of highest pheromone concentration (Kurjan, 1985; Michaelis and Herskowitz, 1988; Jackson and Hartwell, 1990a,b; Segall, 1993). We have used two assays to determine if *far1-s* mutants respond to a gradient of pheromone. The first of these is the "pheromone confusion" assay, which uses a high concentration of exogenous pheromone to saturate cell surface receptors (Dorer et al., 1995). Two wild-type cells mated in the presence of exogenous α -factor exhibit inhibition of mating efficiency (Fig. 4; Table III; Marcus et al., 1991), which is thought to reflect their inability to sense the α -factor gradient normally experienced by the **a** cell: the **a** cell is confused about the location of the α cell, polarizes at random, and consequently mates at a reduced level. An orientation mutant, which is unable to respond to a pheromone gradient by polarizing towards its mating partner, should show no inhibition in mating efficiency in the presence of exogenous α -factor since the process disrupted in the pheromone confusion assay is the same as that defective in the mutant.

Wild-type **a** and α cells mated on a plate saturated with α -factor showed a dramatic inhibition of mating efficiency (Fig. 4). The *far1-s* mutants, D1 and H7, mated to wild-type α cells at low levels, but their mating efficiency was not inhibited in the presence of α -factor (Fig. 4). These observations suggested the *far1-s* mutants may be defective in responding to a gradient of pheromone.

To quantitate the pheromone confusion assay, known numbers of cells were mated in the presence and absence of pheromone. Because the *far1-s* mutants mate at much lower levels than wild type, it was important to compare the inhibition of mating efficiency rather than the absolute level of mating. A wild-type strain mated at 10.5% efficiency in the absence of α -factor and at 0.11% in the presence of α -factor, yielding a 96-fold inhibition of mating (Table III).

One expectation for the pheromone confusion assay is

that supersensitive strains should have a high degree of confusion because they already experience an increased level of pheromone, even in the absence of exogenous pheromone. Consistent with this prediction, the mating of a supersensitive *bar1-1* strain (which fails to degrade α -factor; Kurjan, 1992) was only inhibited 1.6-fold (Table III).

All of the *far1-s* mutants exhibited significant defects in the pheromone confusion assay (Table III), which suggests that they may be defective in polarizing towards a source of pheromone. Each of the *far1-s* mutants behaved quantitatively differently in this assay (Table III). Mutants H7 and D1 showed little inhibition of mating efficiency (2.8- and 1.6-fold, respectively). The two *far1-s* mutants which mate more efficiently, B4 and G18, exhibited greater inhibition in mating (5.6- and 15-fold). Two other mutants isolated in the same genetic screen (Chenevert et al., 1994), M4 and M5, which are *FAR1*⁺, exhibited low mating frequencies similar to the *far1-s* mutants but were inhibited for mating by pheromone (120- and 55-fold; Table III).

Table III. *far1-s* Mutants Are Defective in the Pheromone Confusion Assay

a cell*	Mating - α -factor	Mating + α -factor	Fold inhibition by pheromone
	%	%	
WT	10.5	.11	95.5
<i>bar1-1</i>	0.18	.11	1.6
B4	.67	.12	5.6
D1	.11	.07	1.6
G18	1.9	.12	15
H7	.17	.06	2.8
M4	1.2	.01	120
M5	1.1	.02	55

*The **a** strains used were as follows: WT (NVY180), *bar1-1* (JC2-1B), B4 (NVY162), D1 (NVY163), G18 (NVY165), H7 (NVY164), M4 (NVY145), and M5 (NVY146). The α strain was IH1793.

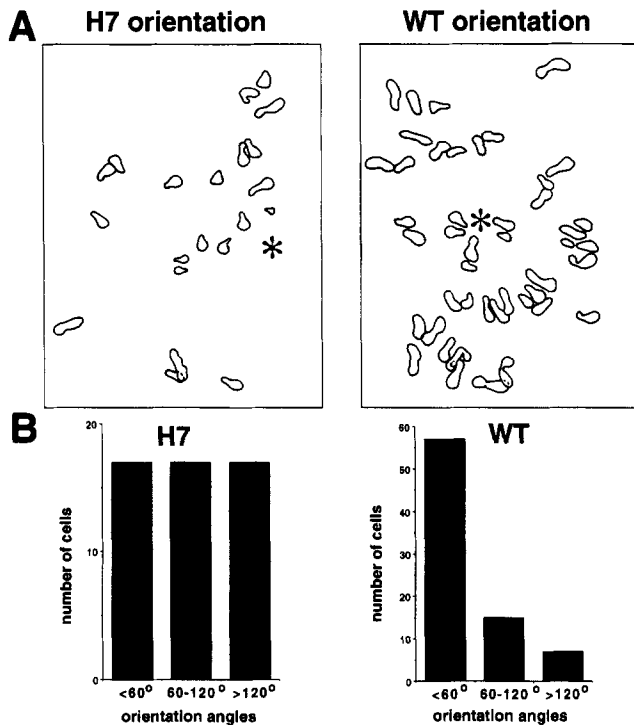


Figure 5. Orientation in a gradient of pheromone. (A) Traces of the H7 mutant (*a far1-s*) cells (NVY164) and wild-type *a* cells (NVY180) exposed to α -factor for 5 h. (B) Orientation was quantitated by measuring the angle between the direction of the needle (the source of pheromone, indicated by the asterisk) and the direction of the shmoo tip: strongly oriented cells grew towards the needle ($<60^\circ$), weakly oriented cells grew between $60-120^\circ$, and poorly oriented cells grew away from the needle ($>120^\circ$). Wild-type cells were generally oriented towards the needle ($>70\%$ oriented within 60°), whereas the *far1-s* mutant H7 was unoriented and exhibited a uniform distribution of cells over the three directions assayed.

The behavior of these mutants indicates that cells which are poor maters can be inhibited for mating by pheromone. These data support the hypothesis that *far1-s* mutants are defective in locating the mating partner.

Orientation Assay

To determine if *far1-s* mutants are able to polarize towards a source of pheromone, we used a direct microscope assay (Segall, 1993). A micropipette filled with α -factor was placed in a field of *a* cells, and the cells were followed over several hours. Wild-type *a* cells polarized their growth towards the source of α -factor, the tip of the needle (Fig. 5). To quantitate orientation, the angle between the direction of the pipette and the direction of cell growth was measured. Wild-type and H7 cells initiated projection formation with similar timing. After 5 h, the majority of wild-type cells (72%) had an orientation angle of less than 60° (Fig. 5 B). In contrast, the *far1-s* mutant H7 failed to orient towards the pipette (Fig. 5), as indicated by the even distribution of orientation angles across all possible angles (Fig. 5 B).

Cells orient best near their threshold of response to pheromone (Segall, 1993), that is, just above the concen-

tration of α -factor that arrests the cells in G1. At higher concentrations of α -factor, cells are presumed to experience saturating levels of pheromone over their surface, which obscures the position of the α -factor source and results in poorer orientation. It was therefore important to determine whether the concentration of α -factor used in these experiments was near threshold for each strain and whether the threshold for wild type and the H7 mutant was similar.

Three observations suggest that the wild-type and H7 cells experienced a concentration of α -factor near their threshold for response. First, the cell morphology near the α -factor source displayed by both strains was the "peanut" shape seen for cells near the threshold of response (Moore, 1983); pear-shaped shmoos with tight projections seen at higher concentrations of α -factor were rarely seen ($<10^{-4}$). Second, budding cells were seen at the periphery of the field of view for both strains, which indicates that they were below the threshold for response and supports the conclusion that the pheromone emerging from the needle was present as a gradient. Finally, the concentration of pheromone required to polarize wild-type and H7 cells in a homogeneous field was determined (data not shown; see Moore, 1983). H7 cells were approximately half as sensitive to pheromone as wild type. These data suggest that both wild-type and H7 cells experienced a concentration of pheromone near threshold for response, and that H7 is not less oriented simply because it is more sensitive to pheromone.

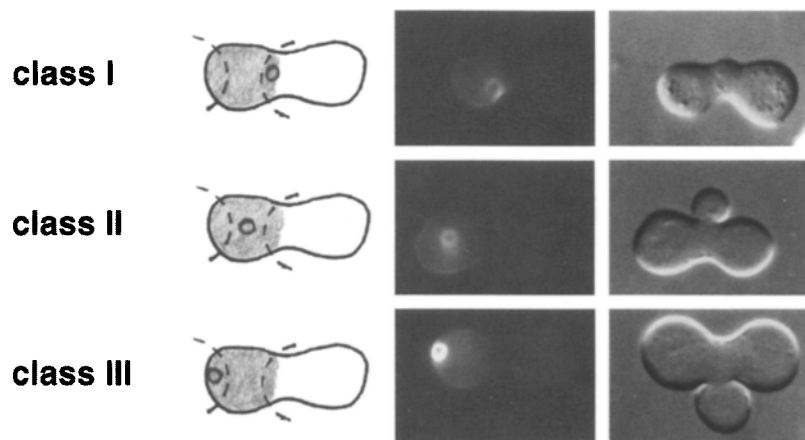
far1-s Mutants Polarize Towards the Incipient Bud Site

If *far1-s* mutants do not polarize towards their mating partner, then how do they choose a direction for polarization? One possibility is that *far1-s* cells polarize towards the other polarity landmark in the cell, the incipient bud site. To address this possibility, the position of bud scars relative to the position of the mating partner was determined for wild-type and *far1-s* zygotes. The bud scars of *a* cells were stained with Calcofluor, and these cells were then mated with wild-type α cells for 3 h. This staining protocol allowed the clear identification of the *a* cell partner, as it was much more brightly labeled with Calcofluor. In addition, it was possible to identify the border of the *a* cell, as areas of new growth (such as the conjugation bridge which forms between the two mating partners) are unlabeled.

As expected, wild-type zygotes had bud scars distributed across the surface of the *a* cell: these cells are able to polarize towards the mating partner irrespective of the position of the last bud (Fig. 6). To quantitate this observation, we used the method of Madden and Snyder (1992). Zygotes with one bud scar were counted, dividing the *a* cell into three regions: class I zygotes had their bud scar adjacent to the mating partner; class II zygotes had their bud scar in the middle of the cell; and class III zygotes had their bud scar at the end of the cell opposite the mating partner. Wild-type cells had the expected distribution among the three classes, with class II slightly larger (55%) than class I (20%) and class III (25%) because it includes the greatest surface area of the cell (Fig. 6 B).

far1-s mutants displayed a striking preference for class I

A



B

<u>a cell</u>	<u>class I</u>	<u>class II</u>	<u>class III</u>
WT	20	55	25
G18	34	51	15
B4	41	38	21
H7	58	29	13
D1	80	13	7

zygotes (Fig. 6 B). For example, the mutant D1 formed 80% class I zygotes and only 13 and 7% class II and class III zygotes, respectively (Fig. 6 B). *far1-s* mutant B4, which has a weaker mating defect than D1 (1.7 vs 0.83%, Table II), had more class II and class III zygotes than D1 (but fewer than wild type). Similarly, G18 has the weakest mating defect (33%) and the zygote class distribution most similar to wild type. These results indicate that the *far1-s* mutants polarize towards the bud site and are able to mate when that position is adjacent to the mating partner.

Molecular Alterations in *far1-s* Mutants

The molecular nature of the defect in the *far1-s* alleles was characterized. Far1p produced in each mutant was probed by Western blot in the absence and presence of α -factor (Fig. 7 A). Each mutant produced full-length Far1p except H7, which yielded a slightly truncated protein. Far1p is normally expressed at low levels in vegetative cells and is induced 4–5-fold upon pheromone treatment (Fig. 7 A; Chang and Herskowitz, 1992; Peter et al., 1993). All four *far1-s* mutants exhibited wild-type levels of expression, which suggests that their mating defects are not due to altered levels of protein. Finally, Far1p in all of the mutants appeared normally phosphorylated as judged by its characteristic mobility shift (Chang and Herskowitz, 1992).

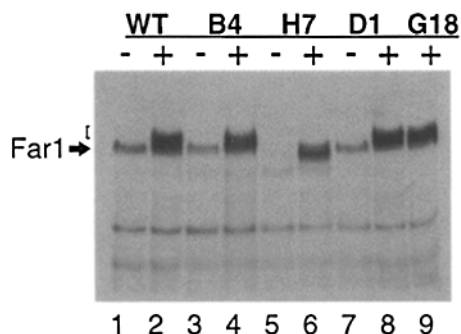
To characterize the nature of these mutations, the *far1-s* alleles were recovered and their sequences determined. Each *far1-s* allele carried a mutation(s) in *FAR1* (Fig. 7 B). It was previously known that the amino terminus of Far1p (amino acids 1–390) is sufficient for cell cycle arrest; trun-

cation of the carboxy terminus of Far1p does not allow efficient mating although it can promote cell cycle arrest (Chang and Herskowitz, 1990a). Similarly, three of the mutations, H7, G18, and D1, affected the carboxy terminus of Far1p, which supports the earlier conclusion that the COOH terminus is involved in orientation. The H7 mutant contained a nonsense mutation at position 756, which predicts a truncated protein as observed (Fig. 7 A). D1 carried two point mutations, both of which fall within the carboxy terminus and lead to amino acid changes at two positions: glycine 646 was changed to an aspartic acid and proline 671 was changed to a leucine. G18 also carried a point mutation in the COOH terminus which changed a glycine at position 650 to an arginine. In contrast, the B4 mutant contained a mutation in the LIM-like segment of the amino terminus, changing a conserved cysteine to tyrosine (Fig. 7 B). These results show that the *far1-s* mutants harbor mutations in *FAR1* and indicate that two regions of the protein are required for oriented polarization: the COOH terminus and the NH₂-terminal LIM-like domain.

far1-s Mutations Exhibit Intragenic Complementation

If Far1p has two functions, one involved in cell cycle arrest and the other in orientation towards the mating partner, it may be possible to coexpress mutant proteins defective in each of these functions and restore wild-type mating and cell cycle arrest. For this analysis, we have used two mutations of *FAR1*, *far1-60F3* and *far1s-D1*. The *far1-60F3* mutant carries a point mutation in the amino terminus which

A



B

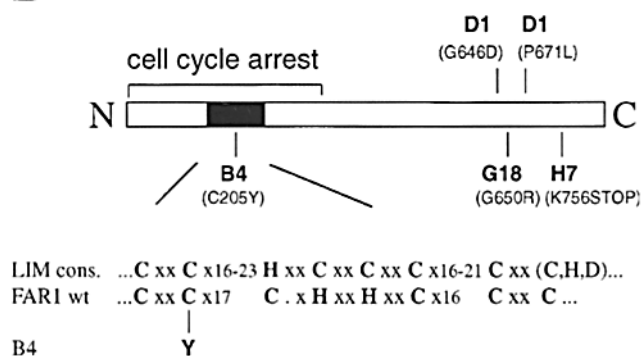


Figure 7. Molecular analysis of the *far1-s* mutants. (A) Whole-cell extracts prepared from either wild-type (lanes 1 and 2) or *far1-s* mutants (lanes 3–9) treated (indicated by “+”, lanes 2, 4, 6, 8, and 9) or not treated (indicated by “–”, lanes 1, 3, 5, and 7) with α -factor were analyzed for the expression of Far1 protein by immunoblotting. The arrow indicates the position of full-length, unphosphorylated Far1 protein; the bracket marks the position of phosphorylated forms of Far1p. (B) Schematic representation of the amino acid changes in the *far1-s* mutants. The bracket indicates the segment of *FAR1* which is both necessary and sufficient for cell cycle arrest (Peter et al., 1993); the black bar marks a segment with similarity to LIM domains. The homology between the LIM domain consensus sequence (Sanchez-Garcia and Rabbitts, 1994) and Far1p is indicated below. Strains were as follows (see Table 1): wild type (JC2-1B), B4 (NVY80), H7 (NVY98), D1 (NVY87), and G18 (NVY97).

does not allow cell cycle arrest but allows mating (Peter et al., 1993); when a plasmid carrying this mutation was transformed into a *far1* deletion strain, the cells did not arrest in G1 but mated with increased efficiency (Fig. 8 D). These cells did not mate at wild-type levels presumably because they were not all in G1, the mating-competent stage of the cell cycle (Reid and Hartwell, 1977). In contrast, the *far1s*-D1 mutant did not mate efficiently but exhibited cell cycle arrest (Fig. 8 B). Strikingly, cells that carry both of these mutant *far1* alleles arrested in G1 and mated as well as a wild-type strain (Fig. 8 C). Control strains (wild-type, Fig. 8 A, and *far1* Δ , Fig. 8 E) exhibited the expected behaviors. These data support the conclusion that Far1p has two separable, independent functions during mating.

Discussion

far1-s mutants exhibit the phenotype expected of a mutant defective in orienting towards the mating partner. These mutants correctly transduce the pheromone signal, as they exhibit normal cell cycle arrest, gene induction, and morphological changes in response to pheromone. Their defect in the pheromone confusion assay and their bilateral mating defect are consistent with the hypothesis that they are orientation mutants. However, it is the orientation assay which directly demonstrates that these mutants are unable to orient towards a source of pheromone. Instead of polarizing towards the mating partner, *far1-s* mutants appear to polarize towards the incipient bud site. Thus, *FAR1* is required for two functions during mating: cell cycle arrest and polarization towards the mating partner.

Polarization Towards the Mating Partner Is Required for Efficient Mating

Yeast polarization towards the mating partner has been observed for several decades. It is believed that this polarization directs the deposition of mating-specific proteins to their site of action, where the mating cell contacts its partner. However, it has not been directly demonstrated that polarization towards the mating partner is required for efficient mating. Since the only known defect of the *far1-s* mutants is in orientation, it seems reasonable to conclude that the mating defect of *far1-s* mutants is due to failure to locate the partner. Thus, polarization towards the mating partner appears critical for efficient mating.

FAR1 May Link the Membrane and the Cytoskeleton

How a pheromone gradient establishes a landmark for polarization remains obscure, as is the molecular nature of the landmark itself. The pheromone gradient probably induces a high local concentration of activated pheromone receptors on the cell surface next to the mating partner, resulting in local signaling activity. Which components of the pheromone signal transduction pathway are involved in mating site selection remain to be determined. Candidates include the receptor itself, the G protein, its unidentified effector, and several signaling proteins—*STE50* (Ramezani Rad et al., 1992), *STE20*, and *STE5*—whose precise functions are not clearly understood.

Budding yeast relies on polarity for two phases of its life cycle, budding, and mating. It appears that budding and mating use different molecules to specify the landmarks for polarization but use the same proteins to organize the cytoskeleton towards the landmark (Chenevert, 1994). During budding, the bud site selection proteins are thought to cooperate to build a landmark recognized by the polarity establishment proteins (Drubin, 1991; Madden et al., 1992; Chant, 1994). The incipient bud site is ignored during mating, and cells instead polarize towards the mating partner. It is not clear if the information at the bud site is destroyed or masked during mating or if the new signal at the mating site is simply more powerful than the bud site and can compete away the polarity establishment proteins.

far1-s mutants do not recognize the position of the mating partner and instead polarize towards the incipient bud site. This behavior is consistent with at least three possible

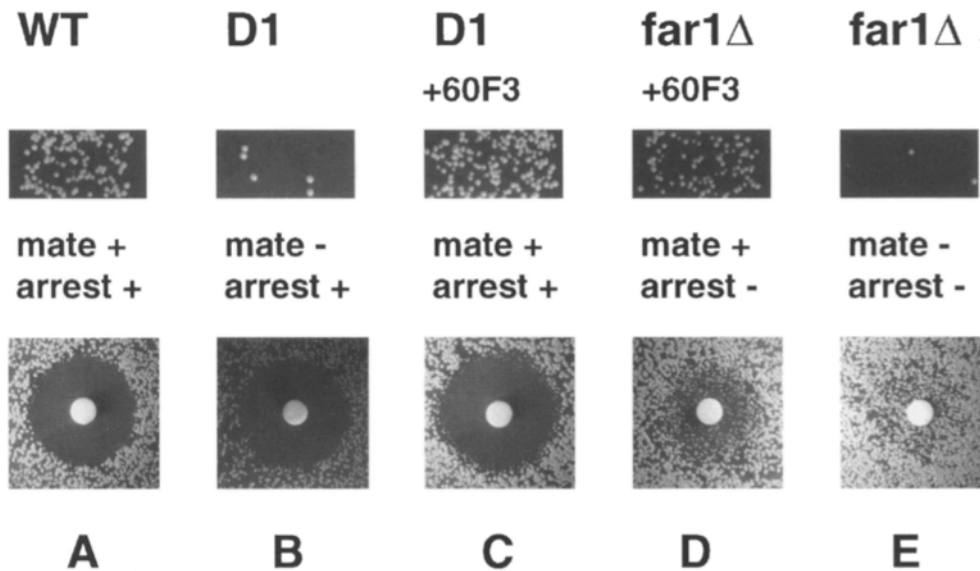


Figure 8. Intragenic complementation by two *far1* mutants, D1 and 60F3. (A) Wild-type cells exhibit normal mating (upper panel) and cell cycle arrest in response to pheromone (lower panel). (B) The *far1-s* mutant D1 does not mate efficiently but can cell cycle arrest. (C) The *FAR1* mutant 60F3 is defective in cell cycle arrest but competent for mating; when a low copy plasmid carrying this mutation is transformed into the D1 mutant, wild-type mating is restored. (D) A *far1Δ* strain producing the mutant *FAR1*-60F3 protein exhibits mating but only very poor cell cycle arrest; (E) the *far1Δ* strain cannot mate or cell cycle arrest. Strains were as follows (see Table I): wild type (JC2-1B), D1 (NVY80), and *far1Δ* (YMP18).

roles for Far1p during orientation. One possibility, by analogy with the Bud proteins, is that Far1p is involved directly in creating a landmark which the polarity establishment proteins can recognize, perhaps by binding to a component of the pheromone response pathway. Another possibility is that Far1p erases the polarity information at the bud site and frees the polarity establishment proteins to recognize the mating-site landmark. Finally, Far1p may function to strengthen interactions between the landmark and the polarity establishment proteins to allow maintenance of polarity. The existence of a mutation affecting the LIM-like domain (mutant B4) suggests that Far1p may interact with another protein in order to orient polarization (Schmeichel and Beckerle, 1994). Determining the localization of Far1p and identifying interacting proteins may shed light on how Far1p functions to link an external signal, a pheromone gradient, to the internal organization of the cytoskeleton.

Far1 Is a Bifunctional Protein with Two Distinct Roles during Mating

We have demonstrated here that Far1p is required for two different events during mating: cell cycle arrest and polarization towards the mating partner. Our observation that mutants defective separately in cell cycle arrest and polarization exhibit intragenic complementation illustrates that this protein has two independent functions during mating.

One striking feature of Far1p is its size: at 120 kD, it is significantly larger than other CKI proteins such as mammalian p16 and p21 (Sherr and Roberts, 1995). Far1p is larger than necessary to function as a CKI (Peter et al., 1993), its additional size being accounted for by its additional function, orienting polarization during mating. Although it was originally surprising to find that *FAR1* performs two different functions, both are involved in the

same differentiation process, mating. Joining of two activities in a single polypeptide may serve to coordinate different events during mating.

Far1p is a CKI that plays a key role in yeast cell differentiation during mating, controlling cell cycle progression and morphogenesis. Recent data indicate that other CKIs may also be bifunctional. For example, p21 exhibits PCNA-dependent inhibition of DNA polymerase δ (Flores-Rozas et al., 1994; Waga et al., 1994). Differentiation of various mammalian tissues is correlated with induction of the CKI p21 (Halevy et al., 1995; Parker et al., 1995; Skapek et al., 1995). The involvement of CKI proteins with multiple functions may thus be a general molecular strategy used to coordinate different events during differentiation.

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