Identification of Genes Required for Normal Pheromone-Induced Cell Polarization in *Saccharomyces cerevisiae*

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ABSTRACT

In response to mating pheromones, cells of the yeast *Saccharomyces cerevisiae* adopt a polarized "shmoo" morphology, in which the cytoskeleton and proteins involved in mating are localized to a cell-surface projection. This polarization is presumed to reflect the oriented morphogenesis that occurs between mating partners to facilitate cell and nuclear fusion. To identify genes involved in pheromone-induced cell polarization, we have isolated mutants defective in mating to an enfeebled partner and studied a subset of these mutants. The 34 mutants of interest are proficient for pheromone production, arrest in response to pheromone, mate to wild-type strains, and exhibit normal cell polarity during vegetative growth. The mutants were divided into classes based on their morphological responses to mating pheromone. One class is unable to localize cell-surface growth in response to mating factor and instead enlarges in a uniform manner. These mutants harbor special alleles of genes required for cell polarization during vegetative growth, *BEM1* and *CDC24*. Another class of mutants forms bilobed, peanut-like shapes when treated with pheromone and defines two genes, *PEA1* and *PEA2*. *PEA1* is identical to *SPA2*. A third class forms normally shaped but tiny shmoos and defines the gene *TNY1*. A final group of mutants exhibits apparently normal shmoo morphology. The nature of their mating defect is yet to be determined. We discuss the possible roles of these gene products in establishing cell polarity during mating.

MANY cell types generate cellular asymmetry in response to environmental signals. The signals and signal transduction mechanisms which cause these responses are known in many cases, but the intracellular components that participate in the oriented establishment of cell polarity are not well understood (reviewed by NELSON 1992; LUNA and HITT 1992; STOSSEL 1993). Cells of the yeast *Saccharomyces cerevisiae* polarize toward an extracellular signal during conjugation. When two cells of opposite mating type come into contact, they localize new cell-surface growth and orient their cytoskeletons toward each other (BYERS and GOETSCH 1975; BYERS 1981; FORD and PRINGLE1986; HASEK *et al.* 1987), which facilitates efficient cell and nuclear fusion (reviewed in CROSS *et al.* 1988). This localized growth is directed by a spatial signal emanating from the mating partner which is apparently a high concentration of mating pheromone (KURJAN 1985; MICHAELIS and HERSKOWITZ 1988; JACKSON and HARTWELL 1990a, b; SEGALL 1993). These pheromones are secreted peptides recognized by cell-surface receptors in a cell-type specific manner: **a** cells produce **a**-factor, which binds to a receptor on α cells, and α cells produce α-factor which binds to a receptor on **a** cells. These receptors belong to the large family of G-protein-coupled receptors with seven transmembrane domains (reviewed in MARSH *et al.* 1991; KURJAN 1992).

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The addition of purified pheromone to cells of the opposite cell type causes a variety of mating responses, including cell cycle arrest, gene induction, and formation of a pear-shaped cell ("shmoo"). The shmoo is a manifestation of the pheromone-stimulated morphogenesis which normally occurs during mating but which becomes exaggerated in the absence of a mating partner. The projection of the shmoo (or "shmoo tip") is formed by deposition of new membrane and cell wall material to a localized region of the cell surface (LIPKE *et al.* 1976; TKACZ and MACKAY 1979; FIELD and SCHEKMAN 1980). This growth reflects the polarized orientation of the cytoskeleton (reviewed by BARNES *et al.* 1990; READ *et al.* 1992). Actin accumulates at the growing region of the cell cortex, and actin cables align along the growth axis (FORD and PRINGLE 1986; HASEK *et al.* 1987; GEHRUNG and SNYDER 1990). The spindle pole body (the yeast analog of the centrosome or microtubule organizing center) orients toward the shmoo tip, and microtubules emanating from it extend into the tip (ROSE and FINK 1987; GEHRUNG and SNYDER 1990; MELUH and ROSE 1990). Most organelles, including the nucleus, accumulate on the side of the cell where the projection forms (BABA *et al.* 1989; BYERS and GOETSCH 1975; TKACZ and MACKAY 1979; HASEK *et al.* 1987; ROSE and FINK 1987; GEHRUNG and SNYDER 1990). Many molecules with roles in mating localize to the shmoo tip; these include **a**-agglutinin (WATZELE *et al.* 1988), Fus1p (TRUEHEART *et al.* 1987), Spa2p (GEHRUNG and SNYDER 1990), Ste2p (JACKSON *et al.*

1991), Fus2p (E. ELION, personal communication), and Ste6p (KUCHLER *et al.* 1993).

Yeast cells also grow in a polarized manner during vegetative growth, by budding from distinct sites on the cell surface. Unlike sites of growth during mating, which are directed by external signals, bud sites are defined by some intracellular landmark, perhaps a remnant of the previous cell division, and are determined by the genetic makeup of a cell. Two classes of genes which regulate the budding process have been identified, bud-site selection genes (*BUD1-5*) and polarity-establishment genes (such as CDC24, *CDC42*, *BEM1*) (reviewed in DRUBIN 1991). Mutations in the bud-site selection genes cause improper positioning of the bud but no growth defect (BENDER and PRINGLE 1989; CHANT and HERSKOWITZ 1991; CHANT *et al.* 1991). Mutants in the polarity-establishment genes are unable to localize growth to form a bud but rather enlarge in a uniform manner and grow slowly or are inviable (SLOAT and PRINGLE 1978; SLOAT *et al.* 1981; ADAMS *et al.* 1990; JOHNSON and PRINGLE 1990; CHENEVERT *et al.* 1992; CHANT *et al.* 1991; BENDER and PRINGLE 1991). Studies on the functions of these genes and the interactions among them have led to a model of hierarchical assembly of components involved in bud formation (CHANT and HERSKOWITZ 1991): the *BUD* gene products are proposed to recognize the cell-surface landmark and guide the positioning of the polarity-establishment gene products, which in turn organize the cytoskeleton and initiate bud growth at the proper site.

Less is known about the genes involved in polarizing the cell in response to mating pheromones. Some of the polarity-establishment genes important for budding are also necessary for shmooing. Temperature-sensitive *cdc24* mutants exhibit defects in mating (REID and HARTWELL 1977) and shmooing (FIELD and SCHEKMAN 1980) at the nonpermissive temperature. Special mutations in *BEM1* have been identified which do not affect budding but which cause defects in shmooing and mating (CHENEVERT *et al.* 1992; this study). The gene encoding the α-pheromone receptor, *STE2*, is required for shmooing in some capacity in addition to its role in the signal transduction pathway. A mutant form of the receptor which lacks the intracellular C terminus is proficient for signaling but produces uniformly enlarged cells rather than polarized shmoos (KONOPKA *et al.* 1988). Finally, yeast strains in which *SPA2* is deleted do not form shmoo tips but instead form round cells when treated with pheromone (GEHRUNG and SNYDER 1990). We hypothesize that, during shmoo formation or mating, activated pheromone receptors generate an intracellular spatial signal which is recognized by molecules capable of locally organizing the cytoskeleton. To identify genes involved in this process, we devised a genetic screen to isolate mutants defective in polarizing in response to mating pheromones.

MATERIALS AND METHODS

Strains, media and genetic methods: Yeast strains are listed in Table 1. Crosses, sporulation and tetrad dissection were performed as described previously (SHERMAN *et al.* 1982). Matings involving mating-deficient strains were performed by selection for prototrophy generally following a period of growth under nonselective conditions. Yeast rich medium (YEPD) and synthetic minimal medium (SD) were prepared as described previously (HICKS and HERSKOWITZ 1976).

Mutagenesis: Strain JC2-1B was mutagenized by exposure to ethyl methanesulfonate (EMS; Sigma). The genotype of this strain (see Table 1) is such that morphological response to α-factor can be easily scored and mutants defective in *SIR* genes will not be isolated. Ten 1.5-ml overnight cultures inoculated from separate single colonies of JC2-1B were grown and treated separately as mutagenesis series A–J to assure independent isolation of mutants. Cells were washed and resuspended in 1.5 ml 10 mM phosphate buffer, pH 7, and sonicated for 5 sec to disrupt cell clumps. An 0.5-ml aliquot was removed to another tube as an untreated control, and 20 μl EMS were added to the remaining 1-ml culture. The treated tubes were incubated at 30° with aeration for either 45 min (pools A–D) or 60 min (pools E–J). Cells were pelleted and the EMS inactivated by addition of 1.5 ml 5% sodium thiosulfate. The mutagenized cells were then washed twice with water, once with YEPD, and resuspended in 1 ml YEPD. These cultures were frozen for 2 days while the titer was determined, and then cells were plated for screening. The frequency of survivors was between 89% (45-min EMS treatment) and 68% (60-min EMS treatment). The frequency of mutation to canavanine resistance was approximately 1.5×10^{-3}.

Assays of phenotype: Pilot experiments revealed that a 4–6-hr permissive mating was best for distinguishing between the abilities of wild-type and mutant colonies to mate to the enfeebled tester strain. Mutagenized yeast cells were plated on YEPD at a density of 100–200 cells per plate and incubated at 30° for 4 days. Each plate was then replica plated to two YEPD plates on which a confluent lawn of fresh α *far1* (IH2514) or α *fus1 fus2* (IH2351) mating testers had been spread (approximately 10^7 cells per plate). These plates were incubated at 30° for 4–6 hr to allow mating and then replica plated to conditions selective for diploids (SD for matings to the *far1* strain and SD supplemented with uracil for matings to the *fus1 fus2* strain). After further incubation for 1–2 days, mating-defective mutants exhibited reduced or no formation of diploids. Identification of mating-defective mutants on crowded plates was aided by a color distinction: diploid mating products became Ade^+ and thus white, whereas mating-defective mutant colonies remained Ade^- and gave imprints of dead and dying cells on the selective plate which were easily seen as red shadows in a background of white diploid prototrophs. These mating-defective candidates were picked from the original plate and retested.

The ability of mating-defective mutants to respond to pheromone was determined by assaying cell cycle arrest. A conventional halo assay (HERSKOWITZ 1988) was modified in the following way so that many mutants could be tested (usually six per plate). A suspension of each strain to be tested was made in 1 ml YEPD at approximate density 10^7 cells/ml. A drop of suspension was streaked with a pipette on a YEPD plate and allowed to dry. Then fresh α-factor-producing cells (strain IH1793, Table 1) were patched in the middle of each streak with a toothpick. After overnight incubation at 30°, a halo of growth inhibition appeared in streaks of strains capable of responding to pheromone. More sensitive pheromone response tests were carried out for mutants which gave ambiguous results (neither completely sensitive nor completely resis-

TABLE 1

Yeast strains

Strain	Genotype	Source	Comments/Reference
JC2-1B	*MAT***a** *HML***a** *HMR***a** *ura3-52 ade2-101 met1 bar1-1*	This study	Parent strain for mutant hunt
JC-G11	*MAT***a** *HML***a** *bem1-s1*	This study	Mutant derived from JC2-1B
JC-J9	*MAT***a** *HML***a** *pea1-2*	This study	Mutant derived from JC2-1B
JC-A10	*MAT***a** *HML***a** *pea2-2*	This study	Mutant derived from JC2-1B
JC-G16	*MAT***a** *HML***a** *tny1-1*	This study	Mutant derived from JC2-1B
IH2514	*MAT***a** *far1-c (Tn3::URA3) trp1 leu2 ura3 his4*	F. CHANG	Enfeebled mating tester
JC31-7D	*MATα far1-c lys1*	This study	Enfeebled mating tester
JC31-1D	*MAT***a** *far1-c lys1*	This study	Enfeebled mating tester
IH2351	*MATα fus1-Δ1 fus2-Δ3 ura3–52 trp1-Δ1*	G. FINK	Enfeebled mating tester
IH2353	*MAT***a** *fus1-Δ1 fus2-Δ3 ura3–52 trp1-Δ1*	G. FINK	Enfeebled mating tester
IH1793	*MATα lys1*	IH laboratory collection	Mating tester
IH1792	*MAT***a** *lys1 cry1*	IH laboratory collection	Mating tester
IH993	*MATα sst2-1 met1 his6 can1 cyh2*	IH laboratory collection	Halo tester
IH414	*MAT***a** *bar1 arg9 ilv3 ura1* killer$^+$	IH laboratory collection	Halo tester
IH2356	*MATα trp1 leu2 ura3 his4 FUS1::lacZ(URA3)*	IH laboratory collection	
IH2431 (E187Jα)	*MATα cdc24-3(ts)*	J. PRINGLE	SLOAT *et al.* (1981)
IH2433 (CJ198)	*MATα cdc43(ts) ura3 trp1*	J. PRINGLE	
KO1-1A	*MATα bem1::URA3 trp1 leu2 ura3 his4*	This study	
KO2-5B	*MATα bem1::LEU2 trp1 leu2 ura3 his4*	This study	CHENEVERT *et al.* (1992)
JC108	*MATα trp1 ade2 ura3 bem1-s1* (G11 mutation)	This study	Used for complementation tests
NVY14	*MATα leu1 trp5 ura3 bar1 pea1-1* (D6 mutation)	This study	Used for complementation tests
NVY20	*MATα leu1 trp5 ura3 bar1 pea2-1* (I14 mutation)	This study	Used for complementation tests
NV52a	*MATα leu1 trp5 ura3 bar1 tny1-1* (G16 mutation)	This study	Used for complementation tests
JC117	*MATα bem1-s1 his4 leu2 ura3 FUS1::lacZ(URA3)*	This study	Used for quantitative matings
NVY123	*MATα leu1 trp5 bar1*	This study	Used for quantitative matings
NVY124	*MATα pea1-1 leu1 trp5 bar1*	This study	Used for quantitative matings
NVY126	*MATα tny1-1 trp1 leu5 ade2 bar1*	This study	Used for quantitative matings
NVY118	*MATα pea2-1 leu1 trp5 ade2 bar1*	This study	Used for quantitative matings

tant). (1) Quantitative pheromone-response assays were performed by pipetting different amounts of pheromone onto filter discs placed on lawns of the mutant **a** cells. (2) Individual mutant cells were observed microscopically by plating dilutions on YEPD slabs, streaking an α-factor-producing strain on the slab, and then scoring the response of individual cells as a function of distance from the pheromone source.

The test for **a**-factor production was essentially as described by MICHAELIS and HERSKOWITZ (1988). Patches of the mutant **a** strains were replica plated to lawns of a strain supersensitive to **a**-factor (strain IH993, Table 1). The ability to produce **a**-factor was seen as a zone of growth inhibition surrounding the patch.

To observe the morphological response of the mutant strains to pheromone, cultures of each were grown to mid-log phase in YEPD at 30° (OD_{600} = 0.7), α-factor was added to a final concentration of 10^{-6} M, and the cultures were returned to 30° to grow with shaking. Aliquots were removed at various times (2, 4, 6 hr), sonicated for 3 sec, and viewed in the phase-contrast microscope. Morphology was also observed on solid medium by the slab assay for pheromone response as described above.

The budding patterns of the mutants were determined by plating single cells on YEPD slabs, allowing them to divide, and observing microcolonies at the four-cell stage as described by CHANT and HERSKOWITZ (1991).

Complementation tests: Since mating is a haploid-specific phenotype, complementation had to be scored in diploid yeast strains that exhibited an **a** or α phenotype. The procedure for generating such diploid strains is essentially as described by HERSKOWITZ and JENSON (1991) and had the following steps. (1) A *MAT*α mutant segregant was obtained by crossing the original *MAT***a** mutant to a wild-type α strain and analyzing meiotic progeny. (2) The mutant α was transformed with a plasmid containing the *HO* gene under control of the *GAL* promoter. (3) This transformant was mated to the *MAT***a** strains carrying a different mutation, and diploids were selected. (4) The diploids were grown on galactose medium for a limited time to allow switching from *MAT***a**/*MAT*α to *MAT***a**/*MAT***a**. The cells were then plated for single colonies and tested for pheromone production. Colonies producing **a**-factor are expected to be *MAT***a**/*MAT***a** diploids containing both original mutations; these were then tested in the original mating assay.

Quantitative mating assays: Assays were performed essentially as in NEIMAN *et al.* (1992) except that the mating temperature was 30° instead of 37°. Approximately 3 × 10^6 cells from exponentially growing cultures of each strain were mixed and then filtered onto 0.45 μm nitrocellulose filters (Millipore). The filters were rinsed with 5 ml YEPD, placed on YEPD plates and incubated for 4 hr at 30°. Cells were resuspended in 5 ml SD by vigorous vortexing for 30 sec followed by sonication for 3 sec. Dilutions were plated on YEPD to determine total colony-forming units and on SD minimal plates to select for diploids. Mating frequency was calculated as the ratio of diploid cells to total cells.

Cloning of *BEM1*: A *bem1-s1* strain (JC-G11) was transformed with a genomic DNA library in a low copy number vector (YCp50) (ROSE *et al.* 1987). Transformants were plated on SD plates lacking uracil at a density of about 100 colonies per plate and mated to an α *far1* mating tester (IH2514) as described above. Four mating-proficient colonies were identified from 1700 transformants tested. Restriction analysis revealed that three of these plasmids were identical; the fourth contained DNA fragments in common with the others. Loss of the plasmids correlated with loss of mating ability. Subcloning identified a 1.6-kb *Hin*dIII fragment which was able to complement the *bem1-s* mutant in one orientation. The sequence of this fragment revealed that it contained most of the *BEM1* gene, which was simultaneously identified and sequenced in the PRINGLE laboratory (BENDER and PRINGLE 1991; CHANT *et al.* 1991).

***FAR1*, *FUS1* and *FUS2* plasmids:** pTP41 was a gift of M. PETER and contains the 3.8-kb *Bgl*II fragment containing *FAR1* from pFC21 (F. CHANG, unpublished) cloned into the

*Bam*HI site of YCp50 (JOHNSTON and DAVIS 1984). YCp50-based plasmids containing *FUS1* (pSB245) or *FUS2* (pSB265) were gifts of G. FINK.

RESULTS

Rationale for mutant isolation: During sexual conjugation of *S. cerevisiae*, the mating partners exhibit cell polarization in response to mating pheromones. We were interested in identifying genes involved in this polarization and predicted that such genes might be required in only one partner for cell fusion to occur. Thus a mutant defective in pheromone-induced polarization may show little or no defect in mating with a wild-type strain but a dramatic defect in mating with an enfeebled mutant defective in the same function. We therefore isolated mutants unable to mate to strains already enfeebled for mating.

The enfeebled partner strains used in our screen were defective in either the *FAR1* gene or in the *FUS1* and *FUS2* genes and may be defective in pheromone-induced cell polarization. *FAR1* is required for cell-cycle arrest in response to mating pheromone and also for some other function involved in mating (CHANG and HERSKOWITZ 1990). Yeast strains carrying the *far1-c* mutation respond to pheromone and arrest well but have a mating defect, perhaps due to inability to orient toward a mating partner (CHANG 1991). *FUS1* and *FUS2* are required for cell fusion; mutants defective in these genes form "prezygotes" in which the two cell membranes of the mating partners do not fuse (TRUEHEART *et al.* 1987; MCCAFFREY *et al.* 1987). The *FUS1* and *FUS2* genes are both highly pheromone inducible, and their gene products are localized to the shmoo tip (TRUEHEART *et al.* 1987; E. ELION, personal communication). We imagined that proteins important for cell-cell fusion must be properly localized to the shmoo tip in at least one partner for mating to occur. The second reason for choosing these mutants is that they exhibit bilateral mating defects of the type predicted for mutants defective in mating polarization: *far1* mutants can mate to a *FAR1* strain quite well (efficiency of mating *ca.* 10^{-1}); in contrast, mating between two *far1* mutants occurs at a frequency of $<10^{-7}$ (CHANG and HERSKOWITZ 1990). Similarly, a *fus1 fus2* strain mates well to a *FUS* strain (mating frequency of 0.23) but poorly to another *fus1 fus2* strain (a frequency of 5.9×10^{-4}; TRUEHEART *et al.* 1987). In addition *far1* mutants mate to *fus1 fus2* mutants at a very low frequency (10^{-5}; CHANG 1991; J. CHENEVERT, unpublished observations). We hoped that mutants directly involved in polarizing in response to mating pheromone would be found among those unable to mate to these crippled partners.

Initial characterization of mutants: In the primary screen of 26,000 mutagenized colonies from 10 independently mutagenized pools (A–J), 137 were defective in mating to the enfeebled tester strains (Table 2, Figure 1). Most of these (88) were defective in mating to both the *far1* tester and the *fus1 fus2* tester, but some were able to mate to the *fus1 fus2* strain (39 mutants) or to the *far1* strain (10 mutants). 86 of the mutants (including representatives from each of the aforementioned mating-defective classes) were not arrested by pheromone (Figure 1, Table 2); they may be defective in known sterile (*STE*) genes required for signal transduction or in genes involved in cell cycle arrest and were not studied further. Of the 51 pheromone-sensitive mutants, 9 were deficient in a-factor production and were not studied further.

TABLE 2

Demographics of mutant hunt

Mutant phenotype	No. of isolates[a]
Defective in arrest by pheromone	86
Defective in pheromone production	9
Reduced vegetative growth or abnormal vegetative morphology	9
Aberrant budding pattern	2
Other mutants	31
Total mating-defective	137

[a] 26,000 total colonies were screened.

Because we were interested in morphological defects specific to pheromone treatment, we analyzed the mutants for growth rate and morphology in the absence of pheromone and studied further only those mutants that were normal in these tests. Nine mutants grew substantially slower than a wild-type strain or produced aberrantly shaped cells (A1, A2, C5, C6, C8, E13, I3, I13, J16). Only one of the mutants (I13), which exhibited interesting morphology in the presence of pheromone (see below), was studied further. Two mutants exhibited abnormal budding patterns but no other vegetative defects: F16 displayed a random budding pattern and I15 displayed a bipolar pattern (S. SANDERS, personal communication). The final tally of candidates with potential defects in mating-related polarization was 34 (see Table 2). All mated well to a wild-type strain, indicating that they differ from mutants defective in standard *STE* genes.

Five classes of pheromone-induced morphology: The 34 mutants studied further are those that mated to a wild-type strain, failed to mate to a *far1* or a *fus1 fus2* strain, and exhibited normal morphology during vegetative growth. Among these 34 mutants, a range of relative mating efficiencies to the two enfeebled testers was displayed; in some cases the mating defect was more dramatic with one tester than with the other. These mutants were treated with pheromone and characterized microscopically. A variety of shapes was observed, falling into five broad categories (Table 3 and Figure 2). (1) One class of mutants (shmooless mutants, 3 isolates) appeared to be unable to form shmoos but instead enlarged in a uniform manner (Figure 2B). (2) A second class (peanut mutants, 4 isolates) formed cells that were

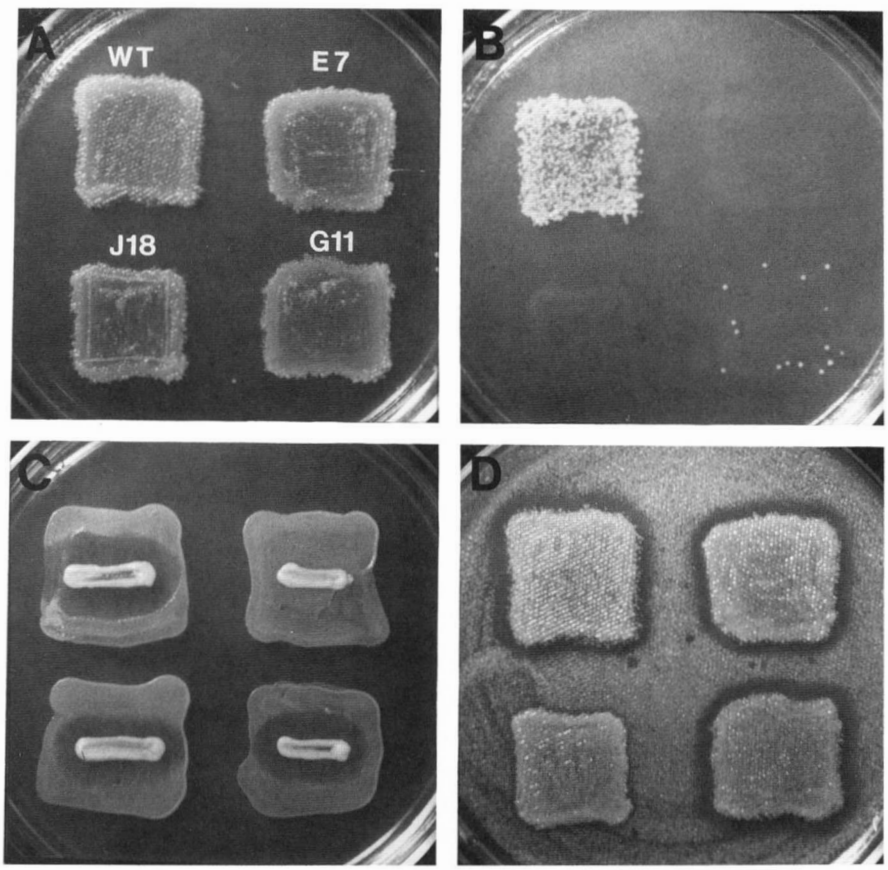

FIGURE 1.—Mutant phenotypes. Three representative mating-defective mutants (A, B) and the basis for the elimination of two of them (C, D) are shown. (A) Strains JC2-1B (wild-type), G11, E7 and J18 are patched onto a YEPD plate. (B) The patches in A were replica-plated to a lawn of JC31-7D (*MATα far1*) cells spread on a YEPD plate and then replica-plated to an SD plate. Prototrophic colonies result from mating. (C) Mini-lawns of the suspensions of the strains from A were streaked out as described in MATERIALS AND METHODS and a wild-type α strain was patched on top. Mutant E7 was eliminated as pheromone-resistant. (D) The patches in A were replica-plated onto a lawn of the **a**-factor tester strain IH993. Mutant J18 was eliminated as defective in **a**-factor production.

TABLE 3

Morphologies of mutants in the presence of pheromone

Mutants	No.	Designations	Genes
Homogeneous terminal morphologies:			
Class 1. Shmooless	3	F5, G11	*BEM1*
		I13	*CDC24*
Class 2. Peanut shmoo	4	D6, J9	*PEA1*
		A10, I14	*PEA2*
Class 3. Tiny shmoo	2	G16, H9	*TNY1*
Class 4. Wild-type shmoo	18	B4, D1, G18, H7	*FAR1*
		F20	*FUS2*
		A5, B6, E15, F16, G3, G17, I5, I11, I15, J3, J10, J21, J26	?
Heterogeneous terminal morphologies:	7		
Total:	34[a]		

[a] These 34 mutants include one with reduced vegetative growth rate (I13), two which exhibit aberrant budding patterns (F16 and I15), and the 31 "other" mutants indicated in Table 2.

roughly symmetrical and had two lobes (Fig. 2D). (3) The third class (tiny mutants, 2 isolates) contained about 50% shmoos of unusually small size (Figure 2C). (4) The fourth class (18 mutants) formed shmoos that were completely normal or that exhibited more subtle defects, such as forming shmoos more slowly than wild-type or with projections that were shorter or blunter than wild-type shmoos. These four classes are described as "homogeneous" because affected cells exhibited uniform morphological aberrations (50% for tiny mutants, >90% for the others). (5) The final class (7 mutants) is termed "heterogeneous" because cells of a particular mutant strain formed a diverse set of morphologies upon pheromone treatment. This class of mutants was not studied further.

We focussed on the shmooless, peanut shmoo, and tiny shmoo mutants because they displayed abnormal but homogeneous morphologies in the presence of pheromone. For each of the nine mutants in the shmooless, peanut shmoo, and tiny shmoo classes, genetic analysis showed that the morphological abnormality cosegregated with the mating defect, demonstrating that both phenotypes are due to a defect in the same gene (see below and data not shown).

Shmooless mutants are defective in polarity establishment genes *BEM1* and *CDC24*: Complementation tests were performed to determine whether the three

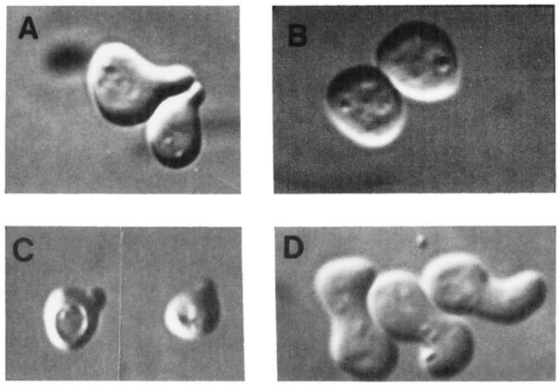

FIGURE 2.—Morphologies of yeast strains in the presence of pheromone. (A) Wild-type **a** strain JC2-1B; (B) shmooless mutant G11 (*bem1-s1*); (C) tiny shmoo mutant G16 (*tny1-1*); (D) peanut-shmoo mutant A10 (*pea2-1*). The percentage of cells which manifest the mutant phenotype is about 50% for the tiny shmoos and >90% for the others. Cells were viewed under Nomarski optics and photographed with the Zeiss axioscope automatic exposure camera using Technical Pan film.

TABLE 4

Complementation test of shmooless mutants

Test	Mating[a]
A. *MAT***a**/*MAT***a** diploids derived from mating between	
JC108 (mutation from G11) and JC2–1B (wild-type)	+
JC108 (G11) and mutant G11	–
JC108 (G11) and mutant F5[b]	–
JC108 (G11) and mutant I13	+
	Growth at 37°
B. Haploids:	
Mutant I13	–
IH2431 (*cdc24*)	–
IH2433 (*cdc43*)	–
KO2-5B (*bem1*)	–
Diploid derived from mating between:	
Mutant I13 and IH2431 (*cdc24*)	–
Mutant I13 and IH2433 (*cdc43*)	+
Mutant I13 and KO2-5B (*bem1*)	+

[a] Mating to *far1-c* strain JC31-7D.
[b] **a**/**a** diploids formed between F5 and other mutants exhibited mating, excluding the possibility that F5 contains a dominant mutation.

mutants which exhibited the shmooless phenotype were defective in the same gene. A *MAT*α segregant containing the G11 mutation (JC108) was mated to each of the *MAT***a** shmooless mutants, G11, F5 and I13, and to the wild-type parent JC2-1B as a control for dominance. The resulting *MAT***a**/*MAT*α diploids were converted to *MAT***a**/*MAT***a** diploids as described in MATERIALS AND METHODS and then tested for mating ability to *far1* and *fus1 fus2* strains. By these tests, the mutation in G11 is recessive to wild type. G11 and F5 are defective in the same complementation group, whereas I13 is defective in a different complementation group (Table 4).

A gene with the ability to restore mating and shmooing to the G11 and F5 shmooless mutants was cloned by complementation of the mating defect of G11 as previously reported (CHENEVERT *et al.* 1992; see MATERIALS AND METHODS), and proved to be *BEM1*, a gene involved in budding polarity (CHANT *et al.* 1991; BENDER and PRINGLE1991). Tetrad analysis verified that the G11 mutant harbored a mutation in *BEM1*: when a diploid formed by mating G11 to a strain deleted for *BEM1* (KO1-1A) was sporulated and dissected, wild-type recombinants were not recovered (cross 1, Table 5).

Two observations on mutant I13 gave clues to its identity. First, it was unable to form colonies at high temperature (>33°) and grew slower than a wild-type strain at 30°. Second, it did not yield prototrophic diploids when mated with a *cdc24-ts* mutant at 37° (data not shown). To determine whether I13 was defective in the *CDC24* gene, it was mated to a *cdc24-ts* mutant (strain IH2431) at permissive temperature (25°) to yield a diploid. This strain was unable to grow at nonpermissive temperature (37°), indicating that I13 contains a mutation in *CDC24*. This mutation is recessive and did complement *cdc43* and *bem1* mutations (Table 4). The diploid formed by mating I13 to the *cdc24-ts* strain IH2431 yielded meiotic segregants all of which were temperature sensitive (Table 5), demonstrating that mutant I13 contains a mutation of *CDC24*.

Peanut shmoo mutants define two genes, *PEA1* and *PEA2*: Because complementation tests for the peanut class of mutants were ambiguous (data not shown), allelism was tested by segregation analysis (Table 5). A *MAT*α segregant containing the mutation present in D6 was obtained (strain NVY14, Table 1) and mated to the three other **a** mutants that exhibited the peanut phenotype. The diploids were sporulated and dissected, and tetrads were analyzed for both mating ability (all segregants) and shmoo morphology (*MAT***a** segregants only). In all cases, the mating defect and peanut morphology cosegregated. Wild-type recombinants were readily recovered when NVY14 (which contains the mutation from D6) was crossed to mutant I14 or mutant A10 but not when NVY14 was crossed to mutant J9. When a *MAT*α strain containing the mutation from I14 (strain NVY20) was crossed to mutant J9, a high frequency of wild-type segregants was observed but not when NVY20 (I14) was mated to mutant A10. We conclude that the mutations in D6 and J9 are allelic and that the mutations in I14 and A10 are allelic. These studies thus identify two genes which when mutated can give the peanut shmoo phenotype. We name these genes *PEA1* (defective in mutants J9, D6) and *PEA2* (defective in mutants A10, I14). The data in Table 5 also show that *PEA1* and *PEA2* are linked. We calculate the genetic distance between them to be approximately 30 cM.

Tiny shmoo mutants define one gene, *TNY1*: To determine if the two mutants with the tiny shmoo phenotype were defective in the same gene, a *MAT*α strain containing the G16 mutation (NV52a) was crossed to the independently isolated H9 mutant. Tetrad analysis

TABLE 5

Allelism analysis

Cross	PD	NPD	T	Percent wild-type segregants	Phenotype scored
(1) G11 × KO1-1A (*bem1*)	10	0	0	0[a]	Mating
Conclusion: mutant G11 contains a mutation in *BEM1*					
(2) I13 × IH 2431 (*cdc24*)	12	0	0	0[a]	Growth at 37°
Conclusion: mutant I13 contains a mutation in *CDC24*					
(3) NVY14 (D6) × I14	3	0	3	17	Mating[b] and peanut morphology
(4) NVY14 (D6) × A10	4	0	4	14	(as above)
(5) NVY14 (D6) × J9	13	0	0	0	(as above)
(6) NVY20 (I14) × J9	2	0	6	20	(as above)
(7) NVY20 (I14) × A10	8	0	0	0	(as above)
Conclusion: D6 and J9 contain mutations that are allelic; A10 and I14 contain mutations that are allelic					
(8) NV52a (G16) × H9	18	0	0	0	Mating[b] and tiny morphology
Conclusion: H9 and G16 contain mutations that are allelic					

[a] Strains deleted for *bem1* or defective in *cdc24* exhibited poor spore viability. Thus the number of complete tetrads was relatively low. All germinating segregants were analyzed: 128 spores for cross (1) and 95 spores for cross (2).
[b] Mating to *far1-c* strains JC31-7D or JC31-1D or *fus1 fus2* strains IH2351 or IH2353.

of the resultant diploid (Table 5) indicates that they are defective in the same gene, which we call *TNY1*.

Mutants in *BEM1* may exhibit bilateral sterility: The original rationale for mutant identification was that mutants defective in polarizing toward a mating partner may mate well to a wild-type partner but poorly to another strain defective in polarization. Thus we anticipated that a mutant defective in a gene required for cell polarization might exhibit a much reduced mating frequency to a strain defective in the same gene as compared to the mating frequency of the mutant when mated to a wild-type strain. We tested the ability of a *MAT***a** strain containing a mutation in *BEM1*, *PEA1*, *PEA2* or *TNY1* to mate to a *MAT*α strain containing the same mutation. The results are given in Table 6. Two mutants defective in *BEM1* display a slight mating defect when mated to a wild type strain (about 1/7 wild-type levels, 1.8 *vs.* 8.7 for one strain; 0.2 *vs.* 8.7 for the other). The mating frequency between a *MAT***a** *bem1* mutant and a *MAT*α *bem1* mutant (0.00001), however, is lower than the product of the mating frequencies observed for a *MAT***a** *bem1* mutant to a *MAT*α *BEM1* strain (0.018) and a *MAT***a** *BEM1* strain to a *MAT*α *bem1* mutant (0.002). Thus a mutation in *BEM1* confers an apparent bilateral mating defect. We have observed in this and other experiments that *MAT***a**/*MAT*α *bem1/bem1* diploids grow substantially slower than *MAT***a** *bem1* or *MAT*α *bem1* haploids (data not shown). The growth defect may lower the observed frequency of diploid formation.

Strong bilateral sterility was not observed for mutants defective in *PEA1*, *PEA2* or *TNY1*, although mutants defective in any of these genes do show reduced mating frequencies relative to wild-type strains. The mating frequency between a *MAT***a** *pea1* mutant and a *MAT*α *pea1* mutant, for example, is not significantly lower than the product of the mating frequencies observed for a *MAT***a** *pea1* mutant to a *MAT*α *PEA1* strain and a *MAT***a** *PEA1* strain to a *MAT*α *pea1* mutant.

TABLE 6

Do *bem1*, *pea1*, *pea2*, or *tny1* mutants exhibit a bilateral mating defect?

*MAT***a** strain	Relevant genotype	*MAT*α strain	Relevant genotype	Efficiency of mating (%)
JC2-1B	+	IH1793	+	8.7[a]
JC-G11	*bem1*	IH1793	+	1.8
JC2-1B	+	JC117	*bem1*	0.2
JC-G11	*bem1*	JC117	*bem1*	0.001
JC2-1B	+	NVY123	+	22
JC-J9	*pea1*	NVY123	+	2.6
JC2-1B	+	NVY124	*pea1*	7.9
JC-J9	*pea1*	NVY124	*pea1*	1.6
JC2-1B	+	NVY123	+	12
JC-A10	*pea2*	NVY123	+	1.2
JC2-1B	+	NVY118	*pea2*	3.6
JC-A10	*pea2*	NVY118	*pea2*	0.12
JC2-1B	+	NVY123	+	21
JC-G16	*tny1*	NVY123	+	11
JC2-1B	+	NVY126	*tny1*	1.8
JC-G16	*tny1*	NVY126	*tny1*	0.2

[a] Each set of three matings involving mutant strains is preceded by a wild-type mating which is the positive control for that set.

New alleles of *FAR1* and *FUS2*: We expected to isolate mutants defective in *FAR1*, *FUS1* and *FUS2*, since strains mutated in these genes are known to be defective in mating to our crippled partners. Four mutants (B4, D1, G18 and H7) which exhibited normal shmoos and which failed to mate to both the *far1* and *fus1 fus2* mating testers were defective in the same complementation group (data not shown). Mating was completely restored to the three mutants tested from this group (B4, D1 and H7) when they were transformed with a single-copy plasmid containing the *FAR1* gene (pTP41). We conclude that these three and probably all four stains carry mutations in *FAR1*. These mutants appar-

ently do not affect the activity of *FAR1* necessary for cell-cycle arrest but affect another activity of *FAR1* necessary for mating (CHANG and HERSKOWITZ 1990; PETER *et al.* 1993). Plasmids containing the *FUS1* (pSB245) or *FUS2* (pSB265) genes were transformed into the remaining 14 mutants, and the transformants were tested for mating ability. Mating was restored only to mutant F20, when transformed by the *FUS2* plasmid. F20 thus appears to contain a mutation in *FUS2*.

DISCUSSION

We have identified yeast genes required for normal pheromone-induced cell polarization. Use of a novel screen for mating-defective mutants made it possible to isolate 34 mutants defective in mating but proficient for pheromone-induced cell cycle arrest, pheromone production, and vegetative growth. Some of the mutants contain special alleles of genes *BEM1* and *CDC24*, which are required for polarity of vegetative cells. Three other genes *PEA1*, *PEA2* and *TNY1* were also identified. Another class of mutants (with 13 representatives) exhibit normal shmooing morphology. The nature of their mating defect is mysterious.

A sensitive screen to reveal subtle mating functions: By assaying mating to a partner enfeebled for mating, mutants with only subtle effects on mating efficiency were isolated which would have been missed in previous screens for defects in mating (MACKAY and MANNEY 1973; WILSON and HERSKOWITZ 1987; ASHBY *et al.* 1993) or pheromone response (HARTWELL 1980; JENNESS *et al.* 1987; A. NEIMAN, personal communication). This initial screen provided an enrichment for mutants with altered morphological responses to pheromone, thereby decreasing the amount of tedious microscopic analysis that would have been required for a direct microscopic screen. More importantly, it provided a colony assay for scoring this phenotype, which proved very useful during genetic manipulations. Interestingly, when mutants defective in pheromone response or production were eliminated, all of the remaining mutants mated well to a wild-type partner, although this was not a requirement of our screen.

Many of the mutants, including some defective in pheromone production or pheromone-induced arrest, were not equally defective in mating with both enfeebled mating testers. This behavior may not be surprising, since the bases for the mating defects of *far1* mutants, of *fus1 fus2* mutants, and of the mutants isolated in this study are not completely understood and diverse cellular functions may be affected. Some of the mutants identified in this study may be defective in functions important but not essential for mating or they may contain non-null alleles of genes essential for mating. Thus their mating defects would not be absolute and could vary depending on the tester used.

Polarity establishment and selection of the mating site: The identification of *BEM1* and *CDC24* in a screen for genes involved in polarizing the cell in response to pheromone lends support to the notion that the same set of polarity-establishment proteins is used for both bud formation and shmoo formation. Since many of the same cytoskeletal components and secretory functions are shared between these two processes (FIELD and SCHEKMAN 1980; BARNES *et al.* 1990; BYERS 1981; READ *et al.* 1992), it is not surprising that a common set of proteins organizes these functions during budding and shmooing. Mutants harboring the shmooless alleles of *BEM1* bud normally at all temperatures and are thus distinct from *bem1* null mutants, which are defective in budding and inviable at high temperature (CHANT *et al.* 1991; CHENEVERT *et al.* 1992, BENDER and PRINGLE 1991); these alleles are thus denoted *bem1-s1* and *bem1-s2* to signify that they cause a "shmooless" phenotype. The *bem1-s* mutations might identify a mating-specific domain of the *BEM1* protein or decrease the amount of functional Bem1p to a level adequate for budding but not for shmooing. The mutation in *CDC24* (now called *cdc24-6*) causes a temperature-sensitive growth defect which appears to be more severe than that of other *cdc24* alleles (HARTWELL *et al.* 1973; SLOAT and PRINGLE 1978; SLOAT *et al.* 1981). *cdc24-6* strains grow normally at 25° but slower than wild-type or *cdc24-4* at 30°. Thus the function of the *CDC24* protein in the *cdc24-6* strain is compromised at 30°, which could explain why it exhibits a defect in shmooing and mating at 30°.

According to a model for bud formation (CHANT and HERSKOWITZ 1991), the polarity establishment proteins (Bem1p, Cdc24p and Cdc42p) respond to spatial information provided by bud-site selection proteins (*BUD1-5*). The *BUD* gene products are hypothesized to recognize an intracellular landmark and identify it as the site for growth. We propose that during conjugation, the polarity establishment proteins are guided to perform their function at a site which is dictated by an extracellular signal, the mating pheromone, and that the bud-site selection program is overridden. *BUD* proteins do not appear to be required for polarization during mating: *bud* mutants exhibit no defects in shmooing or mating (J. CHANT, personal communication; J. CHENEVERT, unpublished observations). We propose that mating-site selection proteins exist which detect the pheromone signal and mark the proper site for organization of the polarity establishment proteins.

In response to pheromone, cells containing the shmooless mutations in *BEM1* and *CDC24* grow throughout their cell surface instead of at a single site and thus do not form a projection. In the *bem1* mutants, this isotropic growth reflects disorganized actin and delocalized cell wall deposition (CHENEVERT *et al.* 1992). One interpretation of the shmooless phenotype is that the actin cytoskeleton and secretory apparatus are severed from information which marks the proper site for growth to occur. The wild-type function of Bem1p or

Cdc24p in this scenario would be to organize the growth machinery at a unique position on the cell surface. The class of mutants which forms shmoos of normal morphology but still fails to mate may affect genes involved in marking the proper site. These mutants are able to establish polarity but may be unable to do so in the correct place and thus choose a site at random. Current work is addressing this possibility.

Function of *TNY1*: Mutants defective in the *TNY1* gene form small shmoos and do not appear to be defective in polarization *per se*, but rather are defective in growth in response to pheromone. The phenotype of small shmoos has been reported previously, in yeast cells grown in calcium-free medium (IIDA *et al.* 1990) and in cells defective in calcineurin, a Ca^{2+}/calmodulin-dependent phosphoprotein phosphatase (M. CYERT, personal communication). *TNY1* may be part of a pathway or complex which monitors calcium concentration in pheromone-treated cells and permits growth. The *TNY1* protein, for example, might be a substrate for calcineurin. It is conceivable that calcium levels affect cell polarity in yeast by regulating polarity establishment proteins. *CDC24* encodes a putative Ca^{2+}-binding protein (MIYAMOTO *et al.* 1987) and was identified as a calcium-sensitive mutant (OHYA *et al.* 1986). It may also be functionally relevant that calmodulin (BROCKERHOFF and DAVIS 1992) and Bem1p (K. CORRADO and J. PRINGLE, personal communication) both localize to the site of bud emergence.

A strain carrying a mutation in the *SLK1/BCK1* gene, which is involved in growth control and cell morphogenesis, is also reported to form small shmoos (COSTIGAN *et al.* 1992). Mutations in this gene were identified as being synthetically lethal with *spa2* mutations (COSTIGAN *et al.* 1992) and independently as dominant suppressors of a defect in protein kinase C (LEE and LEVIN1992). *SLK1/BCK1* is predicted to encode a protein kinase, raising the possibility that the *TNY1* protein could be a substrate.

Function of *PEA1* and *PEA2*: Four mutants isolated in our screen form shmoos which resemble peanuts–they are broader at the neck and the tip than wild-type shmoos. We envision two sorts of defects which could lead to this behavior. According to one explanation, these mutants are defective in limiting the shmoo site to a small region. The *PEA1* and *PEA2* gene products could be part of a complex which restricts growth of the initial area of the shmoo tip to a precise area of the cell surface. Another explanation is that the *pea1* and *pea2* mutants are defective in a later stage of shmoo formation, for example, in inhibition of the shmoo tip after it has been formed. When treated with pheromone for long periods, wild-type yeast cells eventually arrest shmoo tip growth and form a second projection elsewhere. *PEA1* and *PEA2* gene products may be components of a pathway that arrests shmoo tip growth when productive mating is not achieved and allows a second projection to form.

We have recently cloned the *PEA1* gene and obtained partial sequence information (N. VALTZ, unpublished) which shows that it is identical to *SPA2* (GEHRUNG and SNYDER 1990). The discrepancy between the phenotype of pheromone-treated strains deleted for the *SPA2* gene (the round shapes described by GEHRUNG and SNYDER 1990) and the phenotype of pheromone-treated *pea1* mutants (peanut shapes) is under study. The *SPA2* protein localizes at the tip of a bud or a shmoo (SNYDER 1989) and thus is in an appropriate position to be involved in defining the shmoo site or limiting shmoo tip growth. The existence of a second gene, *PEA2*, with a mutant phenotype identical to *pea1* mutants, raises the possibility that these two genes interact or are components of a common pathway. The *PEA2* gene product may be responsible, for example, for localization of the *PEA1/SPA2* gene product.

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