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AFR1 Acts in Conjunction with the α-Factor Receptor To Promote Morphogenesis and Adaptation

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Mating pheromone receptors activate a G-protein signaling pathway that induces changes in transcription, cell division, and morphogenesis needed for the conjugation of *Saccharomyces cerevisiae*. The C terminus of the α-factor pheromone receptor functions in two complex processes, adaptation and morphogenesis. Adaptation to α-factor may occur through receptor desensitization, and α-factor-induced morphogenesis forms the conjugation bridge between mating cells. A plasmid overexpression strategy was used to isolate a new gene, *AFR1*, which acts together with the receptor C terminus to promote adaptation. The expression of *AFR1* was highly induced by α-factor. Unexpectedly, cells lacking *AFR1* showed a defect in α-factor-stimulated morphogenesis that was similar to the morphogenesis defect observed in cells producing C-terminally truncated α-factor receptors. In contrast, *AFR1* overexpression resulted in longer projections of morphogenesis, which suggests that this gene may directly stimulate morphogenesis. These results indicate that *AFR1* encodes a developmentally regulated function that coordinates both the regulation of receptor signaling and the induction of morphogenesis during conjugation.

During yeast conjugation, haploid cells of mating types **a** and α signal each other with polypeptide pheromones to induce mating functions that lead to the formation of a diploid zygote (39, 41). The **a** or α mating type is determined by the *MAT* locus (30). *MAT***a** cells secrete **a**-factor pheromone, which activates a receptor on the surface of *MAT*α cells; *MAT*α cells secrete α-factor pheromone, which activates a receptor on the surface of *MAT***a** cells. Pheromone signaling induces the wide range of physiological responses needed for mating, including transcriptional activation of mating genes (20), arrest of cell division in G_1 to synchronize cell cycles, and morphogenesis to facilitate the fusion of mating cells (21, 47). Once cell fusion occurs, the nuclei fuse to complete the formation of a diploid zygote.

Mating pheromones activate a signal transduction pathway in the yeast *Saccharomyces cerevisiae* that shows a remarkable similarity to the signaling pathways used to sense a wide range of stimuli in multicellular organisms, including light, odor, taste, and hormones (19, 29). The pheromone receptors for α-factor, encoded by *STE2* (4, 52), and for **a**-factor, encoded by *STE3* (25, 52), belong to a large family of receptors that are distinguished by possessing seven transmembrane domains. The best-studied receptors in this family are the β-adrenergic receptor and rhodopsin (19). Receptors in this family transduce their signal by stimulating the α subunit of a heterotrimeric G protein to bind GTP. The G_α subunit then dissociates from the $G_{\beta\gamma}$ subunit; either G_α or $G_{\beta\gamma}$ goes on to activate an effector (29). The pheromone-responsive G_α subunit is encoded by *GPA1* (*SCG1*) (17, 50); G_β and G_γ are encoded by *STE4* and *STE18* (68). In yeast cells, free $G_{\beta\gamma}$ transmits the pheromone signal by activating an unknown effector (3, 13, 52, 70). Subsequent steps in the signaling pathway require the action of *STE5*, *STE50*, and five protein kinases (6, 22, 43, 55, 65). Phosphorylation of STE12, a pheromone-responsive transcription factor, stimulates the transcription of the genes that function in mating (63). Pheromone signaling stimulates cell division arrest through inactivation of the *CDC28* protein kinase and associated cyclin proteins by transcriptional and posttranscriptional mechanisms (9, 22).

Pheromones also signal spatial orientation, which enables yeast cells to identify an appropriate mating partner with high efficiency and fidelity (34). Since yeast cells are nonmotile, intracellular reorganization occurs to polarize cell growth toward an appropriate partner cell (2, 5). Polarized cell growth forms projections of morphogenesis that allow the mating cells to make contact and form a conjugation bridge that fuses the cells together. The mechanism that stimulates cell polarization is unclear, but pheromone receptor signaling plays a key role in this process through G-protein-dependent and G-protein-independent signal pathways (35). The G-protein signal pathway must be properly regulated because adaptation mutations that result in increased sensitivity to pheromone are defective in mating partner discrimination (34). Polarized cell growth is mediated by cytoskeletal elements, such as actin, and by many of the same genes that mediate polarized morphogenesis during bud formation (21, 47). A similar mechanism may occur in leukocytes, since activation of the fMet-Leu-Phe receptor stimulates G-protein signaling that promotes actin reorganization and cell polarization (16, 66).

Pheromone signal transduction is regulated by several distinct adaptation mechanisms that are needed for efficient mating (34). For example, α-factor signaling stimulates *MAT***a** cells to increase expression of *BAR1* (*SST1*), which encodes a secreted protease that specifically degrades α-factor in the medium (46). The ligand-bound α-factor receptors are down-regulated by endocytosis (38, 72). Pheromone-induced expression of *SST2* stimulates a mechanism that acts intracellularly in both cell types to confer adaptation (18). The GTP-bound G_α subunit has been proposed to stimulate a unique adaptation pathway (32), but this is controversial (42). The receptor (40, 57) and G_β subunit (12) are also targets for regulatory mechanisms that confer adap-

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TABLE 1. Strains used

Strain	Genotype	Source
DJ211-5-3	*MATa ade2-1° cry1 his4-580ᴬ leu2 lys2° trp1ᴬ tyr1° ura3 SUP4-3ᵗˢ bar1-1*	D. Jenness
DJ147-1-2	*MATa ade2-1° cry1 his4-580ᴬ leu2 lys2° trp1ᴬ ura3 SUP4-3ᵗˢ*	D. Jenness
JK7441-4-2	*MATa ade2-1° cry1 his4-580ᴬ leu2 lys2° trp1ᴬ ura3 SUP4-3ᵗˢ bar1-1 ste2-T326*	J. Konopka
JK7440-1	*MATa ade2-1° cry1 his4-580ᴬ leu2 lys2° trp1ᴬ ura3 SUP4-3ᵗˢ ste2-T326*	J. Konopka
JK27-1	*MATa ade2-1° cry1 his4-580ᴬ leu2 lys2° trp1ᴬ ura3 SUP4-3ᵗˢ afr::URA3*	J. Konopka
JK33-9-1	*MATα ade6 leu2 lys2° trp1ᴬ tyr1° ura3 SUP4-3ᵗˢ*	J. Konopka
JK33-6-1	*MATα ade6 his4-580ᴬ leu2 lys2° trp1ᴬ tyr1° ura3 SUP4-3ᵗˢ afr1::URA3*	J. Konopka
JK33-5-2	*MATα cry1 his4-580ᴬ leu2 lys2° trp1ᴬ ura3 SUP4-3ᵗˢ afr1::URA3*	J. Konopka
JK33-13-4	*MATα cry1 his3 his4-580ᴬ leu2 lys2° trp1ᴬ ura3 SUP4-3ᵗˢ afr1::URA3*	J. Konopka
7417-5-4	*MATα ade2-1° cry1 his4-580ᴬ leu2 lys2° trp1ᴬ ura3 SUP4-3ᵗˢ*	J. Konopka
SF301	*MATα ade6 his4 leu2 lys2 trp1 ura3*	S. Fields
SF496	*MATa ade1 cdc34ᵗˢ his3 leu2 ura3*	S. Fields
mfα1 mfα2	*MATα ade2-1 can1 trp1-1 leu2-3,112 ura3-1 his3-11,15 mfα1::LEU2C mfα2::URA3D*	J. Kurjan
JK7423-2-2	*MATα ade2-1° his4-580ᴬ leu2 lys2° trp1ᴬ tyr1° ura3 SUP4-3ᵗˢ bar1-1 ste2::LEU2*	J. Konopka
7646-3-1	*MATa cry1 his3 leu2-3,112 lys2° trp1ᴬ tyr1° ura3-52 scg1 (gpa1)::LEU2 ste2::LEU2 ste5-3ᵗˢ SUP4-3ᵗˢ*	C. Jackson
708-1-1	*MATa ade2-1° ade3 cry1 his4-580ᴬ leu2 lys2° trp1ᴬ ura3 SUP4-3ᵗˢ ste5-3ᵗˢ*	D. Jenness
DJ777-13-1	*MATa ade2-1° cry1 his4-580ᴬ leu2 lys2° trp1ᴬ ura3 SUP4-3ᵗˢ ste2::LEU2 STE4ᴴᴾᴸ ste5-3ᵗˢ*	D. Jenness
RS893	*ade2 his3 leu2 trp1 ura3 HML::TRP1*	R. Sternglanz
JK35	*MATa/MATα ade2-1°/ade2-1° cry1/cry1 his4-580ᴬ/his4-580ᴬ leu2/leu2 lys2°/lys2° trp1ᴬ/trp1ᴬ TYR1/tyr1° ura3/ura3 SUP4-3ᵗˢ/SUP4-3ᵗˢ BAR1/bar1*	J. Konopka

tation. These mechanisms also enable cells that fail to mate to adapt to the pheromone signal and resume the normal division cycle.

The analysis of α-factor receptor function by mutagenesis has demonstrated that the N-terminal domain mediates ligand binding and signaling and that the cytoplasmic C terminus acts as a regulatory domain that is needed for efficient mating. Truncation of the cytoplasmic C terminus causes a defect in adaptation (40, 57) and a defect in α-factor-induced morphogenesis (40). The cytoplasmic C-terminal sequences of the related receptors, rhodopsin and the β-adrenergic receptor, are regulatory domains that mediate receptor desensitization. Receptor desensitization results from phosphorylation of the C-terminal sequences by a receptor-specific protein kinase and binding of an arrestin protein which blocks further G-protein activation (19). The genes that mediate α-factor receptor regulation have not been identified. Therefore, a plasmid overexpression strategy was used to identify a new gene, *AFR1*, that acts in conjunction with the α-factor receptor C terminus to negatively regulate pheromone signaling. The rationale of this approach was based on the previous demonstration that high-copy-number plasmid overexpression of adaptation genes, such as *SST2*, negatively regulates signaling and confers resistance to pheromone-induced cell division arrest (17, 18). Surprisingly, *AFR1* was also found to act in conjunction with the receptor C terminus to promote morphogenesis in response to α-factor.

MATERIALS AND METHODS

Strains and media. The yeast strains used in this study are described in Table 1. Cells were grown in media described by Sherman (60). Plasmid-containing cells were grown in synthetic medium containing adenine and amino acid additives but lacking uracil to select for plasmid maintenance. Plasmids were transformed into yeast strains by using lithium acetate (33).

Isolation of plasmids that confer α-factor resistance. Yeast strain DJ211-5-3 was transformed with a genomic plasmid library in vector YEp24 (7). The minimum concentration of α-factor (Sigma Chemical Co.) required to maintain the arrest of wild-type cells was 10^{-9} M. Therefore, transformants were replica plated to agar plates containing 3-fold-higher (3×10^{-9} M) and 10-fold-higher (10^{-8} M) concentrations of α-factor and then incubated at 30°C. One hundred five α-factor-resistant transformants were identified after over 5,000 independent transformants were screened. The candidates were then tested for the ability to produce pheromone in order to eliminate plasmids that interfere with normal *MAT***a** cell type regulation. Ninety-five candidates showed normal cell type regulation, since they produced **a**-factor but not α-factor. These candidates also did not produce the α-factor-degrading protease (BAR1 [46]), which may have caused resistance by degrading α-factor in the medium. Plasmids were recovered from these strains and transformed into *Escherichia coli*. After purification from *E. coli*, 50 plasmids were able to confer α-factor resistance to a fresh culture of the yeast strain DJ211-5-3. Four plasmids were eliminated from the pool because they derepressed the expression of a *TRP1* gene integrated at *HML* (strain RS893), which indicates that they cause α-factor resistance by derepressing *MAT*α information at *HML*. Obvious duplicate plasmids were eliminated by comparing the restriction fragments generated after digestion with *Eco*RI and *Hin*dIII on agarose gels. Comparison of restriction fragments also identified *SST2* four times and *GPA1* two times; these known genes were eliminated from the pool. *AFR1* was isolated four times. After these steps, 25 apparently distinct plasmids were identified that ranged in ability to confer α-factor resistance from threefold to those that were essentially resistant to α-factor.

DNA sequence analysis. DNA sequence analysis of both strands of *AFR1* was facilitated by the use of the modified transposon Tn*10*-LUK (31). The *AFR1* gene was initially localized on plasmid pAFR1 by identifying the sites of Tn*10*-LUK insertions that inactivated the α-factor resistance function. pAFR1 plasmids carrying Tn*10*-LUK insertions were then used as templates for DNA sequence analysis using the dideoxy-chain termination method as modified for use with double-stranded plasmids and [α-^{35}S]ATP (NEN/DuPont, Boston, Mass.). Universal *lacZ* primer (−40) was

hybridized to *lacZ* sequences within Tn*10*-LUK which are adjacent to the terminal repeat sequence, and then DNA sequence reactions were performed by using a Sequenase kit as instructed by the manufacturer (United States Biochemical). This permitted the identification of DNA sequences adjacent to the Tn*10*-LUK insertion. Approximately 80% of the reported sequence was determined in this manner, and then oligonucleotides were synthesized and used as primers to complete the remaining sequence analysis. The open reading frame was identified, and structural predictions were made for the AFR1 protein by using the Genetics Computer Group program (15). The DNA sequence was compared with sequences in the GenBank and EMBL data bases, and the predicted AFR1 protein sequence was compared with sequences in the GenPept, Swiss-Prot and PIR protein data bases, by using the BLAST program (1) (calculations were done through the National Center for Biotechnology).

Construction of modified *AFR1* genes. To construct a deletion allele of *AFR1*, the *Bam*HI-*Sal*I fragment of pAFR1-Tn10-9 was subcloned into the Bluescript plasmid (Stratagene). In pAFR1-Tn10-9, the *URA3* gene is inserted into the *AFR1* gene as part of a Tn*10*-LUK transposon (31) inserted at base 877 of the sequence. This plasmid was digested with *Eco*RI and was then allowed to self-ligate, resulting in the loss of a 3-kb portion of Tn*10*-LUK and nearly all of the *AFR1* coding sequence. Only 50 of the C-terminal codons remained. Thus, this plasmid (pJK38) contained the *URA3* gene flanked by the 5′ and 3′ sequences of the *AFR1* gene to generate the *afr1*::*URA3* allele. The *afr1*::*URA3* deletion allele was used to replace the endogenous wild-type *AFR1* allele in yeast cells by one-step recombination (59). The *AFR1-lacZ* fusion gene was constructed by inserting a 3-kb *Eco*RI fragment, containing the 5′ sequences through base 2143 of *AFR1*, into YEplac195 (24). The *Bam*HI site in the polylinker region was filled in with Klenow fragment of DNA polymerase to create a translational fusion when the *lacZ* gene was inserted. An *Xba*I fragment containing the *E. coli lacZ* gene, derived from pSGMU31 (23), was then inserted into the *Xba*I site in the polylinker to complete the construction of the *AFR1-lacZ* fusion gene (pJK33).

Chromosomal map position of *AFR1*. The *AFR1* gene was localized to chromosome IV by hybridization of an *AFR1* probe to a blot containing electrophoretically separated yeast chromosomes (Clontech, Palo Alto, Calif.). A 3.2-kb *Eco*RI fragment containing *AFR1* was labeled for use as a probe by incorporation of [α-^{32}P]dATP, using random primers. The map position was further defined by hybridizing the *AFR1* probe DNA to a panel of phage λ clones that covers 82% of the yeast genome (58). Specific hybridization to λ clone 4012 demonstrated that *AFR1* maps between *STE5* and *CDC34*. These results were confirmed by meiotic mapping. *AFR1* and *CDC34* were determined to be about 8.6 centimorgans (cM) apart by tetrad analysis of a cross between JK33-5-2 (*MAT*α *afr1*::*URA3 CDC34 ura3*) and SF496 (*MAT***a** *AFR1 cdc34*^{ts} *ura3*), yielding 24 parental ditypes:0 nonparental ditypes:5 tetratypes. *AFR1* and *STE5* were determined to be about 13.2 cM apart by tetrad analysis of a cross between JK33-13-4 (*MAT*α *afr1*::*URA3 STE5 ura3*) and 708-1-1 (*MAT***a** *AFR1 ste5*^{ts} *ura3*), which yielded 14 parental ditypes:0 nonparental ditypes:5 tetratypes. The complete genotypes of the strains used for meiotic mapping are listed in Table 1.

Pheromone response assays. Halo assays for α-factor-induced cell division arrest were performed by spreading approximately 10^5 stationary-phase cells from an overnight culture on the surface of an agar plate. α-Factor was added to sterile filter disks (Difco, Detroit, Mich.), which were then applied to the surface of the agar plate and incubated at 30°C for 2 days. All other assays were performed with mid-logarithmic-phase cells. Projection formation was analyzed with *bar1 MAT***a** cells in YPD medium treated with α-factor as described by Moore (51). The altered morphology of α-factor-treated *afr1*::*URA3* was observed in independent isolates and cosegregrated with *afr1*::*URA3* after meiosis. Cells were photographed with Kodak TMAX film, using an Olympus BH2 microscope equipped with differential interference (Nomarski) optics. Pheromone induction of gene expression was analyzed in logarithmic-phase cells carrying an *afr1-lacZ* (pJK32) or *fus1-lacZ* (pSL1580 [26]) plasmid. The cells were permeabilized with chloroform and sodium dodecyl sulfate, and then β-galactosidase assays were performed at 30°C as previously described (49). All assays were performed in duplicate, and the values reported represent the averages of two independent experiments. The standard deviation was usually less than 10% within a single experiment but was more variable when independent experiments were compared. Cell-free supernatant from *MAT***a** strain DJ147-1-2 was concentrated by using a YM-30 membrane (Amicon Corp., Danvers, Mass.) as previously described (48) and used as a source of **a**-factor to induce *MAT*α cells.

Mating assays. For standard mating tests, 1.5×10^6 cells of each parent were collected on a Millipore filter, and then zygote production was analyzed by resuspending cells off the filter and plating dilutions on plates that select for the growth of diploid cells as previously described (28, 40). Mating partner discrimination assays to detect mismating were performed by using 10^5 *MAT***a** test cells mixed with 2.5×10^6 cells of pheromone-producing *MAT*α strain SF301 and 2.5×10^6 cells of nonproducing strain *mf*α*1 mf*α*2*. The diploids formed with each *MAT*α strain were quantitated as previously described (34). The values reported are the averages of two independent experiments.

Nucleotide sequence accession number. The *AFR1* sequence was assigned GenBank accession number L21702.

RESULTS

Isolation of multicopy plasmids that confer resistance to α-factor. Genetic analysis indicates that the C terminus of the α-factor receptor mediates adaptation by a mechanism that is independent of the known adaptation genes (40, 57). Since the adaptation genes were identified primarily because their mutation results in increased sensitivity to pheromone (8, 64), adaptation genes that are redundant in function or are essential for viability cannot be identified in this manner. These limitations were circumvented by using an alternative approach, a plasmid overexpression strategy, to identify adaptation genes that act on the receptor. This approach is based on the previous observation that overexpression of adaptation genes makes cells more resistant to α-factor-induced cell division arrest (17, 18). Overexpression of genes in yeast cells is readily achieved by cloning genes into a high-copy-number plasmid vector. Therefore, candidate adaptation genes were identified by introducing an *S. cerevisiae* genomic library on the multicopy plasmid vector YEp24 (7) into an α-pheromone-responsive *MAT***a** strain (DJ211-5-3). Pheromone-resistant transformants were identified by replica plating cells to pheromone-containing plates as described in Materials and Methods.

From a screen of over 5,000 independent transformants, 105 candidates that exhibited an increase in resistance to

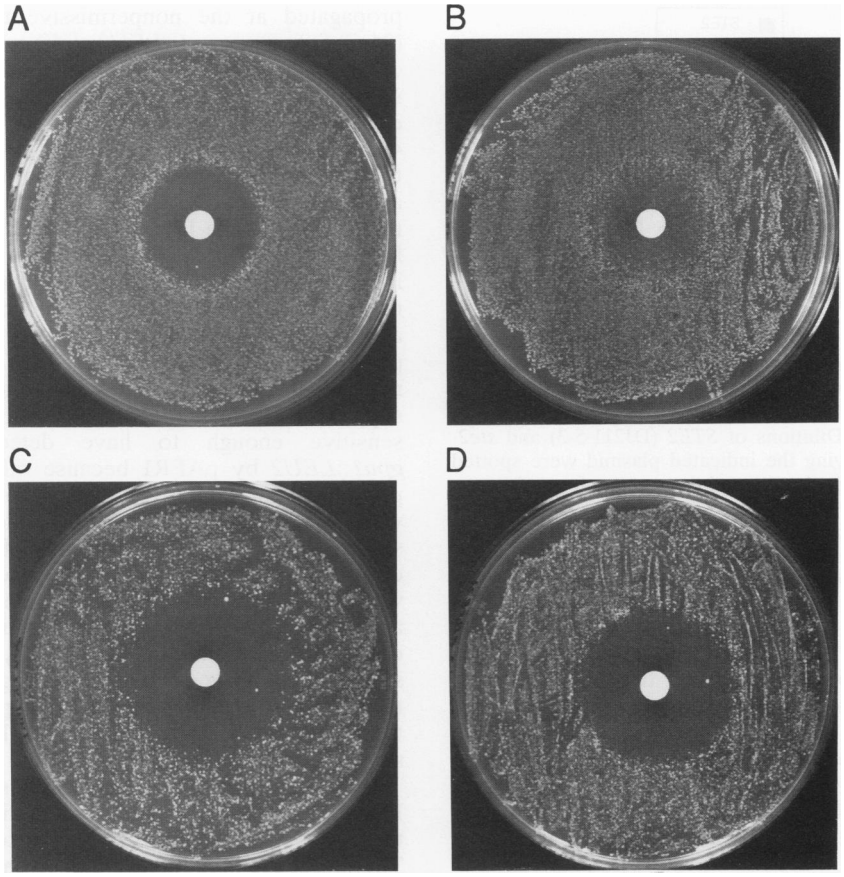

FIG. 1. Halo assays demonstrate the effects of pAFR1 on α-factor sensitivity. Comparison of the sensitivities of *STE2*$^+$ cells (strain DJ211-5-3) carrying plasmid vector YEp24 (A) and pAFR1 (B) and *ste2-T326* cells (strain 7441-4-2) carrying YEp24 (C) and pAFR1 (D). Cells were spread as a lawn on agar plates, and then a filter disk containing 1 μg of α-factor was added to the plate to induce division arrest. After a 48-h incubation, a zone of growth inhibition surrounding the filter disk was observed that reflects the sensitivity to α-factor.

α-factor ranging from about threefold to complete resistance were identified. Since a genomic mutation in one of the signal transduction genes could also cause cells to become resistant to α-factor, it was important to demonstrate that the resistance was plasmid linked. Therefore, the plasmids were shuttled into *E. coli* and then retested for the ability to confer α-factor resistance when transformed into a fresh culture of the host yeast strain. After elimination of false positives, the *SST2* and *GPA1* adaptation genes, and obvious duplicates as described in Materials and Methods, 25 plasmids remained as candidates for new adaptation genes and were designated pAFR (plasmid conferring α-factor resistance).

pAFR1 requires the receptor C terminus in order to confer α-factor resistance. The genes carried on the pAFR plasmids could act by antagonizing any stage of the pheromone signal pathway that leads to cell division arrest. To identify adaptation genes that act on the α-factor receptor, the pAFR plasmids were analyzed in a *ste2-T326* strain that produces C-terminally truncated α-factor receptors (40). The rationale of this experiment is that genes that act on the C terminus of the α-factor receptor are expected to have no effect in the *ste2-T326* strain because the C-terminal domain is not present. Plasmid pAFR1 was unique in that it failed to confer the same relative degree of resistance in the *ste2-T326* strain as seen in an *STE2* strain. The other 24 pAFR plasmids conferred resistance in both strains. The relative resistance to α-factor-induced cell division arrest was examined by using a halo assay for cell division arrest (Fig. 1). This assay measures the halo (zone of growth inhibition) that forms on a lawn of cells due to α-factor-induced cell division arrest. As expected, *STE2* and *ste2-T326* strains carrying the control vector YEp24 produced a distinct halo (Fig. 1A and C). The size of the halo is larger in the *ste2-T326* strain because of its 10-fold-increased sensitivity to α-factor. In contrast, *STE2* cells carrying pAFR1 do not form a distinct halo (Fig. 1B). This indicates that pAFR1 causes cells to become strongly resistant to α-factor-induced cell division arrest. However, pAFR1 had only a weak effect in the *ste2-T326* strain and did not confer strong α-factor resistance, since a distinct halo of cell division arrest was observed (Fig. 1D).

Cells carrying pAFR1 were heterogeneous in their ability to adapt to α-factor, which made α-factor resistance difficult to quantify by the halo assay. This heterogeneity is expected since the number of plasmid copies each cell carries is variable because of the unequal partitioning of the YEp24 plasmid vector at mitosis. Therefore, the relative degree of α-factor resistance was determined by plating dilutions of cells on agar plates containing various concentrations of α-factor. At least 10% of *STE2* cells carrying pAFR1 were resistant to the highest concentration of α-factor tested (3×10^{-7} M), which corresponds to a saturating dose of α-factor (Fig. 2). In contrast, *ste2-T326* cells carrying pAFR1 showed essentially the same degree of α-factor resistance in this

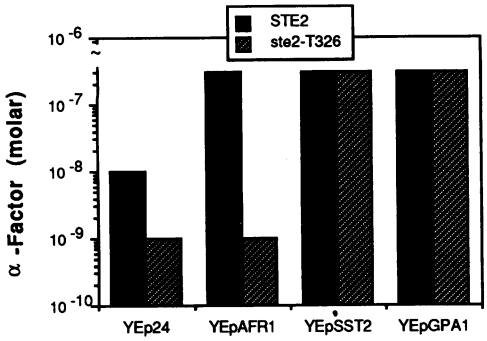

FIG. 2. Colony formation in the presence of α-factor. The effects of multicopy plasmids YEp24, YEpAFR1, YEpSST2, and YEpGPA1 on the ability of cells to form colonies in the presence of α-factor were examined. Dilutions of *STE2* (DJ211-5-3) and *ste2-T326* (7441-4-2) cells carrying the indicated plasmid were spotted onto agar plates containing α-factor, and then colony formation was observed after 4 days at 30°C. The highest concentration of α-factor at which at least 10% of the cells can form a colony is shown. The highest concentration of α-factor tested was 3×10^{-7} M since it corresponds to an essentially saturating dose of α-factor.

assay as do *ste2-T326* cells carrying the YEp24 vector plasmid. This differential ability to confer α-factor resistance was specific for pAFR1; overexpression of adaptation gene *SST2* or *GPA1* conferred a high degree of α-factor resistance in both the *STE2* and *ste2-T326* strains (Fig. 2). These results demonstrate that pAFR1 is unique in that it acts together with the receptor C terminus in order to confer α-factor resistance.

The failure of pAFR1 to function in the *ste2-T326* strain was not just an indirect consequence of the 10-fold-elevated α-factor sensitivity of this strain. pAFR1 conferred α-factor resistance to the following strains that show comparable levels of α-factor supersensitivity: $bar1^-$ (46), $gpa1^{Ser\text{-}302}$ (33), and $ste4^{\Delta 310\text{-}346}$ (12) (data not shown). In a 100-fold-supersensitive $sst2^-$ strain (18), however, pAFR1 had only weak effects and overexpression of *GPA1* showed 10-fold-reduced ability to confer α-factor resistance. Although this finding could suggest a specific genetic interaction, it also seems likely that these plasmids are simply unable to counteract the extreme α-factor supersensitivity caused by the $sst2^-$ mutation. $sst2^-$ mutants are defective even in adapting to a short pulse of α-factor (8, 40). In addition, *SST2* acts by a mechanism that is independent of the α-factor receptor C terminus since YEpSST2 conferred increased resistance in a *ste2-T326* strain (Fig. 2). Furthermore, the $sst2^-$ and *ste2-T326* mutations have independent effects on adaptation (40).

pAFR1 acts prior to the G-protein step. The stage of the pheromone signal pathway that is antagonized by pAFR1 was examined by using mutant strains that activate G-protein signaling independently of the α-factor receptor. The expectation is that if pAFR1 acts on the receptor, it should not suppress the constitutive cell division arrest caused by deletion of the G_α gene (*gpa1::LEU2*). The *gpa1::LEU2* mutation is thought to mimic the effects of pheromone signaling by raising the pool of free $G_{\beta\gamma}$ subunits in the cell (3, 12, 53, 70). Suppression of *gpa1::LEU2* would indicate that the resistance function acts on or after the G-protein step and is unlikely to act directly on the receptor. For this analysis, the pAFR plasmids were introduced into a *gpa1::LEU2* test strain (7646-3-1) that carried a $ste5^{ts}$ mutation which blocks G-protein signaling and allows cells to be propagated at the nonpermissive temperature for $ste5^{ts}$ (34°C) (28). The *gpa1::LEU2* $ste5^{ts}$ cells were then incubated on agar plates at the permissive temperature for $ste5^{ts}$ (23°C) in order to initiate constitutive G-protein signaling. As expected, cells carrying YEp24 failed to form colonies after 4 days of incubation as a result of constitutive activation of the cell division arrest signal. Microscopic analysis of the agar plates showed that cells carrying YEp24 were present mainly as a single layer of cells and were not piling up to form microcolonies (Fig. 3A). Cells carrying pAFR1 were indistinguishable from cells carrying YEp24, which indicates that pAFR1 does not suppress the constitutive cell division arrest signal (Fig. 3B). In contrast, the 24 other pAFR plasmids were able to suppress *gpa1::LEU2* with the same relative efficiency as that with which they were able to suppress α-factor-induced cell division arrest. This assay is sensitive enough to have detected suppression of *gpa1::LEU2* by pAFR1 because other plasmids, such as pAFR14, that confer weak α-factor resistance (≤3-fold) were able to confer partial suppression of *gpa1::LEU2*. As shown in Fig. 3C, cells carrying pAFR14 had undergone cell division and piled up to form microcolonies. Similar results were obtained when the pAFR plasmids were tested for the ability to suppress constitutive signaling caused by a special dominant mutation in the G_β subunit, $STE4^{HPL}$ (3) (data not shown). Therefore, temporal ordering of pAFR1 function in the pheromone signal transduction pathway indicates that the pAFR1 function acts prior to the G-protein step and suggests that it acts on the receptor.

Effects of pAFR1 on the initial sensitivity to α-factor. Resistance to α-factor-induced cell division arrest is a long-term assay that requires ≥2 days to observe the results. Therefore, the induction of a pheromone-responsive *FUS1-lacZ* reporter gene (26) was analyzed in order to investigate the effects of pAFR1 on the initial response to α-factor. Plasmid-carrying cells were induced with 10^{-8} M α-factor and then assayed for the induction of the *lacZ*-encoded β-galactosidase activity after 30, 60, 90, and 120 min (Fig. 4). The results demonstrate that pAFR1-carrying cells were only slightly inhibited in the ability to induce the *FUS1-lacZ* reporter gene relative to cells carrying the YEp24 vector plasmid. A similar effect was seen for cells overexpressing *SST2* or *GPA1*. The inhibition of *FUS1-lacZ* at the earliest time points measured (<45 min) was 24% (±3) for YEpAFR1, 29% (±13) for YEpSST2, and 48% (±14) for YEpGPA1 (Fig. 4 and data not shown). Similar results were obtained at other concentrations of α-factor (data not shown). The slight decrease in the initial sensitivity to α-factor seen for cells carrying YEpAFR1 or YEpSST2 may be due to an elevated basal level of adaptation activity. The stronger decrease in initial sensitivity seen for cells overexpressing *GPA1* is consistent with this gene encoding the G_α subunit, which negatively regulates the signaling activity of the $G_{\beta\gamma}$ subunits. These results demonstrate that pAFR1 cells are initially sensitive to α-factor but can later adapt to high concentrations of α-factor.

DNA sequence of *AFR1*. Transposon insertional mutagenesis was used to localize the specific gene which confers α-factor resistance within the 8.5-kb insert of pAFR1 (Fig. 5A and Materials and Methods). DNA sequence analysis identified an open reading frame of 1,860 bases which will be called *AFR1*. Assuming that the initiation codon corresponds to the first methionine within the open reading frame, the predicted AFR1 protein contains 620 amino acids (molecular weight, 71,319). Hydropathy analysis of the AFR1 protein did not detect any obvious hydrophobic segments

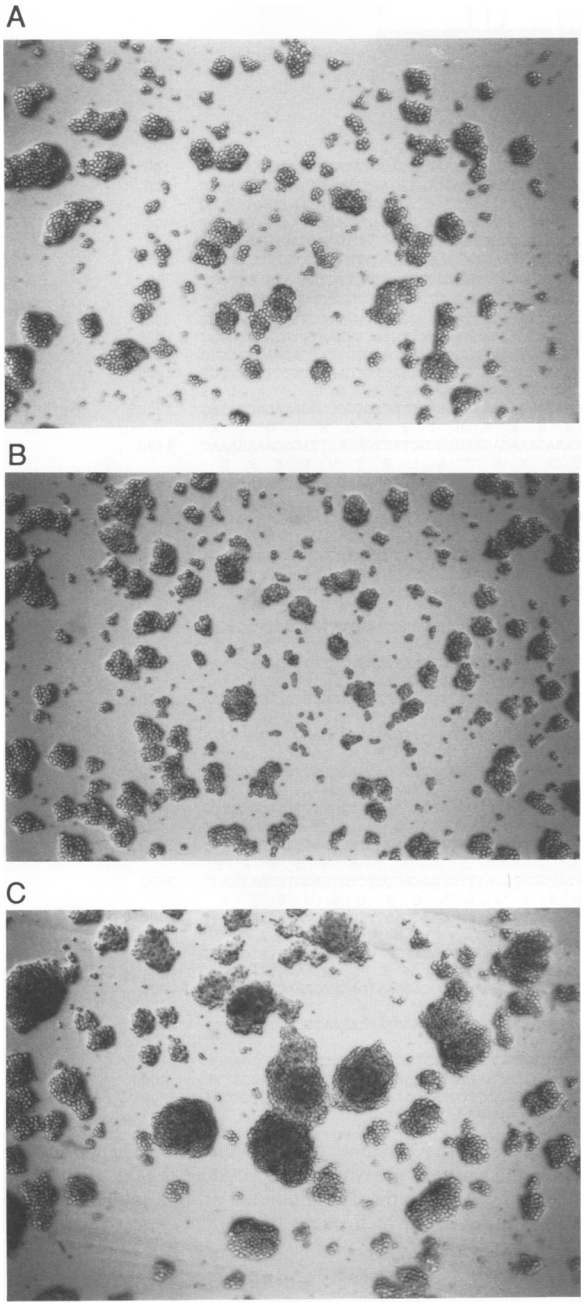

FIG. 3. Suppression of constitutive signaling caused by *gpa1* mutation. Plasmids YEp24 (A), pAFR1 (B), and pAFR14 (C) were tested for the ability to suppress the constitutive signal for cell division arrest caused by the *gpa1*::*LEU2* mutation (G_α subunit) in strain 7646-3-1. Cells were spread on agar plates, incubated for 4 days at 23°C, and then examined for evidence of cell division as an indicator of suppression of *gpa1*::*LEU2*. (A) Cells carrying plasmid vector YEp24 demonstrate the expected effects of the *gpa1*::*LEU2* mutation; cells accumulate in the unbudded stage and do not form colonies. (B) Plasmid pAFR1 does not suppress the constitutive signaling because cell division was arrested as observed in panel A. (C) Cells carrying pAFR14 formed small microcolonies, which indicates that this plasmid causes partial suppression of the constitutive cell division arrest signal. Photographs were taken of cells on agar plates through a 20× objective. Strain 7646-3-1 also carries a *ste5*^{ts} mutation, which permitted cell cultures to be propagated at 34°C and then shifted to 23°C for this analysis.

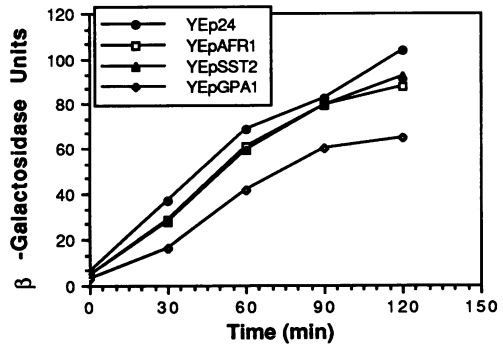

FIG. 4. Effects of AFR1 overexpression on the induction of the *FUS1-lacZ* gene. The effects of YEp24, YEpAFR1, YEpSST2, and YEpGPA1 on the induction of the pheromone-responsive *FUS1-lacZ* reporter gene were examined. Strain DJ211-5-3 carried the *FUS1-lacZ* gene integrated into the *MFA2* locus as described previously (26). Cells were incubated in the presence of 10^{-8} M α-factor for the amount of time indicated on the *x* axis. The level of β-galactosidase activity was quantitated as described in Materials and Methods.

that could act as transmembrane domains. This finding suggests that the AFR1 protein is not an integral membrane protein as is the α-factor receptor. Computer-assisted searches of the GenBank and EMBL sequence data bases indicate that *AFR1* is a new gene that has not been previously reported. Furthermore, *AFR1* maps to a novel position on chromosome IV that does not correspond to the map position of any reported genes. *AFR1* was found to map 8.6 cM from *CDC34* and 13.2 cM from *STE5*, using a combination of physical and meiotic mapping techniques as described in Materials and Methods. Comparison of the predicted AFR1 protein with data base sequences by using the BLAST method (1) did not detect any highly homologous protein sequences. The relationship between *AFR1* and other known sequences will be described further in Discussion.

The 5′ noncoding sequences were examined for sequences that would indicate that *AFR1* expression is transcriptionally regulated. Significantly, several close matches to the PRE consensus (ATGAAACA) were observed (Fig. 5B). The PRE sites are binding sites for the transcription factor STE12, which mediates pheromone-induced transcription (20, 26). PRE sequences are found upstream of all pheromone-induced genes, including the adaptation genes *BAR1* and *SST2*. In a similar manner, PRE sites are clustered around 400 to 600 bases before the presumed ATG initiator of the *AFR1* open reading frame. The upstream region containing the PRE consensus sites is required for *AFR1* function. A transposon which inserted 230 bases upstream of the ATG initiator (base 877) separated the PRE sites from the coding region of the gene and resulted in a loss of ability to confer α-factor resistance (Fig. 5A and data not shown).

AFR1* expression is induced by pheromone.** Identification of the open reading frame made it possible to construct a hybrid *lacZ* fusion gene for use in monitoring the expression of *AFR1*. An *AFR1-lacZ* fusion gene was constructed by cloning the 5′ regulatory sequences and N-terminal coding sequences of *AFR1* fused in frame to the *lacZ* coding sequence for the enzyme β-galactosidase (see Materials and Methods). *MATa** cells carrying the *AFR1-lacZ* fusion gene were treated with various doses of α-factor, and then expression of the fusion gene was monitored by quantitating the induction of

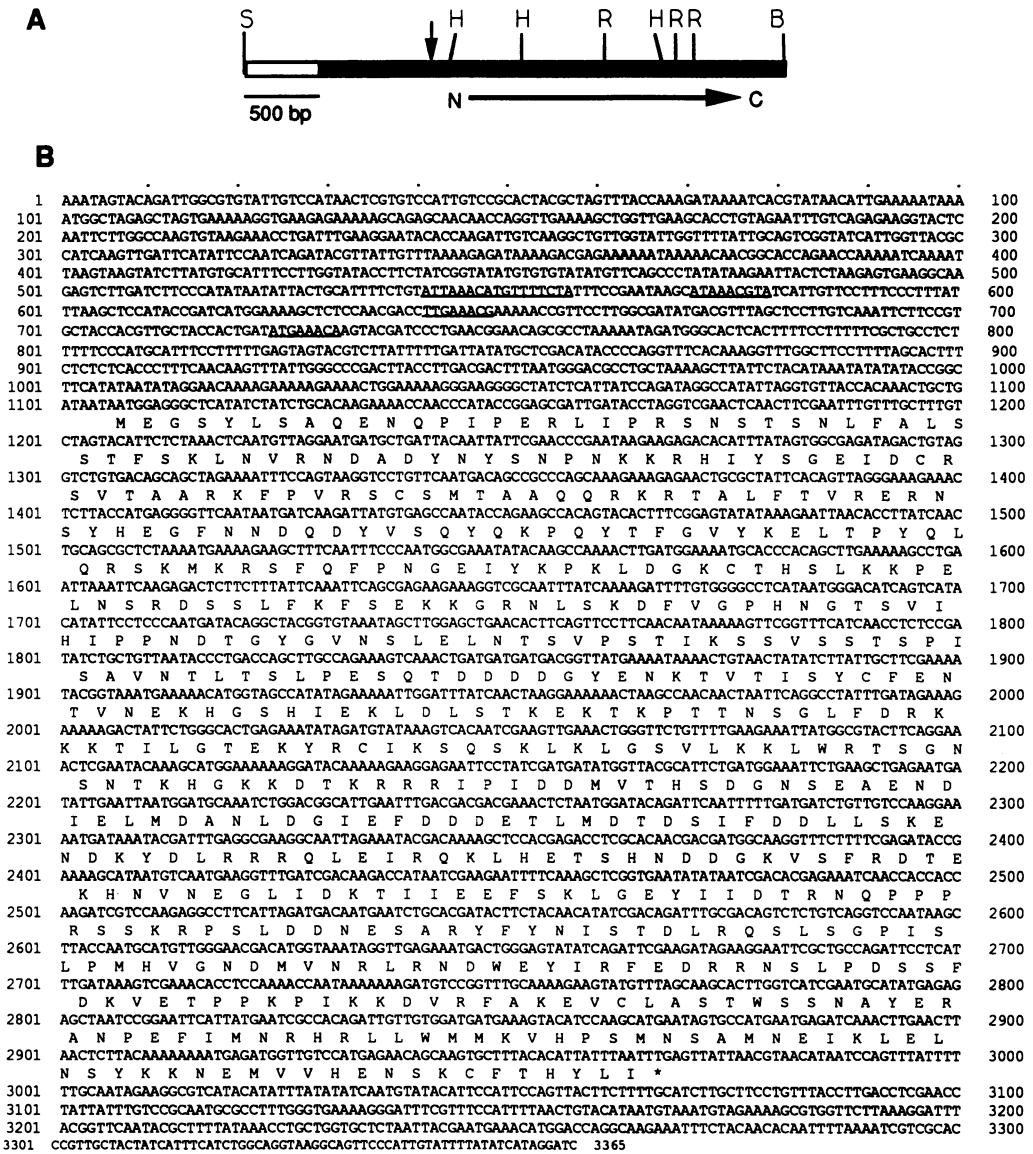

FIG. 5. Structure of the *AFR1* gene. (A) Diagram of the *AFR1* gene which shows the prominent restriction sites. The position of a Tn*10*-LUK insertion in the 5′ promoter region that inactivates the gene is indicated by an arrowhead. The coding region of the gene is indicated by the horizontal arrow below the restriction map. (B) DNA sequence of the *AFR1* gene. The predicted protein sequence is shown below the DNA sequence. Close matches to the PRE consensus sequence (ATGAAACA) which occur in the 5′ region of the gene are underlined. PRE elements are sufficient to mediate pheromone-induced transcription (26).

β-galactosidase activity. As shown in Fig. 6, the expression of *AFR1-lacZ* was low in the absence of α-factor. However, the expression was induced in a dose-dependent manner by α-factor. At saturating α-factor concentrations, *AFR1-lacZ* was induced >50-fold. Similar results were obtained for a *FUS1-lacZ* gene which was previously shown to be highly inducible by pheromone (26, 67). The results demonstrate that the expression of *AFR1* is similar to that of the adaptation genes *BAR1* (46) and *SST2* (18), which are transcriptionally induced by pheromone.

The regulation of *AFR1* expression was examined further by transforming the *AFR1-lacZ* fusion gene into additional yeast strains. Induction of *AFR1-lacZ* by α-factor was readily detected in a *ste2-T326* strain (Fig. 7). Thus, the failure of pAFR1 to confer α-factor resistance in the *ste2-T326* strain is not due to the lack of *AFR1* expression. α-Factor-regulated expression of the *AFR1-lacZ* gene was also detected in *bar1* and *sst2* adaptation mutant *MAT***a** strains. However, *MAT***a**/α diploid cells, which are not responsive to pheromone, produced a low basal level of *AFR1-lacZ* that was not induced by α-factor. In the *MAT*α cell type, *AFR1-lacZ* expression was induced by **a**-factor similar to the induction seen for *MAT***a** cells treated with α-factor. Since truncation of the *STE3*-encoded **a**-factor receptor also causes a defect in adaptation (unpublished data), *AFR1* may affect both the **a**-factor and the α-factor receptors.

Construction of an *AFR1* deletion mutant. A yeast strain lacking the *AFR1* gene was constructed to investigate the normal function of *AFR1*, since the pheromonal regulation of

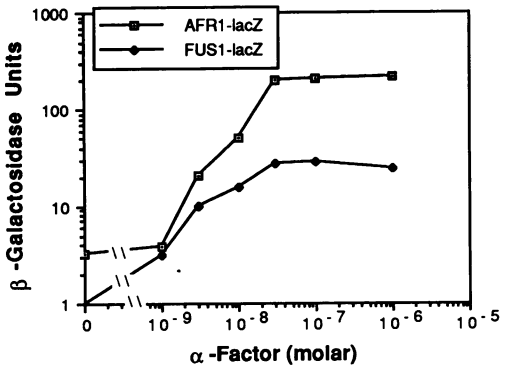

FIG. 6. *AFR1* expression is stimulated by α-factor. Strain DJ211-5-3 carrying pJK33 (*AFR1-lacZ*) or pSL1580 (*FUS1-lacZ* [26]) was incubated in the presence of α-factor as indicated on the *x* axis for 2 h at 30°C. Cultures were then assayed for β-galactosidase activity as described in Materials and Methods.

AFR1 expression and the effects of *AFR1* overexpression strongly indicate that this gene is involved in pheromone signaling. An *AFR1* deletion allele was constructed by modifying a transposon insertion allele that contains a Tn*10*-LUK transposon in the 5′ noncoding region of *AFR1*, 230 bases before the presumed ATG initiation codon (Fig. 5A). In the resultant *afr1*::*URA3* allele, bases 877 to 2916 are replaced by a 3-kb segment of Tn*10*-LUK containing the yeast selectable marker *URA3* (see Materials and Methods). The one-step gene replacement method (59) was used to replace the chromosomal *AFR1* gene with the *afr1*::*URA3* deletion allele. Southern blot analysis confirmed that the normal *AFR1* gene had been effectively replaced by the *afr1*::*URA3* allele (data not shown).

The *afr1*::*URA3* cells grew at a normal rate. Since *AFR1* is not essential for the mitotic cell division cycle, it was possible to examine the *afr1*::*URA3* cells for a defect in pheromone signaling. It was of particular interest to determine whether *AFR1* deletion caused increased sensitivity to α-factor, since overexpression of *AFR1* confers α-factor resistance. However, comparison of α-factor sensitivity by halo assay demonstrated that the *afr1*::*URA3* cells showed at most only a slight increase in sensitivity (<2-fold; data not shown). This finding indicates that the 10-fold-increased sensitivity caused by truncation of the α-factor receptor is not due solely to the inability of *AFR1* to act on the mutant receptor. A likely possibility is that other genes are redundant in function with *AFR1* and can confer normal sensitivity to α-factor in the *afr1*::*URA3* strain (see Discussion). Double-mutant analysis failed to detect any strong synergistic effects on pheromone sensitivity for *sst2*$^-$ *afr1*::*URA3* or *ste2-T326 afr1*::*URA3* mutants (data not shown).

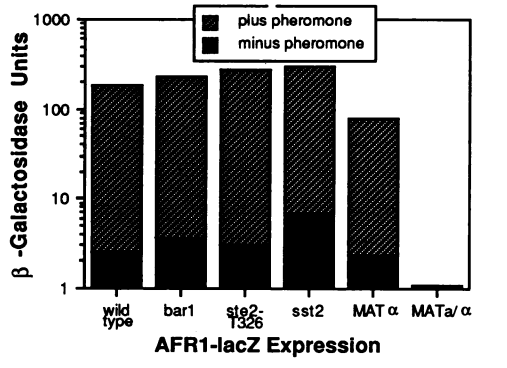

FIG. 7. Pheromone stimulates *AFR1* expression in wild-type and mutant haploid cells. Wild-type (DJ147-2-1), *bar1* (211-5-3), *ste2-T326* (7440-1), and *sst2* (JKY10-2-1) *MAT***a** strains and *MAT***a**/α strain JK35 were incubated in the presence or absence of 10^{-7} M α-factor for 2 h at 30°C. *MAT*α strain 7417-5-4 was incubated with concentrated **a**-cell supernatant as a source of **a**-factor (48). Cell cultures were then harvested and assayed for β-galactosidase activity as described in Materials and Methods.

***AFR1* promotes morphogenesis.** The fact that *ste2-T326* cells are defective in α-factor-induced morphogenesis (40) raised the possibility that the *AFR1* gene is also involved in this process. Therefore, *afr1*::*URA3* cells were examined for ability to induce projections of morphogenesis after incubation in the presence of α-factor (10^{-6} M) for 6 h at 30°C. These conditions highlight the morphological differences between wild-type and mutant cells. Ninety-eight percent of wild-type cells (*STE2 AFR1*) produced at least one projection and 82% produced two or more projections under these conditions of α-factor treatment (Fig. 8A). In contrast, the phenotype of *afr1*::*URA3* cells (Fig. 8E) was similar to that of *ste2-T326* cells (Fig. 8C) in that >80% of *afr1*::*URA3* cells lacked acute projections and less than 4% of the cells appeared to contain multiple projections. Interestingly, overexpression of *AFR1* resulted in the opposite phenotype in that projections not only were formed but also were much longer than normal (Fig. 8B). Twenty-seven percent of the pAFR1 cells contained projections that were greater than one cell body in length and in some cases were two to three cell body lengths long. In contrast, less than 5% of wild-type cells produced projections greater than one cell length, but none were greater than two cell lengths. The gene dosage relationship between *AFR1* and morphogenesis clearly indicates a role for *AFR1* in this process.

The *ste2-T326* and *afr1*::*URA3* cells were similar in that they both were defective in the production of acute projections in response to α-factor. The α-factor-induced morphologies were not identical because *ste2-T326* cells maintained a round or oval shape whereas *afr1*::*URA3* cells developed an irregular outline (Fig. 8C and E). This finding suggests that the morphogenesis defect caused by truncation of the α-factor receptor is more severe than that caused by deletion of *AFR1*. The *afr1*::*URA3* and *ste2-T326* mutants were also similar in that they appeared to increase in volume and become larger than wild-type cells under the same conditions of α-factor treatment. These similarities suggest that the morphogenesis defects of *ste2-T326* and *afr1*::*URA3* cells are related. Therefore, a *ste2-T326* strain carrying pAFR1 was examined to determine whether the receptor C terminus is required for the morphogenetic effects of *AFR1*. The results demonstrate that *AFR1* overexpression does not have a significant effect on morphogenesis in the *ste2-T326* strain (Fig. 8D). The smaller and more rounded appearance of the *ste2-T326* cells in Fig. 8D is due to the effects of growth in synthetic medium to select for the pAFR1 plasmid. However, about 5% of the cells showed atypical projections which are apparently due to high-level expression of *AFR1* in the absence of the receptor C terminus (not shown). Thus, the effects of *AFR1* on adaptation and morphogenesis both require the C terminus of the α-factor receptor.

α-Factor-induced morphogenesis is thought to form the conjugation bridge between mating cells, so *afr1*::*URA3* cells were investigated for a mating defect. In quantitative mating assays, *afr1*::*URA3* cells formed diploids at essen-

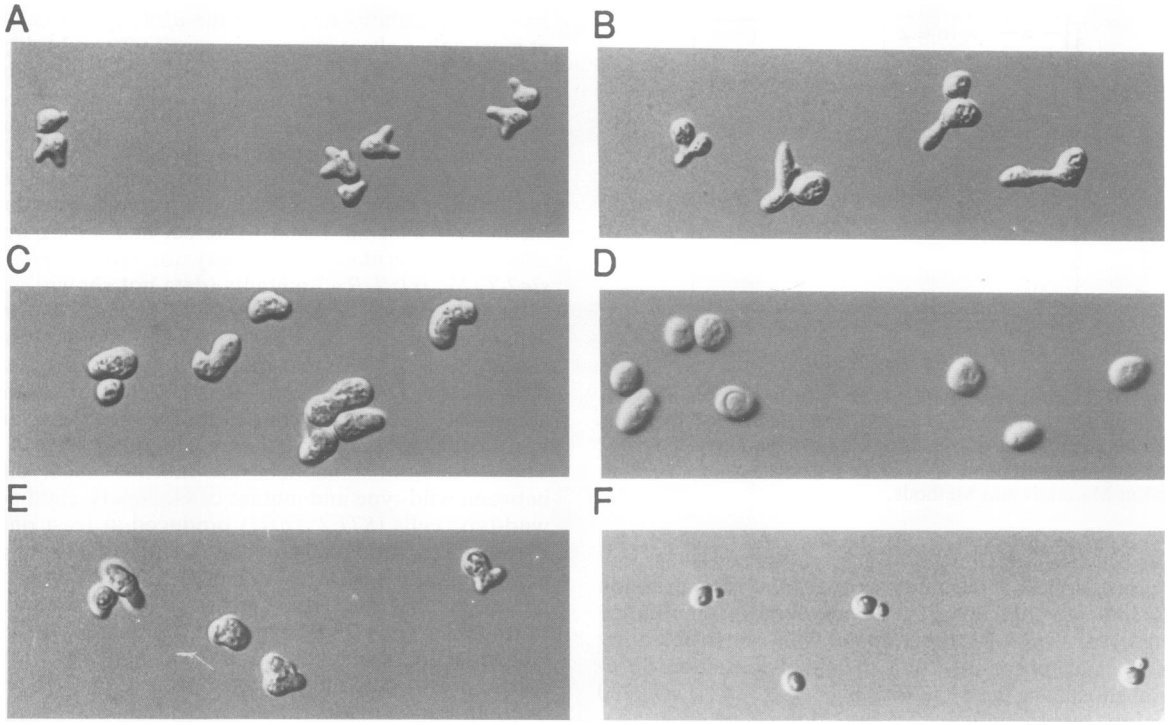

FIG. 8. Effects of *AFR1* on α-factor-induced morphogenesis. Photographs show the morphologies of α-factor-stimulated cells of strains DJ211-5-3 (*STE2 AFR1*) (A), DJ211-5-3 plus pAFR1 (B), 7441-4-2 (*ste2-T326 AFR1*) (C), 7441-4-2 plus pAFR1 (D), and JK26-1 (*STE2 afr1::URA3*) (E). Log-phase cultures were diluted to 5×10^5 cells per ml in YPD medium, and then α-factor was added to 10^{-6} M. The normal bud morphology of *afr1::URA3* (JK26-1) cells in the absence of α-factor is shown in panel F. The cultures were incubated at 30°C for 6 h, and then the cells were fixed with 5% formaldehyde and photographed on glass slides through a 40× objective, using differential interference contrast (Nomarski) optics. Cells were photographed and printed under the same conditions so the relative sizes shown in the photographs correspond to the actual size.

tially the same frequency as wild-type cells did (Table 2). Furthermore, *MAT***a** *afr1::URA3* cells mated with *MATα* *afr1::URA3* cells with nearly the same efficiency as wild-type cells did, which indicates there is no significant bilateral mating defect. The *ste2-T326* cells also do not show a strong overall mating defect but are defective in mating partner discrimination (35). Therefore, *MAT***a** *afr1::URA3* cells were tested for the ability to discriminate between α-factor-producing and nonproducing *MATα* partner cells, using the assay developed by Jackson and Hartwell (34). As expected, wild-type *MAT***a** cells formed diploids with the α-factor-producing *MATα* cells but did not detectably mate with the nonproducing *MATα* cells (≤0.01%; Table 2). This finding demonstrates that wild-type cells can efficiently discriminate between mating partners. Under the same conditions, 0.5% of the *ste2-T326* matings occurred with the non-α-factor-producing cells, which indicates a defect in mating partner discrimination. The *afr1::URA3* cells behaved like wild-type cells and did not detectably mate with the non-α-factor-producing *MATα* cells. The ability of *afr1::URA3* cells to discriminate mating partners implies that *AFR1* is not transmitting a signal for cell polarization. Instead, *AFR1* influences the shape of the morphogenic projection, which is apparently not important in the standard mating assays in which cells are in direct contact.

TABLE 2. Mating efficiencies of *AFR1* mutant strains

*MAT***a**[a]	*MATα*[b]	Mating efficiency[c] (%)	Mismating frequency[d] (%)
AFR1	*AFR1*	100	<0.01
afr1::URA3	*AFR1*	107.5	<0.01
AFR1	*afr1::URA3*	101.1	
afr1::URA3	*afr1::URA3*	94.1	
ste2-T326	*AFR1*	55.9	0.5
ste2-T326	*afr1::URA3*	61.5	

[a] The *MAT***a** *AFR1* strain was DJ147-1-2; the *MAT***a** *afr1::URA3* strain was JK27-1; the *ste2-T326* strain was 7440-1.

[b] The *MATα* *AFR1* strain was JK33-9-1; the *MATα* *afr1::URA3* strain was JK33-6-1.

[c] Average of two separate mating experiments, normalized to 100% for the wild type.

[d] *MAT***a** cells were mated with a mixture of α-factor-producing (SF301) and nonproducing (*mfa1 mfa2*) *MATα* cells. Mismating was determined as the percentage of matings that occurred between the indicated *MAT***a** cells and the non-pheromone-producing *MATα* strain, *mfa1 mfa2*.

DISCUSSION

Identification of *AFR1* by overexpression. *AFR1* is a new pheromone signal transduction gene that functions in adaptation and morphogenesis. This gene was initially identified because its overexpression conferred resistance to α-factor-induced cell division arrest. Previous studies have demonstrated several mechanisms for the ability of overexpressed genes to cause α-factor resistance in yeast cells. Overexpression of the *SST2* adaptation gene or the *GPA1* (*SCG1*) gene, which encodes the G_α subunit, confers resistance to α-factor by increasing the normal adaptation mechanisms (17, 18). The mechanism by which *KSS1* overexpression (14) confers

α-factor resistance is unclear, since *KSS1* is involved in activating the pheromone signal (22). It has been suggested that *KSS1* may also promote adaptation (73). α-Factor resistance can also be caused by overexpression of genes that simply interfere with the synthesis or activation of the signal transduction pathway and do not actively promote adaptation (68). *AFR1* appears to be directly involved in promoting adaptation because the cells overexpressing *AFR1* are initially sensitive to α-factor (Fig. 4 and 8) but can later adapt to higher concentrations of α-factor than can wild-type cells. Furthermore, *AFR1* expression is induced by pheromone similarly to the known adaptation genes *BAR1* and *SST2* (18, 46). Pheromone-regulated expression of adaptation genes presumably permits cells to be initially sensitive to low concentrations of pheromone yet allows adaptation unless the pheromone signal increases in intensity as when two cells come into contact. Since *AFR1* expression is normally proportional to the degree of α-factor stimulation, it is reasonable that *AFR1* overexpression causes cells to hyperadapt.

AFR1 and receptor regulation. Several lines of evidence indicate that *AFR1* acts in conjunction with the α-factor receptor C terminus to promote adaptation. The cytoplasmic C-terminal segment of the α-factor receptor was previously shown to mediate adaptation because deletion of this domain resulted in a defect in the ability of cells to recover from α-factor stimulation (40, 57). The failure of *AFR1* overexpression to cause α-factor resistance in a *ste2-T326* strain, which produces C-terminally truncated receptors, indicates that *AFR1* acts in the same adaptation pathway as the receptor C terminus does. Furthermore, *AFR1* promotes adaptation by acting on an early stage of the pheromone signal, as shown by the ability of *AFR1* overexpression to suppress a signal generated by receptor-ligand interaction but not a constitutive signal caused by mutant G-protein subunits. The G-protein mutations *gpa1*Δ and *STE4*^{HPL} cause constitutive activation of the G-protein signal in the absence of receptor stimulation (3, 17, 50). Thus, *AFR1* may act directly on the α-factor receptor. Since *AFR1* is expressed in *MAT*α cells, it may act on the **a**-factor receptor as well. Unfortunately, concentrated **a**-cell supernatants were not a sufficient source of **a**-factor to perform comparable adaptation assays in *MAT*α cells.

AFR1 is apparently not the only gene that participates in the regulation of pheromone receptor signaling. Cells lacking *AFR1* showed essentially normal sensitivity to α-factor and were not 10-fold more sensitive to α-factor as was seen for *ste2-T326* cells. Genetic redundancy in the functions that mediate adaptation also seems likely because of the failure of previous genetic screens to identify supersensitive mutations that encode adaptation functions that act on the receptor (8, 64). Multiple adaptation mechanisms are used to regulate other G-protein-coupled receptors (19). In the case of the β-adrenergic receptor and rhodopsin, the C-terminal segments mediate adaptation because they are targets for a receptor desensitization mechanism. Activated receptors are phosphorylated on the C terminus by a receptor-specific protein kinase which decreases the ability to activate G proteins (45). Receptor phosphorylation facilitates the binding of an arrestin protein to the receptor, which results in a further decrease in the ability to activate a signal (44, 71). Interestingly, overexpression of β-arrestin or the receptor kinase augments the desensitization of β-adrenergic receptors (54). The β-adrenergic receptor can also be desensitized by other mechanisms which include receptor phosphorylation by protein kinase A or protein kinase C (19).

Since the phenotype of *AFR1* overexpression suggests that the protein product may act on the C terminus of the α-factor receptor, the predicted AFR1 protein sequence was compared with the sequences of the arrestins and receptor-specific protein kinases. AFR1 did not show strong similarity with the receptor-specific kinases and, in fact, is unlikely to encode a protein kinase because the sequence does not contain the highly conserved protein sequence motifs found in protein kinase sequences (27). Arrestin sequences are highly conserved. At least 40% identity exists between photo-arrestins from organisms as diverse as drosophilas and humans (61) and between the arrestins of the rhodopsin and β-adrenergic receptor signaling systems (44). The complete AFR1 protein shows about 20% sequence identity to members of the arrestin family. Although this level of similarity is too low to predict that *AFR1* encodes an arrestin homolog, the pheromone receptors and the G_γ subunit also show a similarly low level of sequence identity with homologous proteins from multicellular organisms (70). In fact, short stretches of stronger sequence similarity between AFR1 and the known arrestin sequences can be identified. Thus, sequence comparisons are not sufficient to confidently predict whether AFR1 functions as an arrestin. Development of biochemical assays for AFR1 will be required to determine whether it functions as an arrestin. Alternatively, AFR1 may function by a novel mechanism to regulate receptor signaling.

AFR1 promotes α-factor-induced morphogenesis. *AFR1* was isolated because of its effects on α-factor sensitivity, so it was unexpected to find that *AFR1* also acts to promote morphogenesis. Cells lacking *AFR1* showed a defect in the formation of α-factor-induced projections of morphogenesis; in contrast, overexpression of *AFR1* resulted in longer projections of morphogenesis. These results indicate that *AFR1* encodes a limiting component for morphogenesis. Thus, the ability of cells to form observable projections may be due to the level to which *AFR1* expression is induced by pheromone. This model of *AFR1* action is consistent with previous observations that cells treated at low concentrations of α-factor arrest cell division but do not form projections (51). Furthermore, the observation that *AFR1* acts in conjunction with the receptor C terminus to promote morphogenesis accounts, at least in part, for the morphogenesis defect of *ste2-T326*. On the basis of these results, it is intriguing to speculate that the AFR1 protein may bind to the C termini of ligand-activated receptors to coordinately regulate receptor desensitization and morphogenesis. The observations that α-factor receptors are localized to the projections of pheromone-induced morphogenesis (35) and that *AFR1* acts in conjunction with the receptor suggest a mechanism that could restrict morphogenesis to a particular domain within the cell during mating. However, this speculation must be reconciled with the observation that *gpa1*$^-$ mutants form projections of morphogenesis as a result of constitutive G-protein signaling in the absence of receptor stimulation (36). Although morphogenesis is observed, other studies have shown that constitutive G-protein signaling causes cells to acquire an abnormal morphology that is distinct from that of wild-type cells treated with α-factor (3, 13, 17, 53, 70). Under the assay conditions used in this study, *gpa1*::*LEU2* mutant cells rarely exhibited multiple projections of morphogenesis that were commonly observed for α-factor-treated cells. The possibility that the AFR1 protein interacts with ligand-activated receptors to promote morphogenesis merits further investigation.

Bud morphogenesis also requires polarized cell growth, so

it is not surprising that many of the genes needed for bud growth also function in morphogenesis during mating (21, 47). For example, actin plays a key role in both bud and α-factor-induced morphogenesis (56), but microtubules do not seem to be necessary for morphogenesis. Other bud morphogenesis mutations are also defective in α-factor-induced morphogenesis. *cdc24* and *bem1* mutants (11), which are defective in cell polarization, and *spa1* mutants, which have subtle defects in budding and in bud site selection (62), are also defective in pheromone-induced morphogenesis. *AFR1* is unique in that it functions specifically in pheromone-induced morphogenesis. *afr1::URA3* cells showed normal bud morphology (Fig. 8F) and medial bud site selection (unpublished data) as described by Chant and Herskowitz (10). Therefore, the action of *AFR1* must be developmentally regulated to promote pheromone-induced morphogenesis without perturbing the ability of cells to complete the formation of buds and arrest as unbudded cells in G_1. Perhaps temporal regulation of *AFR1* expression by pheromone stimulation and spatial regulation of *AFR1* function by the pheromone receptors could serve to restrict the *AFR1* protein from acting inappropriately. Alternatively, since buds and pheromone-induced projections are distinct structures, *AFR1* may not efficiently interact with bud morphogenesis components. Perhaps expression of *AFR1* from a constitutive promoter could be used to distinguish between these possibilities.

Pheromone-induced morphogenesis is thought to polarize cell growth toward an appropriate mating partner and to form the conjugation bridge that connects the cells. The *afr1::URA3* cells, however, did not show a mating defect in spite of their defect in morphogenesis. Furthermore, *afr1::URA3* cells did not show a defect in mating partner discrimination. These results indicate that *AFR1* is not required for pheromone receptors to signal the spatial orientation that allows cells to discriminate between mating partners (35). Perhaps *AFR1* does not appear to have an essential role in mating because cells are placed in direct contact in the typical laboratory mating assays. The acute projections of morphogenesis stimulated by *AFR1* may be important only for mating under nonideal conditions. Alternatively, the genetic regulation of pheromone-induced morphogenesis may be carried out by multiple genes that overlap in function with *AFR1*. The latter possibility is suggested by the complex genetic regulation of bud morphogenesis in that many of the genes involved in the process of budding are not essential (21, 47). Analysis of the genetic interactions between *AFR1* and other known morphogenesis genes may provide insight into the role of *AFR1*-promoted morphogenesis in yeast mating.

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